The Infection Dynamics and Phase Change of the Entomophathogenic Bacterium *Xenorhabdus bovienii* and the associated Nematode Host *Steinernema feltiae.*

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ABSTRACT

Injection infection of *Galleria mellonella* with the entomopathogenic nematode *Steinernema feltiae* and its symbiotic bacterium *Xenorhabdus bovienii* resulted in a mixed population of *Stenotrophomonas maltophilia* and *Xanthomonas axonopidis* on NBTA agar plates. 16S rRNA expression from the general *Eubacteriaceae* population was shown to start 18 hours after infection and it was continued until 72 hours after infection. However, the same infection was shown to result in *X. bovienii* 16S rRNA expression from 2 hours until 48 hours.

Natural infection of 100 and 1000 *S. feltiae* using the same infection model resulted in a mixed population of *Stenotrophomonas maltophilia* and *X. bovienii* on NBTA agar plates. 16S rRNA expression of the *Eubacteriaceae* population was shown to occur between 36 and 72 hours after infection for both the 100 and 1000 *S. feltiae* infections. The 16S rRNA expression of the *X. bovienii* population began at 24 hours and 36 hours for the 100 and 1000 *S. feltiae* infections, respectively, and continued until 72 hours both the infection levels.

The gene fragments partially encoding rpoS and FliC were isolated and cloned from *X. bovienii,* demonstrating that a distinction between Phase I and Phase II *X. bovienii* can be attributed to the expression of the FliC gene and the rpoS gene. Isolation of a *Xenorhabdus*-like bacterium from the total RNA of *G. mellonella* indicated that an unspecified, potentially pathogenic, bacterium could be involved in the infection process in *G. mellonella.*

Isolation of a putative Phase I specific 36.5kDa ABC transporter from Phase I *X. bovienii* using 2-Dimensional gel electrophoresis which could be a potential toxin transporter is a further indication of the distinction between Phase I and Phase II *X. bovienii.*

Declaration

To whom it may concern

This thesis is the sole work of the author. The work reported in this thesis is the result of the author's own research and has not been reported in another thesis. Where material from other sources has been used it has been duly acknowledged in the text and fully listed in the references.

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CHAPTER ONE

Introduction

1.1 Entomopathogenic bacteria: *Xenorhabdus*, an overview

1.1.1 Biological control and life cycle

There are several bacterial pathogens that are currently used as insecticides or have potential future use, they include, *Rickettsiella popillae*, *Bacillus popilliae*, *Bacillus thuringiensis*, *Bacillus lentimorbus*, *Bacillus spaericus*, *Clostridium malacosome*, *Pseudomonas aeruginosa*, *Photorhabdus* spp. and *Xenorhabdus* spp. (Aronson *et al*, 1986). The most widespread is the δ-endotoxin of *B. thuringiensis* (Bt) the application of which leads to gut paralysis, the insect stops feeding and eventually dies from a combination of tissue damage and starvation (Maier *et al*, 2000). Bt is marketed world-wide as a commercial microbial insecticide, however, there is cause for concern over the increase in wide spread resistance to Bt by the target insects. There is documented evidence of several species of insect pests, such as the tobacco hormworm and leather jackets, developing resistance to Bt δ-endotoxins (Rosenheim, 1998). Insects have evolved numerous and effective defence mechanisms to resist infection. The defences include both structural and passive barriers, constituitive cellular and humoral factors in the haemolymph and induced antibacterial proteins (Dunphy & Thurston, 1990).

The introduction of other biological agents which are well adapted for use in soil environments are the entomopathogenic rhabditid nematodes of the genera *Steinernema* and *Heterorhabditis*. Like other nematodes of the Rhabditida order, *Steinernema* and *Heterorhabditis* form infective juveniles (IJs), which are morphologically and physiologically adapted for long term survival in the soil environment (Glazer 1997,). An IJ carries between 0 and 250 cells of its symbiont in the anterior part of the intestine (Treverrow & Bedding, 1993). The symbiotic bacteria are released into the haemolymph after penetration of the IJ into a suitable host insect. Inside the IJ the bacteria are well protected against hostile environmental conditions in the soil.

Xenorhabdus and *Photorhabdus* spp. are Gram negative proteobacteria which form entomopathogenic symbioses with soil nematodes. The nematode bacteria complex is capable of invading and killing the larval stage of various insects. *Xenorhabdus and Photorhabdus* spp. are carried as symbionts in the intestine of the infective juvenile stage of nematodes belonging to the families *Steinernematidae* and *Heterorhabditidae*, respectively. The nematodes enter the digestive tract of the larval stage of a diverse number of insects and subsequently penetrate into the haemocoel of the host insect. The nematode can also gain access to the haemocoel via the respiratory spiracles or by penetrating directly through the insect cuticle. Upon entrance into the haemocoel, the nematodes release the bacteria into the haemolymph. Together the nematode and the bacteria rapidly kill the insect larva, although it has been demonstrated that the bacteria alone are highly virulent (Gotz *et al,* 1981). Within the haemocoel of the larval carcass the bacteria grow to stationary phase conditions while the nematodes develop and sexually reproduce. During the final

stages of development, the nematode and bacteria re-associate and the nematode subsequently develops into its non feeding infective juvenile stage. The infective juvenile, carrying the bacteria in its intestinal tract, then emerges from the insect carcass in search of a new host (Forst *et al,* 1997).

Entomopathogenic nematodes (EPN) are environmentally safe and to date, show no evidence of mammalian pathogenicity (Ehlers *et al,* 1997). Although they are symbiotically associated with bacteria, they are not placed in the category of microorganisms for pest control in most countries. As such, they are usually exempted from registration requirements, making the commercial development of nematode products attractive for industry, as the large costs related to registration can be avoided (Ehlers, 1996).

EPN's are produced in mass culture (Bedding *et a,l* 1993) and are currently marketed for the control of the black vine weevil, *Otiorhynchus sulcans*, the peach borer, *Carposina nipponensus*, and the carpenterworm, *Holocercus insularis* (Ehlers, 1996), however little is known about the mode of action of the entomopathogenic bacteria which these nematodes carry.

1.1.2 *Xenorhabdus* taxonomy

From early studies on *Xenorhabdus* spp. it was concluded that these bacteria are closely related to the family of enteric bacteria, *Enterobacteriaceae* (Poinar & Thomas, 1966). Re-naming of both the bacteria and their nematode hosts has occurred as recently as 1993, DNA-DNA hybridisation studies resulted in the most

recent description of the *Xenorhabdus* genus. Four species have been described: *X. nematophilus*, *X. poinari, X. beddingii* and *X. bovienii* which usually form symbioses with the nematodes *S. carpocapsae, S. glaseri, Steinernema* spp and *S. feltiae*, respectively (Boemare *et al,* 1993).

Although placed in the family *Enterobacteriaceae* on the basis of extensive phyletic analysis, some traits are notably different from those of the other members of the *Enterobacteriaceae*, namely that *Xenorhabdus* spp are catalase negative and do not reduce nitrate to nitrite and that in general, *Xenorhabdus* are much less metabolically diverse than many of their *Enterbacteriaceae* counterparts (Boemare, 1993).

1.2 *Xenorhabdus bovienii* pathogenicity for *Galleria mellonella*

The *Xenorhabdus* bacterial symbionts are carried monoxenically in a special vesicle in the infective stage, infective juvenile (IJ), of members of the Seinernematidae (Bird *et al,* 1983) which provide protection and transport for the bacterial symbionts. The entomopathogenic bacteria are released from within the nematode into the host within 3-5 hours of invasion and the host larvae are usually killed within 48 hours (Akhurst & Dunphy, 1993).

Galleria larvae have been used extensively for studies into the pathogenicity of entomopathogens because this organism is highly susceptible to bacterial infection and will also change colour when infected due to phenoloxidase melanisation, which turns the larvae blackish brown.

In *Galleria*, the *Steinernema/Xenorhabdus* complex successfully kills the host because the nematode evades recognition by the insect immune system which allows time for the nematode to release the bacterium. The bacteria will eventually destroy the host's haemocytes by releasing the hemocytotoxin, lipopolysaccharide (LPS), from the bacterial outer membrane into the haemolymph. The LPS has been shown to prevent the processing of prophenoloxidase to phenoloxidase (Dunphy & Webster, 1988). The toxin is partially bound to the haemocytes by the lipid A moiety, lipid A is known to contain toxic fatty acids that damage the haemocytes. The release of LPS from the bacteria and subsequent damage to the haemocytes, ultimately resulting in a decrease in the number of haemocytes, was show to be correlated (Dunphy & Webster, 1988).

In insects the antibacterial proteins (cecropins) and peptides (attacins) are capable of lysing bacteria such as *Escherichia coli* through alteration of the permeability properties of the outer membrane to inhibit the production of the major outer membrane proteins (Carlsson *et al,* 1991). In *Xenorhabdus* spp. this can be prevented by protein secretions from the nematodes which destroy the cecropins (Dunphy & Thurston, 1990).

Jarosz *et al* (1991), showed that *Galleria* displayed pathological lesions and symptoms of disease when inoculated with larval extracts from insects infected with the nematode/bacterial complex. The inhibition of metamorphosis in insects inoculated with the larvicidal toxin could be related to the lack of change in the function of the corpora allata which during normal development would disappear at the prepupal stage. This would lead to sustained levels of juvenile hormone (JH),

which has to decrease in order for metamorphosis to take place. Pupation may also be blocked as a result of inhibition of ecdysone synthesis because the transformation of larva to pupa is associated with an increase in moulting hormone (MH) (Jarosz *et al*, 1991).

1.3 Production of Antibiotics by *Xenorhabdus* and *Photorhabdus*

Antibiotic production seems to be a crucial feature in infection of insects by the entomopathogen complex. Phase II variants which generally lack this activity are unable to restrict the invasion of the insect carcass by other bacteria; this could be detrimental to the nematodes and ultimately leads to poor nematode reproduction (Aguillera & Smart, 1993). The importance of the antibiotics lies in maintaining dominant cultures of the entomopathogenic bacterium during the nematode growth phase in the infected host and thus avoiding putrefaction of the carcass, which would result in poor nematode growth (Clarke & Dowds, 1995). The outer membrane of Gram negative bacteria, such as *Xenorhabdus*, functions as a selective diffusion barrier, allowing nutrients and other compounds such as antibiotics to passively transfer through water filled diffusion channels or channels formed by porin proteins (Hancock, 1991).

Several different secondary metabolites produced by both *Xenorhabdus* and *Photorhabdus* contribute to the success of the symbiotic relationship (Couche & Gregson, 1987) within the microenvironment of the insect host. Of special significance is the production of antibiotics which maintain the mutualistic

relationship between the bacteria and the nematodes which act as their vectors (McInerney & Gregson, 1991).

It is important to understand how these natural antibiotics inhibit the wide variety of micro-organisms normally encountered in such an environment. Sundar & Chang (1993) investigated the *in vitro* production of antibiotics by *X. nematophilus*, they found that antibody production is highest during the late stationary phase of bacterial multiplication. In agreement with Akhurst (1982), the indole antibiotics were found to be effective in moderate concentrations against a wide variety of both Gram negative and Gram positive bacteria by increasing the intracellular level of the regulatory nuclotide (ppGpp) in the micro-organisms, which in turn leads to severe inhibition of net RNA synthesis.

Results from a previous study by Paul *et al* (1981) into the mode of action of hydroxy-stilbene antibiotics produced by a related species, *P. luminescens* suggest that these bacteria use a similar antibacterial control mechanism. It may be surprising that two structurally dissimilar antibiotics share such a specific mechanism of action . However, given the special role of *Xenorhabdus* spp. in the life cycle of insect-parasitic nematodes (Poinar & Thomas, 1966) it is understandable that the two related species of bacteria have evolved a mechanism by which they can produce rapid and severe growth inhibition of other competing bacteria (Sundar & Chang, 1993).

1.4 Phase variation in *Xenorhabdus* and *Photorhabdus*

Xenorhabdus isolated from the infective stage nematode produce dye adsorbing colonies (Thomas *et al*, 1979). However, when *in vitro* cultures of *Xenorhabdus* are sampled some nonadsorbing colonies are detectable,(Akhurst, 1980). This has been identified as phase variation due to the ability of the secondary (non-adsorbing) form to revert back to the primary form. It is necessary for a reversion from the secondary form to the primary form to take place to be designated as phase change; if no reversion occurs then they are referred to as different forms, (Akhurst & Boemare, 1990). Akhurst first described form variation in *Xenorhabdus* and *Photorhabdus* spp. as a type of variation that involved several factors but which could be confidently delineated by changes in just two biochemical properties, namely the absorption of the dye bromothymol blue and the reduction of triphenyltetrazolium chloride. In subsequent studies, several factors were seen to vary simultaneously in the phase II variants. These included protease, lipase, intracellular crystalline proteins, antibiotic production, pigment production, and for *P. luminescens*, bioluminescence. It is noted however, that considerable variation occurred in several of these characteristics, even between Phase I cells and furthermore, for some species, the strains tested did not show variation in all characters.

Early in the study of phase variation, it was considered that the loss of a phage or plasmid might be the cause of the formation of the phase II forms. This has now been rendered unlikely for several reasons. A second possibility to explain phase variation that has been entertained is that some major DNA rearrangements have occurred. To this end Akhurst *et al* 1992, did extensive RFLP analysis of phase I and II variants of

Xenorhabdus spp. and concluded that no major DNA rearrangements had occurred in any of the phase II forms studied.

At present, it is fair to conclude that the genes are apparently intact but, by one mechanism or another, their gene products are not expressed, and neither the formation nor reversion of phase variation is yet understood. However, it is known that many bacteria, including those with pathogenic characteristics have to withstand various forms of environmental stress, such as high and low temperature, oxidative agents, high and low osmolarity, alkaline and acidic conditions and low oxygen supply. Therefore, most bacteria have developed mechanisms to adapt to these changes in their environment. Phase variation is a common mechanism in pathogenic bacteria to adapt to different environments and to escape the host immune system (Saunders 1986).

A recent study by Krasomil-Osterfeld (1997) suggested that a change in osmolarity could influence a phase-shift in *Photorhabdus luminescens*. The author studied the bacterial cell morphology within the infective juvenile *in vivo* with IJ's that were kept in tissue culture flasks in Ringer's solution at 4°C for at least three months. During storage lipid reserves of the IJ were reduced and the morphology of the bacterial cells resting within the intestines were visible: the cells were observed to be long and to never contain inclusion bodies. They were reported to resemble secondary-phase cells. After being released from the IJ and multiplying within the insect cadaver, the bacteria possessed inclusion bodies and obtained primary-phase characteristics. Therefore, phase variation can be assumed to enable *P. luminescens* to adapt to changing environmental conditions during it's symbiotic lifestyle, enabling the

bacteria to cope with high osmolarity and abundant nutrients within the insect prey during the reproductive stage and low osmolarity and starvation during dormant phases within the intestines of the IJ or outside in the soil environment.

This is further compounded by a study conducted by Smigielski *et al* (1994) who concluded that phase II forms were better suited to survival outside the symbiotic niche, being active with respect to cellular metabolism and respiration. This would fit with other reports that phase II cells forms grow faster than the phase I cells on defined media. Certainly, if the variety of secondary metabolites are not draining cellular metabolism, the phase II cell would be expected to be more competitive (than phase I cells) outside the symbiotic niche.

To this end it could be speculated that if phase II forms were common in soils or other environments they may have been very easily missed because the usual method for identification relies on those properties that are usually strongly expressed in phase I forms.

In many gram-negative bacteria, the transcription factor σS controls regulation that can mediate stress resistance, survival, or host interactions (Hengge-Aronis, 1993). The σS is encoded by the rpoS gene, in *Xenorhabdus* mutants, where the rpoS gene had been disrupted, the rpoS mutant could not colonise the inside of the nematode, thus was unable to contribute further to the symbiotic relationship between bacterium and nematode (Vivas & Goodrich, 2001). The rpoS mutant was shown to be as virulent as the wild type, therefore rpoS is not required to kill the insect host or to produce insecticidal toxins, but is required to colonise the entomopathogenic

nematode. It was concluded that the rpoS gene was expressed as a response to the environmental stresses, such as a decrease in nutrient availability, pH and osmolarity encountered within the nematode host.

One of the major characteristics of Phase I *Xenorhabdus* is the ability to swarm and swim on suitable solid media (0.6 to 1.2% agar) (Moureaux *et al,* 1995). Swarming is a specialised form of bacterial surface movement on solid media which has been observed in gram-negative and gram-positive genera including *Proteus, Vibrio, Bacillus, Clostridium* and *Serratia* (Smyth & Smith, 1992) and more recently in *E. coli* and *Salmonella typhimurium* (Harshey & Matsuyamna, 1994). A study by Givaudan *et al* (1995) purified the flagellar filaments from *X. nematophilus* and estimated the molecular mass by SDS-PAGE to be 36.5kDa, using optical and electron microscopy the authors showed that the Phase II variants had lost their flagella during the phase switch from Phase I. In a separate study it was shown that phase variation led to the differential transcription of two flaggelar genes. The FliC gene encoding for the flagellin and the FliD gene encoding a hook-associated protein (Givaudan *et al,* 1996). The structure of the FliC gene was shown to be the same in both Phase I and II, but it was revealed by Northern blot analysis that the FliC and FliD genes were not transcribed in the Phase II variant (Givaudan & Lanois, 2000).

1.5 Conclusions

Much of the research on entomopathogenic nematodes and bacteria has been carried out using *S. carpocapsae* and its symbiotic bacterium *X. nematophilus*. Characterisation of infection, including populations of bacteria and haemocytes and toxin production by *X. nematophilus*, and also Phase switching by *X. nematophilus* has been investigated with these symbionts as models.

S. feltiae (Strain UK76) in combination with its symbiotic bacterium, *X. bovienii,* is currently in use as a commercial integrated pest management pesticide within the UK, known as Nemasys™, produced by Microbio Ltd. UK. It has been assumed that the mode of action of this EPN is sufficiently similar to that of *X. nematophilus* to allow widespread use within both private and commercial agricultural environments. This study has attempted to investigate the basic population dynamics expressed with infection of *G. mellonella* with *S. feltiae*. Phase change of the symbiotic bacterium, *X. bovienii* has also been explored as has the production of a highly expressed 36.5kDa Phase I protein.

The populations of bacteria during a natural and injection infection of *Galleria* with *S. feltiae* were investigated by recording the number of colony forming units (CFUs) that grew on NBTA agar plates during the time course of infection and comparing the bacterial populations with the number of haemocytes from the same infected individuals. Through quantifying the amount of *Xenorhabdus* 16S rRNA present during the above mentioned time course of infection and comparing it to the amount

of general *Eubacteriaceae* 16S rRNA further insight into characterisation of the infection process within *S. feltiae* infected *G. Mellonella* was gained.

The mechanisms of Phase variation during an infection of *G. mellonella* with *S. feltiae* were investigated through following the expression of *Xenorhabdus* FliC and rpoS genes during a natural and injection infection. It was expected that the transcription of the FliC gene would occur during the early stages of infection when resources within the host were good and *Xenorhabdus* was not placed under environmental stress. It was thought that the reversion to Phase II would occur when resources within the host became scarce, towards the end of the infection process and that this would be highlighted by a reversion to Phase II and the up-regulation of transcription of the rpoS gene.

Further differences between Phase I and II were investigated using 2-dimensional gel electrophoresis, through isolation and characterisation of a 36.5kDa protein expressed during liquid culture by Phase I, and not by Phase II.

1.5.1 Aims

1. To investigate the population dynamics and effects on haemocyte numbers of *X. bovienii* during an infection of *G. mellonella* with *S. feltiae*.
2. To isolate and characterise two of the genes transcribed by the different phases, PI and PII, of *Xenorhabdus bovienii*.
3. To isolate and characterise a PI specific 36.5kDa protein using 2-Dimensional gel electrophoresis.

CHAPTER TWO

Materials and Methods

2.1 Culture of *X. bovienii*

2.1.1 Preparation of Growth Media

Phase I *X. bovienii* were grown in DSMZ recommended medium that contains: 10g Peptone, 5g Yeast Extract, 5g NaCl, dissolved in deinonized water (MQ) (pH to 7.2) and made up to 1l with MQ and autoclaved at 121°C for 15 minutes.

Phase II *X. bovienii* were grown in Low Osmolarity medium (Krasomil-Osterfield, 1995) that contains: 0.5g $NH_4H_2PO_4$, 0.5g K_2HPO_4, $MgSO_4.7H_2O$, 5.0g Yeast Extract (Oxoid), made up to 1l with MQ and autoclaved.

Both Phase I and Phase II *X. bovienii* were grown on NBTA, a selective medium consisting of: 1l of Nutrient agar (Oxoid) plus 25mg of Bromothymol Blue (BTB). The agar was autoclaved and cooled to 42°C prior to the addition of 40mg of Triphenyltetrazolium chloride (TTC). Cells were also cultured on MacConkey agar (Oxoid).

2.1.2 Maintenance of *Xenorhabdus* cultures

An ampoule of dried Phase I and an agar slant of Phase II *Xenorhabdus bovienii* (strains ATCC4766/ ATCC4767 respectively) were obtained from Deutsche Sammlung von Mikroorganismed und Zellkulturen GmbH (DSMZ). The cultures were re-hydrated and grown in DSMZ recommended media (No. 423. Xenorhabdus medium) as instructed by the supplier (DSMZ). Subsequent Phase II cultures were grown in a Low Osmolarity medium to maintain the secondary phase status. Cultures were incubated overnight (O/N) in an orbital shaker at 28°C and 200 rpm.

Growth was monitored by determining the optical density of the cell suspension at 600nm (OD_{600}).

17% Glycerol stocks were stored at -70°C.

2.2 Extraction and Purification of DNA

2.2.1 CTAB Genomic DNA Extraction

DNA was purified using a CTAB Extraction procedure adapted from Sambrook *et al,* 1989. 200ml cultures of *X. bovienii* raised from a frozen glycerol stock was grown overnight in DSMZ Xenorhabdus culture medium. Cells were concentrated by centrifugation at 4000 x g, at 4°C and the cell pellet was re-suspended in 5.67ml of TE.

The cell suspension was transferred to a 50ml teflon Nalgene® centrifuge tube and 0.5% SDS and 0.1mg/ml of Proteinase K were added. The cell suspension was incubated at 37°C until lysis had occured. 1000µl of 5M NaCl and 800µl CTAB solution (pre-heated to 65°c) were added and the lysate was incubated at 65°C for 10 minutes. The lysate was chloroform:iso-amyl alcohol (24:1) extracted and centrifuged at 10,000 X g for 15 minutes in a Sorvall® RC28S centrifuge at 4°C. The aqueous phase was transferred to a fresh Nalgene® tube and extracted with phenol/chloroform/iso-amyl alcohol (24:25:1) prior to recentrifugation at 10,000 X g. Nucleic acids were precipitated following the addition of 0.6 volumes of isopropanol and pelleted for 15 minutes at 10,000 rpm (Sorvall® RC28S centrifuge) at 4°C. The supernatant was aspirated and the pellet was washed with 75% ethanol and centrifuged for 15 minutes at 10,000 rpm. The ethanol was aspirated and the pellet of DNA was air dried, and taken up in 4mls TE and transferred to a Sterilin tube.

2.2.2 CsCl Gradient

4.3g of CsCl was dissolved in DNA solution in 4mls of TE (from section 2.2.1) and 200µl of EtBr was added (from 10mg/ml stock). The solution was transferred to a Beckman polyallomer quick-seal 13x51mm centrifuge tube and centrifuged overnight at 55,000rpm using a Beckman Vti 60 rotor in a Beckman L7-55 ultracentrifuge at 20°C. The DNA band was visualised under UV radiation (305nm) and extracted from the Beckman tube using a 21 gauge needle into a clean microfuge tube. Two volumes of TE buffer were added followed by 200µl of TE saturated butanol. EtBr was extracted by

gentle mixing and then the tubes were centrifuged for 2 minutes at 13,000 rpm (Eppendorf centrifuge 5414C). The top organic layer was discarded and this extraction step repeated until all of the EtBr had been removed. 0.6 volumes of isopropanol were added to the aqueous phase and the DNA pelleted by centrifugation for 15 minutes, 13,000g. The supernatant was aspirated and the pellet was washed in 1ml of 75% EtOH (-20°c) re-pelleted at 13,000g for 5 minutes. The supernatant was aspirated and the pellet left to dry at room temperature. DNA was taken up in either MQ or TE and its integrity and purity was verified by electrophoresis through a 1% w:v TE/agarose gel.

2.2.3 Extraction of Plasmid DNA

A 1.5ml aliquot of an overnight culture of transformed *E. coli* TOP10F' One Shot® (Invitrogen®) cells grown in LB containing 50µg/ml of ampicillin were pelleted by brief centrifugation at 13,000g. Plasmid DNA was extracted using the QIAprep® Spin Miniprep (Qiagen) kit according to the manufacturers recommendations. The plasmid DNA was eluted in 50µl of MQ.

2.3 Manipulation of DNA

2.3.1 Restriction Digest

Confirmation that putative recombinants carried an insert of the expected size was carried out by digesting purified plasmid DNA with the restriction enzyme EcoR1.

Digests were performed as follows: Plasmid: 2µl, Buffer H (Roche): 2µl, Sterile MQ: 15µl, Restriction Enzyme (Eco-R1): 1µl (10U). The digestion was carried out at 37°c for 1-3 hours. The results were checked by electrophoresis through an agarose/TAE gel (see section 2.3.2).

2.3.2 Agarose Gel Electrophoresis

Ultrapure agarose (SeaKem GTG) or low melting point (LMP) agarose (SeaKem NuSieve) at a concentration of 0.6% - 2% was dissolved with 1 x TAE at an appropriate volume, by heating to boiling point in a microwave oven. The solution was then cooled and ethidium bromide (EtBr) was added from a 10mg/ml stock to a final concentration of 0.5µg/ml. The solution was then poured into a gel former, and allowed to solidify at room temperature, before being placed in a gel rig and submerged in 1x TAE buffer, (40mM Tris-acetate, 1mM EDTA pH 8.0). Samples were loaded in 6x bromophenol blue loading buffer, (sucrose 4g, bromophenol blue 2.5g in 10 mls TE), to give 1x concentration.

2.3.3 Recovery of DNA from agarose gels

Individual restriction fragments for sub-cloning were identified and isolated from agarose gels by illuminating the gel with UV light from below, (using a UVP trans-illuminator). Bands were excised from the gel (maximum volume of 300mg) with a sterile razor blade and transferred to a sterile microfuge tube for purification with the Hybaid Recovery™

DNA Purification Kit II according to the manufacturer's recommendations. DNA was eluted in 10-25µl of elution solution (sterile water, Hybaid).

2.3.4 Polymerase Chain Reaction (PCR) and Production of DIG labelled probes

DNA purified by the CsCl density gradient centrifugation was amplified by the PCR in a Techne Cyclogene Dri-Block® Thermal Cycler. Reactions were carried out using the *Taq*PCR Master Mix Kit (Qiagen) in a final reaction volume of 50µl in sterile 500µl microfuge tubes and consisted of: 25µl of PCR Master Mix, 1.5-3.5mM Mg^{2+}, 50 pmoles of each forward and reverse oligonucleotide primer and 2µl of template DNA (~100ng), with the volume made up to 50µl with sterile MQ.

The standard cycling conditions were as follows:
Initial single denaturing step: 95°C for 2 minutes
30 cycles of: denaturing at 94°C for 1 minute
annealing at variable temperature for 1 minute
extension at 72°C for 1 minute
A final extension step of 72°C for 20 minutes
The reactions were then cooled to 4°C.

Successful amplification of PCR products was checked on a TE/agarose gel and fragments of the expected size were isolated from a low melting point TE/agarose gel using the Hybaid recovery kit. Where the purity of the PCR product was high, gel purification was omitted prior to insertion in the T-tailed vector PCR® 2.1-TOPO (Invitrogen®).

A PCR DIG probe synthesis kit (Roche) was used to produce directly labelled Digoxygenin probes according to the manufacturers protocol. Various primers were utilised for amplification depending on the required product using the thermocycle reaction conditions described above.

2.3.5 Plasmid construction

2.3.5.1 Insertion of DNA fragments into the plasmid vector pUC18

pUC18 was the plasmid vector most commonly used for cloning genomic DNA fragments and was digested with the same or compatible enzyme(s) as the genomic DNA. Digests were run out on a LMP gel adjacent to the digested genomic DNA and both were gel purified as described in Section 2.3.3. When single restriction enzymes were used, pUC18 was treated with alkaline phosphatase to prevent self-ligation of the linearised plasmid. Alkaline phosphatase was diluted 1 in 10 in MQ, and 1µl of the diluted enzyme was added to 26µl of linearised gel purified pUC18 and 3µl of alkaline phosphatase buffer (Roche). The reaction mix was incubated at 37°C for 30 minutes, and then extracted with phenol/chloroform, ethanol precipitated and dried before addition to the ligation reaction. Ligations with T4 DNA ligase (Roche) were carried out using the Rapid DNA Ligation kit using an insert:vector ratio of 3:1 as recommended by the manufacturer (Roche).

2.3.5.2 Insertion of PCR Amplified Fragments into pCR®2.1

The recovered PCR product was inserted into pCR® 2.1-TOPO using the TOPO Cloning Kit (Invitrogen®). Reactions were incubated at room temperature for 5-10 minutes and the ligation mix was used to transform TOP10F' One Shot® (Invitrogen®) competent cells by adding 2µl of the ligation reaction to 50µl of the competent cells and incubating on ice for 30 minutes. The cells were heat shocked at 42°C for exactly 30 seconds before the addition of 250µl of SOC medium. The cells were then incubated in an orbital incubator at 37°C and 225rpm for one hour.

50µl and 200µl of the cell suspension were plated on LB plates (LB broth (Difco Bacto tryptone 10g/l, Difco Bacto yeast extract 5g/l, NaCl 10g/l) and 1.5% agar) containing 50µg/ml ampicillin and 40µl of 40mg/ml X-gal. The plates were incubated overnight at 37°C and recombinant colonies were selected for further investigation.

2.3.6 Southern blot analysis and hybridisation

2.3.6.1 Preparation and blotting of agarose gels

Prior to Southern transfer, gels were incubated with gentle agitation in depurination solution (0.25M HCl) for 10 minutes at room temperature. This was followed by two 30

minute washes in denaturing buffer, (1.5M NaCl, 0.5M NaOH), and then in neutralising buffer, (1.5M NaCl, 0.5M Tris pH7.5).

DNA was transferred to a positively charged nylon membrane (Roche) under vacuum (LKB Bromma 2016 Vacugene vacuum blotting unit, and a Hybaid blot processing pump) in 10x SSC (1x SSC is 0.15M NaCl, 15mM NaCitrate, pH7.5). The membrane was air-dried and the DNA immobilised by 15 seconds exposure to UV radiation (305nm) on a UVP transilluminator.

2.3.6.2 Pre-hybridisation and hybridisation

Pre-hybridisation and hybridisation were carried out in 5x SSC, 1% Blocking reagent (Roche), 0.1% Sarkosyl and 0.02% SDS at 68°C unless stated otherwise. The membrane was pre-hybridised for 1 hour in a pre-warmed Hybaid mini hybridisation oven, CAL 9000, and a HB-OV-B5 glass hybridisation tube. 0.5–2µl (5–10ng/ml) of DIG-labelled probe was denatured in 500µl hybridisation solution by heating to 100°C for 10 minutes and added to 10ml of fresh pre-heated hybridisation solution. The pre-hybridisation solution was discarded and replaced with the fresh solution and the membrane was hybridised overnight.

2.3.6.3 Post-hybridisation washes and detection of hybrids

The hybridisation solution was decanted and the membrane transferred to a clean plastic sandwich box. The membrane was stringency washed at room temperature for 2x 5

minutes in 200ml 2x SSC/0.1%(w/v) SDS followed by two 30 minute washes at 68°C at an appropriate stringency in a Techne Hybridiser HB-1 oven. The stringency wash solution was decanted and the membrane was rinsed in maleate buffer (100mM maleic acid, 150mM NaCl, pH7.5) at room temperature for five minutes before being incubated for 30 minutes in 1%(w/v) blocking reagent (Roche) in maleate buffer. This 30 minute incubation was repeated with fresh blocking solution containing anti-digoxygenin-AP, (1:5000). Non-specifically bound antibody was removed by washing for 3x 20 minutes in maleate buffer amended with 0.3% Tween 20. The membrane was then equilibrated in detection buffer 3 (0.1M NaCl, 50mM $MgCl_2$ and 0.1M Tris pH9.6) prior to detection of hybrids by one of the following procedures.

2.3.6.4 Colourimetric detection

The membrane was transferred to a transparent hybridisation bag and 45µl/10ml of X-phosphate (50mg/ml 5-bromo-4-chloro-3-indolylphosphate, toluidium salt in 100% DMF (Roche)) and 35µl/10ml NBT (75mg/ml nitroblue tetrazolium salt in 70% (w/v) DMF (Roche)) were added to 0.5–2ml of detection buffer 3, and the bag was sealed. The membrane was incubated in the dark at room temperature until the colour reaction had developed sufficiently. The reaction was stopped by soaking the membrane in TE (10mM Tris, 1mM EDTA, pH8.0) for 10 minutes, before air-drying.

2.3.6.5 Chemiluminescent detection

The membrane was allowed to equilibrate in the dark for five minutes in a solution of 0.5% CDP-*Star™* (Roche) in Buffer 3 (0.1M NaCl, 0.02M Tris.CL pH9.6). The membrane was blotted briefly on 3MM paper and sealed in a transparent hybridisation bag (Roche). This was exposed to Biomax™ML double emulsion film (Kodak) in a cassette for 30 minutes to 3 hours. The film was developed for three minutes and fixed for two minutes using Kodak GBX reagents. It was then washed in tap water and allowed to air dry.

2.3.7 Sub-genomic library screening

Restriction digests of *Xenorhabdus bovienii* genomic DNA were electrophoresed through a 1% - 2% LMP agarose gel adjacent to lambda DNA Hind III markers. The region of interest was cut from the gel as described in Section 2.3.4 and ligated into pUC18 as described in Section 2.3.5.1, and transformed into competent *E. coli* cells as described in Section 2.3.5.1.

Colonies were transferred to circular nylon membranes following the manufacturers protocol (Roche). The membrane was placed on the surface of the agar plate and left for 1–2 minutes. The orientation of the membrane was marked using a needle, and the colonies were lifted smoothly off the plate. The membrane was transferred colony side up to 1ml of denaturing buffer (1.5M NaCl, 0.5M NaOH) on cling film, and incubated for 15 minutes. The membrane was blotted briefly on 3MM paper and transferred to 1ml of neutralising buffer (1.5M NaCl, 0.5M Tris pH7.5) for 15 minutes. Following blotting,

the membrane was transferred to 1ml 2x SSC for 10 minutes, blotted and allowed to air dry before irradiation with UV (305nm) for 15 seconds to bind the released DNA to the membrane. The membrane was then transferred colonies face down to 1ml of 2x SSC/10% proteinase K spread on aluminium foil. The aluminium foil was sealed around the membrane and the blot was incubated at 37°C for 1 hour. The membrane was then removed and placed on a tray between 2 pieces of 3MM paper wetted with MQ and a bottle was rolled firmly over the sandwiched membrane 3 or 4 times. This procedure removes the digested cell debris from the membrane whilst leaving the bound DNA. The membrane was again blotted on 3MM paper and allowed to briefly air dry. It was then pre-hybridised and hybridised according to the protocol for Southern blots (Section 2.3.6.2). Post hybridisation and detection was carried out as for Southern blots (section 2.3.6.3) and colourimetric detection was used to detect positive hybrids (section 2.3.6.4).

2.3.8 Automated DNA Sequencing

Automated DNA sequencing was carried out with high quality plasmid DNA using the Perkin Elmer Big Dye™ PCR kit. Single stranded plasmid DNA was amplified by linear PCR in a Perkin Elmer Gene Amp PCR system 2400 thermocycler. The reaction volume used was 20µl in sterile, dome lidded 200µl microfuge tubes as follows: 2µl (100ng) plasmid DNA, 3.2pmoles of M13 primer, 8µl Big Dye™ dilution mix (5 x dilution of Big Dye™ in Tris buffer (200mM Tris/HCl, 5mM $MgCl_2$ pH9.0).

A separate reaction was carried out for the forward and reverse primers as follows: denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, extension at 60°C for 4 minutes. The sequencing gel was run on an ABI prism™ 377 DNA sequencer.

2.3.9 Manual Sequencing

The dideoxy-chain termination method (Sanger *et al*, 1977) using the T7 Sequenase v2.0 kit (Amersham Life Sciences) was used to carry out the manual DNA sequencing reactions according to the manufacturers instructions.
Ultra pure Sequagel complete buffer reagent and Sequagel XR (National Diagnostics) were used to prepare a 6% polyacrylamide gel on an Anachem Origo sequencing rig (model v4-11) according to the manufacturers instructions.

2.3.10 Sequence analysis

Automated sequencing output was analysed using Abiview software (University of Leicester). The edited sequence was subjected to a BLAST-X database search using the BLAST Network Service (www.ncbi.nlm.nih.gov/) at the National Centre for Biotechnology Information (NCBI). Alignment of the analysed sequence with published sequences was performed using Clustal X.

2.4 Primer Design

2.4.1 Primer Design

Primers for PCR were designed through carrying out an analysis using Clustal X, of existing fragments of sequences of interest from the BLAST database.

For isolation of the 16S rRNA region from *X. bovienii*:

Eubact 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3'

Eubact 1492R 5'- GGT TAG CTT GTT ACG ACT-3'

To give a fragment of 1465bp.

For isolation of the rpoS sigma factor region from *X. bovienii*:

RpoS2F: 5'-AC(AGCT) TA(CT) GC(AGCT) AC(AGCT) TGG TGG-3'

RpoS2R: 5' TG(CT) TTC AT(AG) TC(AG) TC(AG) TC(CT)-3'

To give a fragment of 225bp.

For isolation of the FliC flaggelar region from *X. bovienii*:

FliCF: 5'-AA(AG) GA(CT) GA(CT) GC(AGCT) GC(AGCT) GG(AGCT) CA-3'

FliCR: 5'-TC(AGCT) AC(AGCT) GC(AG) TA(AG) TC(AGCT) GC(AG) TC-3'

To give a fragment of 725bp.

All Primers were diluted to 100pmoles/µl unless otherwise stated.

2.5 Extraction of RNA

Where possible all solutions and glassware used for RNA work were treated with 0.1% (v/v) di-ethyl pyrocarbonate, 1µl/ml, (DEPC) followed by autoclaving. Solutions containing Tris were prepared in DEPC-treated water and re-autoclaved.
Pipette tips and microfuge tubes for use with RNA were kept separate.

2.5.1 RNA Extraction of Bacterial Cells for Northern Slot Blots

Bacterial cultures were grown until they reached mid-log phase. 30ml of the overnight culture was pelleted at 4000 x g for 10 minutes at 0°C. The cells were re-suspended in 500µl of RNA extraction buffer (100mM LiCl, 50mM Tris pH 7.5, 30mM EGTA, 1% Sodium SDS) and the cell suspension was heated at 65°C until the cells had lysed.

The cell suspension was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube. The aqueous phase was chloroform-isoamyl alcohol (24:1) extracted and 0.1 volumes of sodium acetate and 2.5 volumes of 100% ethanol was added. The RNA was precipitated from solution at –20°C for at least 30 minutes followed by centrifugation at 13000rpm for ten minutes. The supernatant was aspirated and the pellet was washed in 70% ethanol and the pellet was allowed to air dry.

The pellet was dissolved in 200µl of DNase buffer (100mM NaAcetate and 10mM $MgCl_2$ pH 5.6) and1µl of DNase (Roche) was added and the digest was incubated for 60 to 90 minutes at 37°C to hydrolyse the DNA. The digest was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube. The aqueous phase was chloroform-isoamyl alcohol (24:1) extracted and 0.1 volumes of sodium acetate and 2.5 volumes of 100% ethanol was added. The RNA was precipitated from solution at –20°C for at least 30 minutes followed by centrifugation at 13000rpm for ten minutes. The supernatant was aspirated and the pellet was washed in 70% ethanol and the pellet was allowed to air dry. The pellet was taken up in DEPC-MQ.

The purity of the RNA was examined on an agarose/TAE gel against λHind III markers, to check for DNA contamination.

The concentration of the RNA was estimated by determining the OD at 260nm assuming that one OD unit is equivalent to 40µg/ml of RNA.

2.5.2 RNA Extraction of Bacterial Cells for RT-PCR

Total RNA for RT-PCR was extracted as above (Section 2.5.1) except that Lithium SDS was used in the extraction buffer rather than sodium SDS. To further purify the RNA following standard RNA extraction, an equal volume of 4M NaCl was added to the RNA dissolved in MQ and the mixture was held on ice overnight. The sample was centrifuged

for 10 minutes at maximum speed in a bench top centrifuge, the supernatant was discarded and the pellet was air-dried and taken up in MQ and ethanol precipitated again.

Total RNA was quantified by determining the absorbance at 260nm, (A_{260}) and the integrity of samples was verified by electrophoresis in ethidium bromide-stained non-denaturing agarose gels.

2.5.3 RNA extraction of Total RNA from G. mellonella

Ten *Galleria mellonella* weighing ~3 grams were frozen at –70°c and ground with a pre-chilled pestle and mortar (-70°C). The caterpillars were reduced to a powder and while still cold 20mls of tissue guanidinium solution (5M guanidinium isothiocyanate, 50mM Tris.Cl pH 7.5, 10mM Na_2EDTA pH 8.0, 0.72M ß-mercapthoethanol) was added and ground to mix. The homogenate was transferred to a 50ml Greiner tube and centrifuged for 20 minutes at 4000rpm at 5°C. The supernatant was collected, 20µl of 20% Sarkosyl was added and the suspension was heated for 2 min at 65°C. The solution was returned to ice and 2ml of 2M sodium acetate, pH 4, 20ml of water-saturated acid phenol and 8ml of 49:1 chloroform/isoamyl alcohol were added and mixed thoroughly by inversion and incubated for 15 min at 0°C - 4°C. The suspension was centrifuged for 25min at 5000 x g at 4°C and the aqueous phase transferred to a fresh Greiner tube. An equal volume of 100% isopropanol was added and the extracts were incubated at –20°C for at least 30min to precipitate the RNA. The precipitate was centrifuged for 20min at 4000x g at 4°C and the supernatant discarded. The resulting pellet was dissolved in 300µl of denaturing

solution (4M guanidinium isothiocyanate, 25mM sodium citrate pH7, 0.1M ß-mercapthoethanol, 0.5% *N*-lauroylsarcosine) and transferred to a 1.5ml-microfuge tube. The precipitation of RNA was repeated with an equal volume of isopropanol and the microfuge tubes were centrifuged for 10 min at 10,000g at 4°C. The pellet was re-suspended in 75% ethanol, vortexed, and incubated at room temperature for 10–15 minutes to dissolve residual amounts of guanidinium remaining in the pellet. The microfuge tubes were recentrifuged, the ethanol aspirated and the RNA pellet allowed to dry.

The pellet was dissolved in an appropriate volume of DNase buffer (100mM NaAcetate and 10mM $MgCl_2$ pH 5.6), an equivalent volume of DNase (Roche) was added and the digest was incubated for 60 to 90 minutes at 37°c to hydrolyse the DNA. The digest was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube. The aqueous phase was chloroform-isoamyl alcohol (24:1) extracted and 0.1 volumes of sodium acetate and 2.5 volumes of 100% ethanol was added. The RNA was precipitated from solution at –20°C for at least 30 minutes followed by centrifugation at 13000rpm for ten minutes. The supernatant was aspirated and the pellet was washed in 70% ethanol and the pellet was allowed to air dry. The pellet was taken up in DEPC-MQ.

The purity of the RNA was examined on an agarose/TAE gel against λHind III markers, to check for DNA contamination.

The concentration of the RNA was quantified by spectrophotometry at 260nm assuming that one OD unit is equivalent to 40μg/ml of RNA

2.6 Manipulation of RNA

2.6.1 Northern Slot Blots

All hybridisation tubes and boxes and the Bio-dot (Bio-Rad) slot blotting apparatus were soaked overnight in 0.2M NaOH, and then rinsed thoroughly in MQ.

Stock solutions to yield appropriate concentrations of RNA were diluted in RNA loading buffer (500μl formamide; 162μl formaldehyde; 100μl MOPS buffer, pH 7.0 (0.2M MOPS; 0.5M NaAcetate; 0.1M EDTA) and denatured for 15 minutes at 65°C. Following this, 2 x volume of ice cold 20 x SSC was added to each sample. RNA of the appropriate concentrations was transferred to positively charged nylon membranes (Roche) using a Hybaid blot processing pump and a Bio-dot blotter (Bio-Rad). The membrane was washed with DEPC-MQ prior to application of the RNA and with 2 x SSC post application. The membrane was dried at room temperature and the RNA was fixed to the membrane by exposure to UV radiation (305nm) for three minutes.

2.6.1.1 Pre-hybridisation and hybridisation:

Pre-hybridisation and hybridisation were carried out in Dig Easyhyb™ (Roche). The membrane was pre-hybridised for an hour in a pre-warmed mini hybridisation oven

(CAL 9000) and a HB-OV-B5 glass hybridisation tube. Fresh hybridisation solution containing 25pmoles of DIG-labelled oligonucleotide probe (see section 2.6.1.5) was added and the membrane hybridised overnight.

2.6.1.2 Post-hybridisation washes:

Unless stated washes were conducted at room temperature.

The membrane was washed twice for five minutes in 200mls of 2 x SSC, 0.1% (w/v) SDS followed by two 30 minute washes of appropriate stringency and temperature in a Techne Hybridiser HB-1 oven. The membrane was rinsed in maleate buffer (100mM maleic acid, 150mM NaCl, pH 7.5) before transfer to 1% (w/v) blocking reagent (Roche) in maleate buffer for a 30 minutes. Fresh blocking solution was amended with anti-digoxygenin-AP FAB fragments (Roche), (1:5000) and the membrane incubated for a further 30 minutes. Finally the membrane was washed for three x 20 minutes in maleate buffer brought to 0.3% Tween 20. This was followed by equilibration of the membrane in Buffer 3 (0.1M NaCl, 0.1M Tris, pH 9.6) for five minutes prior to the detection procedure.

2.6.1.3 Chemiluminescent detection

Detection was carried out as detailed in Section 2.3.6.5.

2.6.1.4 Quantification of Northern slot blot results:

The developed film was scanned to disk and saved as a TIFF file and analysed using the Gel Works ID (UVP) software package.

2.6.1.5 Dig-labelled Oligonucleotide Probes

All probes were diluted to a concentration of 100pmoles/µl, unless otherwise stated.

XEN2: 5'-ATC AAC AGC GCT ATT TAC GCT-3'

Following analysis of the automated sequence output from *X. bovienii* an oligonucleotide probe was designed from the variable 16S rRNA region. The oligonucleotide was manufactured and labelled at the 5' prime end with Digoxygenin by MWG Biotech.

EUB338: 5'-GCT GCC TCC CGT AGG AGT-3'

Designed by Amann, R.I. *et al* (1990) from conserved regions of the 16S rRNA gene as a universal Eubacteriaceae probe. The oligonucleotide was manufactured as above.

2.6.1.6 Optimisation of hybridisation conditions for Dig-labelled probes

A number of different hybridisation temperatures and post-hybridisation wash stringency's were tried. The temperature/stringency that resulted in the strongest signal

for the positive RNA loadings complemented by the weakest signal from the negative loadings was chosen as the optimal conditions. This was carried out for both XEN2 and EUB338 probes.

Once optimal conditions had been ascertained for both probes, Northern slot blots were constructed to contain loadings of the total RNA extracted from *G. mellonella* over a time period of 0-72 hours (section 2.8). This was carried out for *Galleria* infected both naturally and by the injection method.

2.6.1.7 Construction of Time Series Slot Blots

20μg/well of RNA for each time point (section 2.9) was loaded onto the Slot Blot manifold. In addition uninfected caterpillar and *E. coli* 16S rRNA were used as negative controls and loadings of *X. bovienii* 16S rRNA as a positive control.

2.6.2 RT-PCR

RNA from *Xenorhabdus bovienii* and the time series of infected *Galleria mellonella* were subjected to analysis by RT-PCR. RT-PCR was performed with 0.1–10ng RNA using the Qiagen® OneStep RT-PCR kit following the manufacturers instructions. Primers for the two genes of interest (rpoS and FliC) were utilised (section 2.4.1).

For the rpoS and FliC primers the thermal cycler program was set as follows: 50°C for 30 minutes for reverse transcription followed by 15 minutes at 95°C to inactivate the reverse transcriptase and activate the HotStar Taq DNA polymerase. 30 cycles of a three step cycle were then performed each being 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C. A final extension of 72°C for 10 minutes was performed before the samples were cooled and held at 4°C.

Products were electrophoresed on a 2% agarose gel.

2.7 Extraction of Protein

2.7.1 Extraction of Protein from Bacterial Cells

PI and PII *X. bovienii* were incubated in their respective media until reaching an OD_{600} of 0.8. 15mls of each culture were pelleted and the supernatant removed, the cells were washed three times in 0.1M Tris, pH 8.0. The cells were lysed by re-suspension in 120µl of lysis buffer (0.01M Tris-HCl, Ph7.4, 1mM EDTA, 8M Urea, 0.05M DDT, 10% (v/v) glycerol, 5% (v/v) NP40, 6% (w/v) ampholytes) and incubated for an hour at room temperature. The cellular debris was removed by centrifugation at 13,000 rpm in a bench top centrifuge. 100µl of the lysate was purified using the 2-D Clean-up Kit (Pharmacia) according to the manufacturers instructions. Concentrated protein was resuspended in 237.5µl of re-hydration solution (8M Urea, 2% (w/v) NP-40, IPG buffer (Amersham Pharmacia), a few grains of Bromophenol blue) and 12.5µl of 1M Dithiothreitol (DTT).

2.8 Manipulation of Protein

2.8.1 2-Dimensional Gel Electrophoresis

Re-hydration solution containing soluble protein was applied to an Immobiline™ DryStrip Tray (Amersham Pharmacia) in the Immobiline™ DryStrip Reswelling Tray (Amersham Pharmacia) covered with IPG Cover Fluid (Amersham Pharmacia) and incubated overnight at room temperature.

The first dimension, isoelectric focusing (IEF) was performed on the Mutiphor™ II (Amersham Pharmacia) following the manufacturers instructions. Cooling at 20°C was established using a LVF6 low temperature circulator (Grant Instruments).

Following IEF, the Immobiline™ DryStrips were incubated for 15 minutes on a rocking platform in SDS equilibration buffer (50mM Tris-HCl, pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of bromophenol blue, plus 100mg/10ml of DTT added just prior to use).

The proteins were subsequently separated according to apparent molecular mass on a 10% SDS-PAGE gel (1.5M Tris.Cl pH 8.8, 0.4% SDS, 10% poly-acrylamide) at 300 volts using a Bio-Rad Protean™ II vertical gel unit and a Bio-Rad Power Pac 300 until the dye front was 1mm from the bottom of the gel. The proteins were run with Mark 10 (Invitrogen) markers as a molecular weight reference.

The SDS-PAGE gels were stained overnight with 0.1% Coomassie Brilliant Blue (0.1% (w/v) Coomassie Brilliant Blue, 10% Acetic Acid, 30% Methanol) and de-stained (10% Acetic acid, 30% Methanol) for at least 48 hours. For better definition gels were also Silver stained using the Silver Stain plus Kit (Bio-Rad) according to the manufacturers' instructions.

2.8.2 Protein sequencing

Second dimension separation according to molecular mass was performed using pre-cast 1.5mm NOVEX Bis-Tris 10% (Invitrogen) gels at 200V using a NOVEX vertical gel unit (Invitrogen) and a Bio-Rad Power Pac 300. Gels were stained with SimplyBlue Safe Stain (Invitrogen) according to the manufacturers instructions.

The protein spot of interest was excised from the gel and transferred to a sterile microfuge tube. The sample was transferred under ice to the proteomics unit at the University of Dundee Welcome Trust Centre, Dundee. A trypsin digest was performed on the sample followed by MALDI-TOF analysis.

2.9 Time series of infection of *G. mellonella* with *S. feltiae*

2.9.1 Maintenance of *G. mellonella*

Galleria were obtained from www.livefoodsdirect.co.uk in batches of 250. The *Galleria* were stored at 15°C without food for not more than one week. If not used within this time they were discarded.

2.9.2 Maintenance of S. feltiae

Nematodes were kindly provided by Roma Gwyn at Microbio™, UK. The nematodes were stored, as per manufacturer's instructions, at 4°C. To extract the nematodes from their storage compound, a modified version of the White Trap (Kaya & Stock, 1997) was employed. The lid of a 7cm petri dish was prepared to contain a circle of filter paper (Whatman) moistened with distilled water. The petri dish was floated on 20mls of distilled water contained in a 15cm petri dish. One gram of storage compound, containing the nematodes, was transferred to the 7cm petri dish and was incubated overnight at room temperature. During this time the nematodes migrated out of the storage compound and into the distilled water in the 15cm petri dish. The nematodes were collected from the distilled water by allowing them to settle to the bottom of a 50ml centrifuge tube (Greiner).

2.9.3 Sterilisation of *S. feltiae*

S. feltiae were surface sterilised by incubation in sterilising solution (0.1% (w/v) Thimerosal, 5mg/ml Streptomycin) for 3 hours, as described by Kaya & Stock, 1997. The nematodes were then quadruple rinsed in sterile distilled water. The wash water from each rinsing stage was checked for contamination by plating out 250µl on NBTA agar plates and incubating at 28°C for 72 hours. If no growth occurred the nematodes solution was deemed sterile.

The nematodes were stored at 4°C in sterile Ringer's solution (NaCl, KCl, CaCl, NaH_2CO_3) until required.

2.9.4 Injection infection of *G. mellonella* with *S. feltiae*

Galleria were swabbed with 100% ethanol and injected using a sterile Hamilton syringe with 10µl of sterile Ringer's containing 100 surface sterilised nematodes. As a control, one caterpillar for each time point was sham injected with sterile Ringer's containing no nematodes.

Every 2 hours for the first 12 hours and for 18, 24, 36, 48 and 72 hours following injection 13 *Galleria* were sampled. 10µl of haemolymph from 3 *Galleria* was added to 990µl of ice-cold Ringer's containing a few grains of PTU/E, to prevent melanisation. 50µl was plated onto NBTA plates and incubated at 28°C for 48 hours. The number of

colonies on each plate was counted and colony colour/morphology was noted; photographs of one plate from each time point were taken as a record. The remaining 10 *Galleria* were placed at –70°C for no more than 72 hours until the RNA was extracted (section 2.5.2).

2.9.5 Natural infection of *G. mellonella* with *S. feltiae*

Galleria were incubated on moist filter paper (Whatman) in 7cm petri dishes. The filter paper was moistened with a nematode solution containing either 100 or 1,000 nematodes/caterpillar. It was assumed that 10% of the applied number of nematodes would successfully penetrate the host, resulting in an infection rate of 10 or 100 nematodes/caterpillar, respectively.

13 *Galleria* were sampled at 12, 24, 48 and 36 hours after exposure to the nematodes. Haemolymph was extracted from 3 caterpillars and subsequently treated and plated as previously described (section 2.9.4). The remaining 10 *Galleria* were stored at –70°C for no more than 72 hours until the RNA was extracted (section 2.5.2).

CHAPTER THREE

Time series of Injection and Natural infection of *G. mellonella* with *S. feltiae*: Comparison of natural versus injection infection.

3.1 Introduction

To determine how the population of *X. bovienii* changes within *G. mellonella* infected with *S. feltiae* a time series was performed. Infected *Galleria* were sampled from two to 72 hours following infection with *S. feltiae*, two means of infection were investigated: injection infection, where the infective nematodes were injected directly into the *Galleria* haemocoele, and natural infection where the infective nematodes were allowed to enter the *Galleria* through natural openings, such as the mouth and anus and spiracles (Simoes & Rosa 1996).

At specified time points (Sections 2.9.4 and 2.9.5) during both natural and injection infection samples of haemolymph were taken from the haemocoele, a portion of which was plated onto NBTA agar plates in order to ascertain numbers of colony forming units (CFU) in the haemolymph during the course of infection. The remainder of the haemolymph was used in order to conduct haemocyte counts, as it has been reported that the number of haemocytes present in the circulation system would fluctuate throughout the time series of infection (van Sambeek & Weisner, 1999).

During the injection infection time series particular attention was attributed to the population fluctuations at the beginning of the infection, with sampling occurring every two hours for the first 12 hours, *Galleria* were also sampled at 18, 24, 36, 48 and 72 hours after infection.

During the natural infection time series, which was thought to result in a more stable infection, samples were taken at times 0, 12, 24, 36, 48 and 72 hours after infection. The natural infection time series was further investigated by infecting the *Galleria* with different numbers of nematodes, either 100 or 1000 nematodes per *Galleria*, which, during a natural infection is thought to result in actual infection of 10 or 100 nematodes per *Galleria*, respectively (Kaya & Stock 1997 Westerman, 1998), as only a certain percentage of the nematode population will invade the host.

It was thought that a resulting natural infection of 10 nematodes per larvae would be a fair representation of the infection process within the natural environment, where the nematodes would encounter the host in a random manner. Whereas a natural infection of 100 nematodes per larvae was likely only to occur following application of large numbers of nematodes into the environment, as takes place when the nematodes are used as a biological control agent

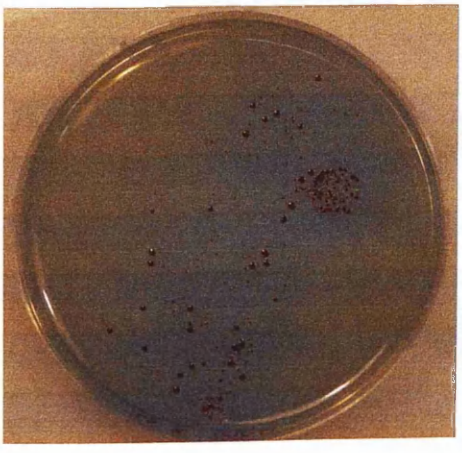
3.2 Injection infection time series

3.2.1 Injection infection time series with 100 *S. feltiae/Galleria*

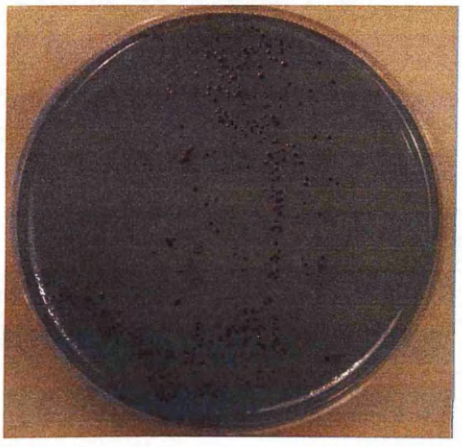
G. mellonella were injected with 100 *S. feltiae*/caterpillar, as described in Section Two (Section 2.9.4). Ten infected caterpillars from each time point were retained for total RNA extraction (Section 4.3). At each time point the haemolymph from ten caterpillars was collected, diluted and plated on to NBTA agar and retained to perform haemocyte counts, as described in Section Two (Section 2.9.4).

The growth on NBTA agar plates from each time point was recorded by digital photography (Figure 3.1) and a plot of the mean number of colony forming units (CFU) against time after infection was constructed (Figure 3.2).

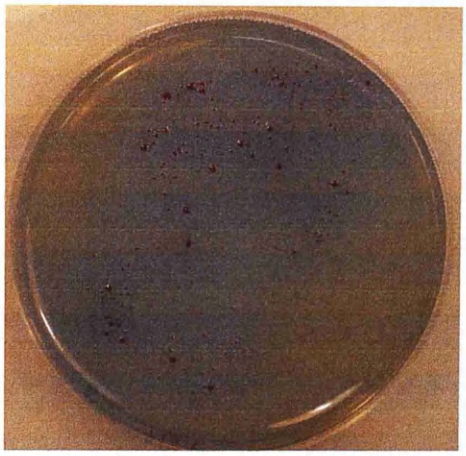
The number of haemocytes at each time point was recorded and a graphical representation of the mean number of haemocytes against time after infection was plotted (Figure 3.3).



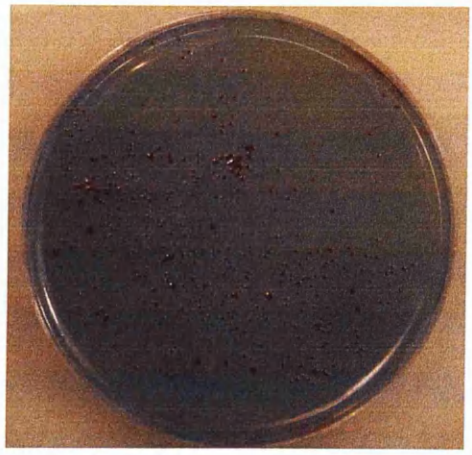
2 hours following injection



4 hours following injection



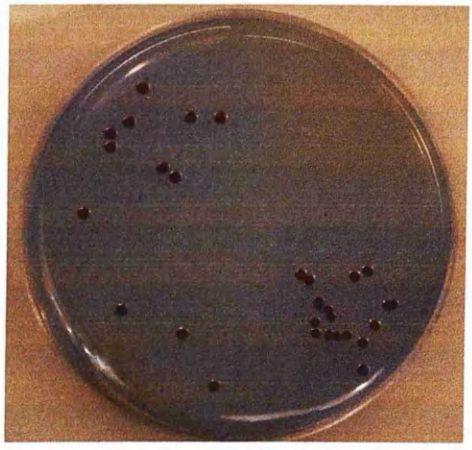
6 hours following injection



8 hours following injection

Figure 3.1 (continued)

10 hours following injection



12 hours following injection

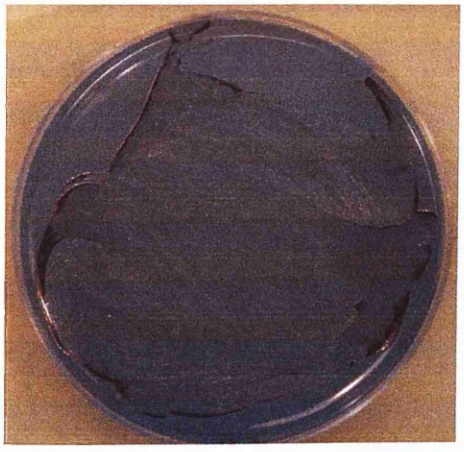
18 hours following injection

24 hours following injection

Figure 3.1 (continued)

36 hours following injection

48 hours following injection



72 hours following injection

Figure 3.1 NBTA agar plates after 48 hours of growth at 28°C showing the number of CFU from *G. mellonella* haemolymph following injection infection with 100 *S. feltiae*/caterpillar over the time period 2, 4, 6, 8, 10, 12, 18, 24, 36, 48 and 72 hours after infection.

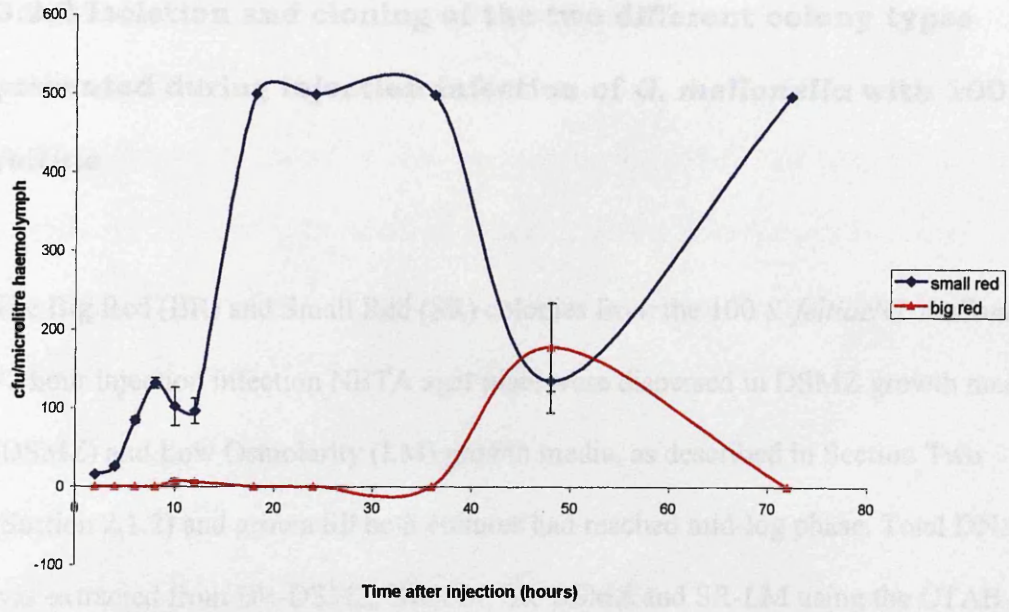


Figure 3.2 Mean number of Big Red and Small Red CFU from time series of 100 *S. feltiae/G. mellonella* injection infection time series. Standard errors are shown. n = 10.

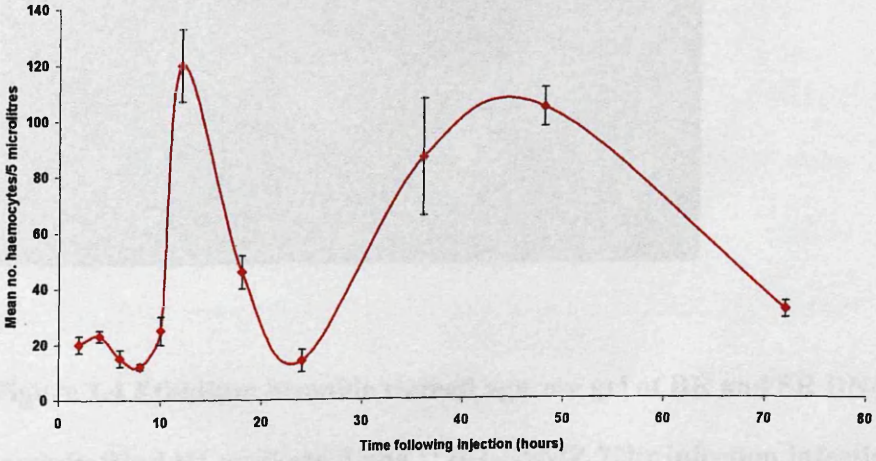


Figure 3.3 Mean number of haemocytes from time series of 100 *S. feltiae/G. mellonella* injection infection time series. Standard errors are shown. n = 10.

3.2.2 Isolation and cloning of the two different colony types presented during injection infection of *G. mellonella* with 100 *S. feltiae*

The Big Red (BR) and Small Red (SR) colonies from the 100 *S. feltiae*/*G. mellonella* 72 hour injection infection NBTA agar plate were dispersed in DSMZ growth media (DSMZ) and Low Osmolarity (LM) growth media, as described in Section Two (Section 2.1.2) and grown till both cultures had reached mid-log phase. Total DNA was extracted from BR-DSMZ, BR-LM, SR-DSMZ and SR-LM using the CTAB extraction method and further purified by CSCl ultracentrifugation (Figure 3.4).

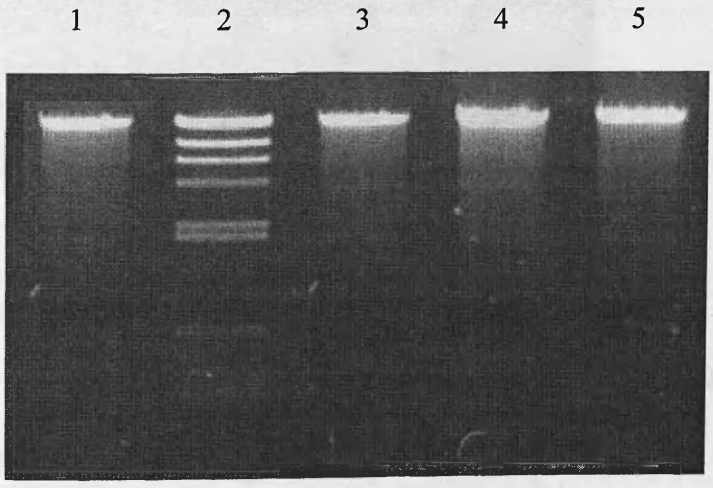


Figure 3.4 Ethidium bromide stained agarose gel of BR and SR DNA. Lane 2: lambda Hind III markers. Lane 1: BR-DSMZ 72hr injection infection genomic DNA, Lane 3: BR-LM 72hr injection infection genomic DNA, Lane 4: SR-DSMZ 72hr injection infection genomic DNA, Lane 5 SR-LM72hr injection infection genomic DNA.

General 16s ribosomal RNA primers, Eubact, as described in Section Two (Section 2.4.1), producing a 1011bp fragment were used to amplify a single band. A number of different reaction conditions were tested using BR-DSMZ, BR-LM, SR-DSMZ and SR-LM 72hr 100 *S. feltiae/G. mellonella* injection infection genomic DNA as templates. An annealing temperature of 56°C was found to be optimal for PCR, using a Mg^{2+} concentration of 1.5mM (Figure 3.5).

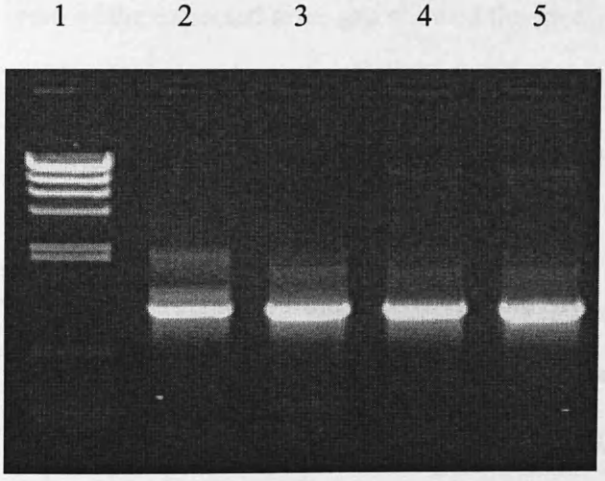


Figure 3.5 Ethidium bromide stained agarose gel of PCR products amplified with EUB 338 forward and reverse primers with BR-DSMZ (lane 2), BR-LM (lane 3), SR-DSMZ (lane 4) and SR-LM (lane 5), 72hr injection infection, genomic DNA templates. Lane 1 contains lambda Hind III markers. Lane 2 contains BR-DSMZ PCR products amplified with 1.5mM Mg^{2+}. Lane 3 contains BR-LM PCR products amplified with 1.5mM Mg^{2+}. Lane 4 contains SR-DSMZ PCR products amplified with 1.5mM Mg^{2+}. Lane 5 contains SR-LM PCR products amplified with 1.5mM Mg^{2+}.

The single band PCR products for all four isolates, shown in lanes two, three, four and five (Figure 3.5) were isolated from a LMP gel and were inserted in pCR®2.1 TOPO and transformed into TOP10F' One Shot® (Invitrogen®). Plasmid preparations and restriction digests with Eco R1 yielded the correct sized inserts, which were subsequently sequenced. Sequencing of these plasmids, named pBR-DSMZ1, pBR-LM1, pSR-DSMZ1 and pSR-LM1 and searching of the NCBI BLAST sequence similarity database revealed that the cloned fragment from BR-DSMZ and BR-LM were of the expected size, and showed the greatest homology with the 16S ribosomal RNA gene from the γ-proteobacterium *Xanthomonas axonopidis*, a causative agent of bacterial spot of tomatoes and peppers (Jones, *et al* 2000), (Figure 3.6).

The cloned fragments from SR-DSMZ and SR-LM were also of the expected size and showed the greatest homology with the 16S ribosomal RNA gene from the γ-proteobacterium *Stenotrophomonas maltophilia* a Psuedomonas-like bacterium (Anzai *et al*, 2000), (Figure 3.6).

A)

GAATTCGCCCTTAAGGAGGTGATCCAGCCGTACCTTCCGATACGGCTACC
TTGTNACGACTTCACCCCAGTNNTCGGTCACACCGTGGCAAGCGCNCTCC
CGAANGTTAAGCTACCTGCTNCTGGTGCAACAAACTCCCNTGGTGTGACN
GGCGGNGTGTNCAAGGCCCGGGAACNTATTCACCGCAGNAATGCTGATCT
GCGATTACTAGCGATTCCGACTTCATGGANTCNANTNGNAGACTCCAATC
CGGACTGAGATAGGGTTTCTGGGATNGGCTTGACCTCGCGGGTTTGNANC
CCTCTGTCCCTACCANNGTANTACNTGTGTACCCTGGTCGTCAGGGCCAT
GATGACTTNGNGTCATCCCCACTTNCTCCGGTTTGTCNNCNGCGGGCTCCT
TAGAGTTCCACCATTACGTGCTGGGCGAACTTNAAGGACAAAGGGTNGNC
GCNTCNTTGCAGGGACTTAACCNCAANATTGTCACGGANNATTGAGGCTT
GANCNACANTCCAATNGAAGCAACCTGGTGGTTCCGGAAGTTTCCCCGAA
ANGGCNCCCAAATTCCAATTCTTCTTGGNAAAAGTTTCTTCGGACNATGG
TTNNANNACCAAGNTNTAAAGGNTACCTTTCGGGNGGTTTGNAATTCCNA
AATTTTAAACCCCCCTNTTNNNTCCAAACAGNGTTTTNTTTCCCNGAANCC
CCCCGTGAAANCCCCCTTTTGNAGGTTTCCAAGTTCTTTTGTGGAACCCGT
ANCTCCCCCCNAAGGTTCGGGGGGAAAACCTTTTTA

Sequences producing significant alignments:		Score (bits)	E Value
gi|31096020|gb|AY288081.1|	Xanthomonas axonopodis pv. vesic...	551	e-154
gi|31096019|gb|AY288080.1|	Xanthomonas vesicatoria strain C...	551	e-154
gi|21110727|gb|AE012082.1|	Xanthomonas axonopodis pv. citri...	551	e-154
gi|21110297|gb|AE012039.1|	Xanthomonas axonopodis pv. citri...	551	e-154
gi|19526281|gb|AF442743.1|	Xanthomonas axonopodis pv. citri...	551	e-154
gi|19526278|gb|AF442740.1|	Xanthomonas axonopodis pv. auran...	551	e-154
gi|4544328|gb|AF123091.2|AF123091	Xanthomonas axonopodis st...	551	e-154
gi|4544327|gb|AF123090.2|AF123090	Xanthomonas axonopodis st...	551	e-154
gi|4544326|gb|AF123089.2|AF123089	Xanthomonas axonopodis st...	551	e-154
gi|4544325|gb|AF123088.2|AF123088	Xanthomonas vesicatoria s...	551	e-154
gi|31096022|gb|AY288083.1|	Xanthomonas gardneri strain CNPH...	543	e-151
gi|31096021|gb|AY288082.1|	Xanthomonas gardneri strain CNPH...	543	e-151
gi|21115099|gb|AE012505.1|	Xanthomonas campestris pv. campe...	543	e-151
gi|21115426|gb|AE012540.1|	Xanthomonas campestris pv. campe...	543	e-151
gi|19526280|gb|AF442742.1|	Xanthomonas axonopodis pv. citri...	543	e-151
gi|19526279|gb|AF442741.1|	Xanthomonas axonopodis pv. citru...	543	e-151
gi|19526277|gb|AF442739.1|	Xanthomonas axonopodis pv. auran...	543	e-151
gi|6525243|gb|AF208315.1|AF208315	Xanthomonas cynarae 16S r...	543	e-151

B)

GAATTCCCCTGANACGCCAAGCTTGGTACCGAGCTCGGATCACTAGTAAC
GGCCGCCAGTGTGCTGGAATTCGCCCTTCAGCAGCCGCGGTAATACGAAG
GGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTT
GTTTAAGTCTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGA
TACTGGGCAACTAGAGTGTGGTAGAGGGTAGTGGAATTCCCGGTGTAGCA
GTGAAATGCGTAGAGATCGGGAGGAACATCCATGGCGAAGGCAGCTACC
TGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGT
GCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGCG
GAANTACGGTCGCAAGACTGAAACTCAAAGGGATTGACGGGGGCCCGCA
CAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAANAACCTTNCC
TGGTCTTNGACATGTCGAGAACTTTCCAAAATGGATTGGTGGCCTTCNGG
AACTCCAACACAGGTTGCTNGCATGGNTTGTCCCTCNAGNTCGTGGTTCC
TNAAGATNGTTNGGGTTAANTNCCGCAANCTAACGCAACCCCTTTGACCN
TNANTTGCCAANCCCCTTNATTGGTTGGGGAACTCTNNANGNAAACCNCC
NGGTTNNCAAAACCGCAANGAAAAGTNGNGGNATNACNTTCANATTCAT
CNTTGC

Sequences producing significant alignments:		Score (bits)	E Value
gi|21654839|gb|AY040357.1|	Stenotrophomonas maltophilia str...	934	0.0
gi|34525879|emb|AJ551165.1|SSP551165	Stenotrophomonas sp. A...	934	0.0
gi|4210845|emb|AJ131117.1|SMA131117	Stenotrophomonas maltop...	934	0.0
gi|21038913|emb|AJ319565.1|UGA319565	Uncultured gamma prote...	934	0.0
gi|18149266|dbj|AB074713.1|	Gamma proteobacterium S-A(2)-11...	934	0.0
gi|18149261|dbj|AB074708.1|	Gamma proteobacterium S-A(1)-4B...	934	0.0
gi|25137120|emb|AJ516049.1|SMA516049	Stenotrophomonas malto...	934	0.0
gi|34329471|gb|AY367030.1|	Stenotrophomonas maltophilia iso...	934	0.0
gi|22217937|emb|AJ244720.1|SSP244720	Stentrophomonas-like s...	934	0.0
gi|4995829|emb|AJ131912.1|SMA131912	Stenotrophomonas maltop...	930	0.0
gi|4995812|emb|AJ131781.1|SMA131781	Stenotrophomonas maltop...	930	0.0
gi|34525882|emb|AJ551168.1|SSP551168	Stenotrophomonas sp. A...	926	0.0
gi|21327148|gb|AF511515.1|	Stenotrophomonas maltophilia iso...	924	0.0
gi|21327155|gb|AF511522.1|	Stenotrophomonas maltophilia iso...	920	0.0
gi|8980462|emb|AJ293462.1|SMA293462	Stenotrophomonas rhizop...	918	0.0
gi|18149289|dbj|AB074736.1|	Gamma proteobacterium S-N(nd)-2...	918	0.0
gi|3646460|emb|AJ011332.1|SMA011332	Stenotrophomonas maltop...	918	0.0
gi|19697907|gb|AY081993.1|	Uncultured bacterium clone KRA30...	912	0.0
gi|15593201|gb|AF408330.1|	Uncultured Stenotrophomonas sp. ...	904	0.0
gi|29570301|gb|AY244913.1|	Uncultured rumen bacterium clone...	902	0.0

Figure 3.6 The 16S ribosomal RNA gene fragment sequence from BR-DSMZ and BR-LM, (A) and SR-DSMZ and SR-LM, (B) isolated from 72hr injection infection. The first 20 BLASTn scores for each species are shown.The EcoR1 restriction site at the beginning of the construct is highlighted in yellow.

The number of SR CFU, which showed the greatest homology to the Psuedomonas-like bacterium, *Stenotrophomonas maltophilia,* undergoes great fluctuation within the time course of the infection (Figure 3.2). An initial rise in the number of *S. maltophilia* is followed by a slight decrease at 10-12 hours, thereafter a sharp increase between 12 and 18 hours shows the number of CFU increasing to over 500. This number remains steady until 36 hours, when the *S. maltophilia* population declines to almost 100 cfu'c at 48 hours. Following this the population increases again to previous levels of over 500 CFU.

The number of BR CFU, which showed the greatest homology to the γ-proteobacterium, *Xanthomonas axonopidis*, remains low during the first few hours of infection (Figure 3.2). The *X. axonopidis* colonies are not seen to emerge until between 36 and 48 hours after injection, where the population peaks at around 100 and then declines steadily to zero after 72 hours of infection.

The population fluctuations are mirrored in the digital images of the NBTA agar plates (Figure 3.1), although in some cases the SR or *S. maltophilia* colonies may be too small to discern on the printed page.

The mean number of haemocytes experiences great fluctuation over the time course of the infection (Figure 3.3). A base level of around 20 haemocytes/5 μl of haemolymph is evident at 2 to 4 hours, this is followed by a slight decrease in haemocyte numbers between 4 and 8 hours, thereafter the number of haemocytes increases sharply to almost 120 haemocytes/5 μl of haemolymph at 12 hours following injection. A sharp decline in haemocyte numbers between 12 and 24 hours

is followed by a steadier increase in numbers between 24 and 48 hours following injection. The number of haemocytes declines between 48 and 72 hours to around 30 haemocytes/5 µl of haemolymph.

3.3 Natural infection time series

3.3.1 Natural infection time series with 100 *S. feltiae*/caterpillar

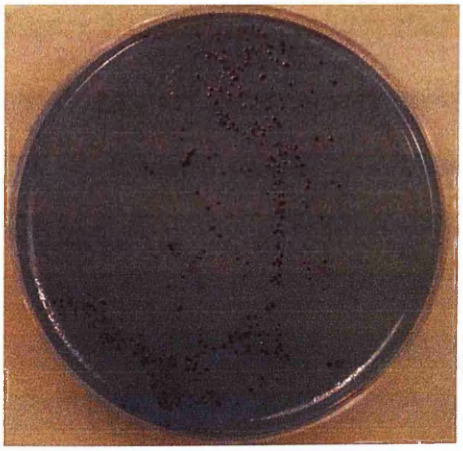
G. mellonella were naturally infected with 100 *S. feltiae*/caterpillar, as described in Section Two (Section 2.9.4). The caterpillars were treated as in Section 3.2.1.

An example of the NBTA agar plates from each time point was recorded as digital photographs (Figure 3.7) and a plot of the mean number of colony forming units (CFU) against time after infection was constructed (Figure 3.8).

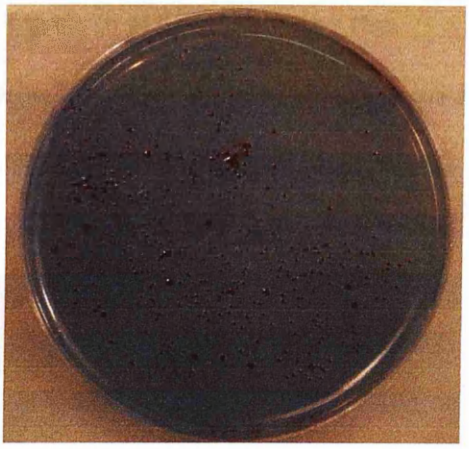
The number of haemocytes at each time point was recorded and a plot of the mean number of haemocytes against time after infection was constructed (Figure 3.9).

12 hours following infection

24 hours following infection

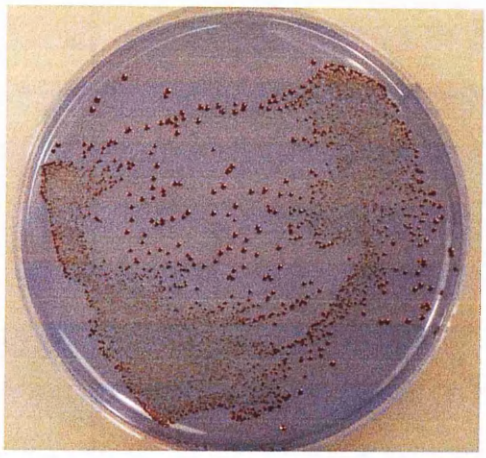


36 hours following infection



48 hours following infection

Figure 3.7 (continued)



72 hours following infection

Figure 3.7 NBTA agar plates after 48 hours of growth at 28°C showing the number of CFU from *G. mellonella* haemolymph following natural infection with 100 *S. feltiae*/caterpillar over the time period 12, 24, 48, 36 and 72 hours after infection.

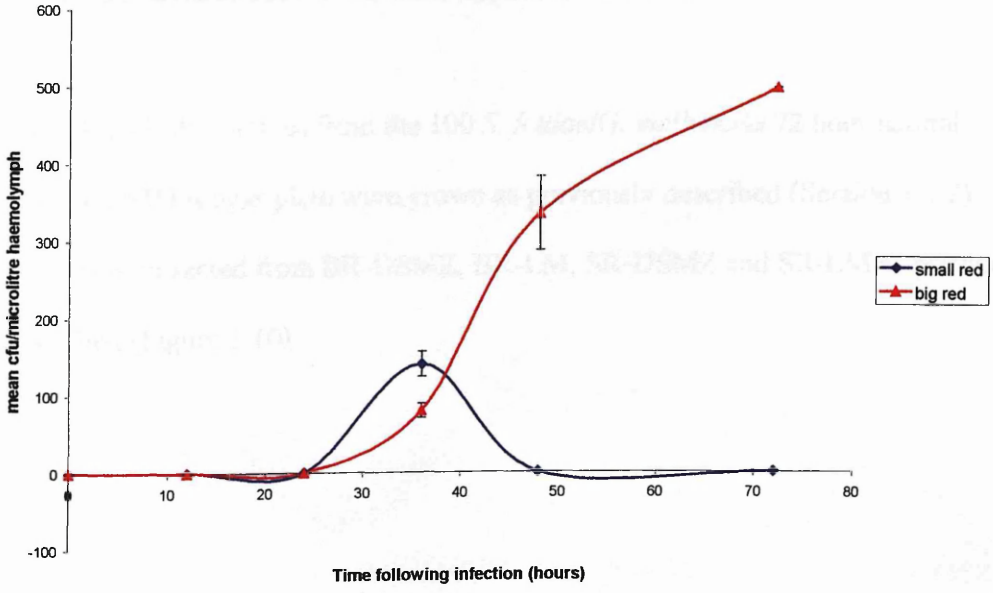


Figure 3.8 Mean number of BR and SR CFU from time series of 100 *S. feltiae/G. mellonella* natural infection time series. Standard errors are shown. n = 10.

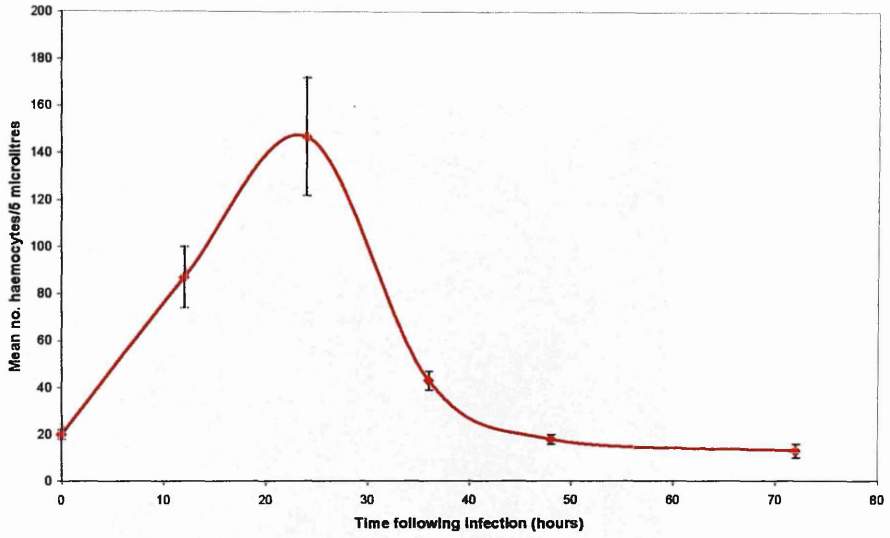


Figure 3.9 Mean number of haemocytes' from time series of 100 *S. feltiae/G. mellonella* natural infection time series. Standard errors are shown. n = 10.

3.3.2 Isolation and cloning of the two different colony types presented during injection infection of *G. mellonella* with 100 *S. feltiae*

The BR and SR colonies from the 100 *S. feltiae/G. mellonella* 72 hour natural infection NBTA agar plate were grown as previously described (Section 3.2.2). Total DNA was extracted from BR-DSMZ, BR-LM, SR-DSMZ and SR-LM as previously described (Figure 3.10).

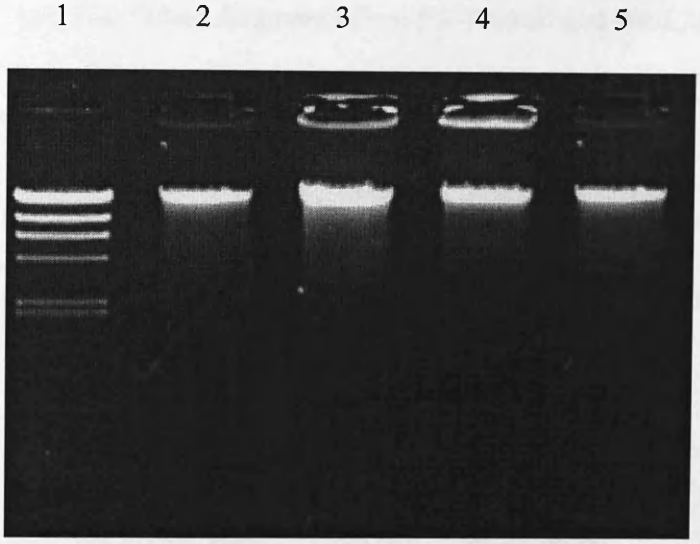


Figure 3.10 Ethidium bromide stained agarose gel of BR and SR 72hr 100 *S. feltiae/G. mellonella* natural infection DNA. Lane 1: lambda Hind III markers. Lane 2: BR-DSMZ genomic DNA, Lane 3: BR-LM genomic DNA, Lane 4: SR-DSMZ genomic DNA, Lane 5 SR-LM genomic DNA.

The same PCR conditions as previously described were used with the Eubact primers to amplify a single band using BR-DSMZ, BR-LM, SR-DSMZ and SR-LM 72 hr 100 *S. feltiae/G. mellonella* natural infection genomic DNA as templates (Figure 3.11).

The single band PCR product for all four isolates, shown in lanes one, two, three and four (Figure 3.11) were isolated and transformed into TOP10F' One Shot® (Invitrogen®). Sequencing of the plasmids, named pBR-DSMZ2, pBR-LM2, pSR-DSMZ2 and pSR-LM2 and a BLASTn search revealed that all the cloned fragments from SR-DSMZ and SR-LM showed the greatest homology with the 16S ribosomal RNA gene from the γ-proteobactereae *Stenotrophomonas maltophilia* (Figure 3.12

A)). The cloned fragments from BR-DSMZ and BR-LM showed the greatest homology with the 16S ribosomal RNA gene from *X. nematophilus* (Figure 3.12 (B)).

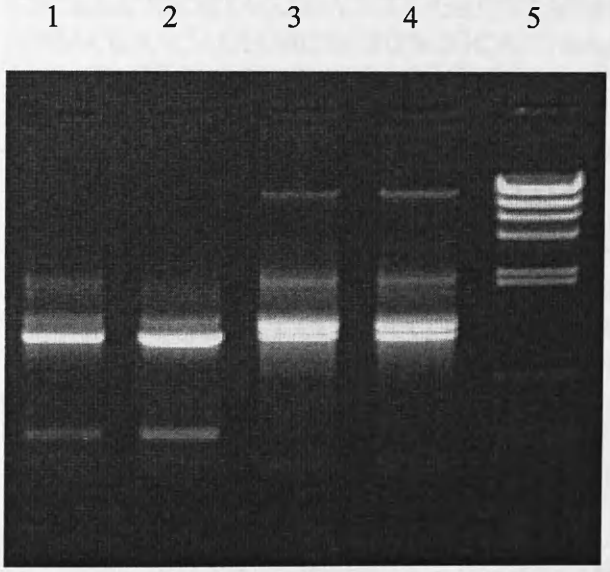


Figure 3.11 Ethidium bromide stained agarose gel of PCR products amplified with EUB 338 forward and reverse primers with BR-DSMZ (lane 2), BR-LM (lane 3), SR-DSMZ (lane 4) and SR-LM (lane 5), 72hr 100*S. feltiae/G. mellonella* natural infection, genomic DNA templates. Lane 5 contains lambda Hind III markers. All lanes were amplified with 2.5mM Mg^{2+}.

A)

GAATTCCCCTGANACGCCAAGCTTGGTACCGAGCTCGGATCACTAGTAAC
GGCCGCCAGTGTGCTGGAATTCGCCCTTCAGCAGCCGCGGTAATACGAAG
GGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTT
GTTTAAGTCTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGA
TACTGGGCAACTAGAGTGTGGTAGAGGGTAGTGGAATTCCCGGTGTAGCA
GTGAAATGCGTAGAGATCGGGAGGAACATCCATGGCGAAGGCAGCTACC
TGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGT
GCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGCG
GAANTACGGTCGCAAGACTGAAACTCAAAGGGATTGACGGGGGCCCGCA
CAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAANAACCTTNCC
TGGTCTTNGACATGTCGAGAACTTTCCAAAATGGATTGGTGGCCTTCNGG
AACTCCAACACAGGTTGCTNGCATGGNTTGTCCCTCNAGNTCGTGGTTCC
TNAAGATNGTTNGGGTTAANTNCCGCAANCTAACGCAACCCCTTTGACCN
TNANTTGCCAANCCCCTTNATTGGTTGGGGAACTCTNNANGNAAACCNCC
NGGTTNNCAAAACCGCAANGAAAAGTNGNGGNATNACNTTCANATTCAT
CNTTGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1657407|emb|X95923.1|SMRR958 S.maltophilia 16S rRNA gene...	874	0.0
gi|4995814|emb|AJ131783.1|SMA131783 Stenotrophomonas maltop...	874	0.0
gi|10334688|gb|AF181569.1|AF181569 Stenotrophomonas sp. JRL...	874	0.0
gi|8980474|emb|AJ293474.1|SMA293474 Stenotrophomonas maltop...	872	0.0
gi|17974204|emb|AJ306833.1|SMA306833 Stenotrophomonas malto...	872	0.0
gi|28569494|gb|AY179327.1| Stenotrophomonas sp. S1 16S ribo...	870	0.0
gi|26324201|gb|AY162052.1| Gamma proteobacterium PI_GH4.1.G...	870	0.0
gi|26324191|gb|AY162042.1| Gamma proteobacterium PI_GH2.1.C...	870	0.0
gi|22135621|gb|AF529130.1| Uncultured gamma proteobacterium...	870	0.0
gi|19697906|gb|AY081992.1| Uncultured bacterium clone KRA30...	870	0.0
gi|2832587|emb|AJ002814.1|SSPAJ2814 Stenotrophomonas sp. 16...	870	0.0
gi|4995815|emb|AJ131784.1|SMA131784 Stenotrophomonas maltop...	870	0.0
gi|4995813|emb|AJ131782.1|SMA131782 Stenotrophomonas maltop...	870	0.0
gi|16151153|gb|AY050496.1| Stenotrophomonas maltophilia SB5...	870	0.0
gi|32351723|gb|AY307922.1| Stenotrophomonas sp. LUP 16S rib...	870	0.0
gi|31540961|gb|AY307998.1| Uncultured Stenotrophomonas sp. ...	870	0.0
gi|3152413|dbj|AB008509.1| Stenotrophomonas maltophilia gen...	870	0.0
gi|4165410|dbj|AB021404.1| Pseudomonas geniculata DNA for 1...	866	0.0
gi|8980471|emb|AJ293471.1|SMA293471 Stenotrophomonas maltop...	864	0.0
gi|8671577|gb|AF100732.1|AF100732 Stenotrophomonas maltophi...	864	0.0

B)

GAATTCCTTGCCCAACGACGGCCAGTGAATTGTAATACGACTCACTATA
GGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATG
GATATCTGCAGAATTCGGCTTAGAGTTTGATCCTGGCTCAGATTGAACGC
TGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGGGGAAGCTT
GCTTCCCCGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGC
CCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACC
TCTGTGGAGTAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACC
CAGATGGGATTAGCTAGTANGTGGGGTAATGGCTCACCTAGGCGACGATC
CCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGC
CCANACCTCCTACGGGANGCAACAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCANCATGCCCCCGTGTATTAAGAAGGCCTTCGGGTTGTAA
AGTACTTTCNACCGGGAGGAAGGCAACANCGTAAATAGCGCCGTTGATTG
ACNTTACCCGCANNAANAACCCCGGCTTAANNCTTNCGNGCNNNNNCCA
TNANTTGNANGGCG

Sequences producing significant alignments:	Score (bits)	E Value
gi|30984071|gb|AY286478.1| Xenorhabdus nematophila 16S ribo...	636	e-179
gi|1754501|dbj|D78010.1| Xenorhabdus poinarii DNA for 16S R...	626	e-176
gi|1754499|dbj|D78008.1| Xenorhabdus japonicus DNA for 16S ...	622	e-175
gi|24459172|gb|AY057392.1| Candidatus Phlomobacter betae 16...	587	e-165
gi|436816|emb|X75275.1|YR16SRRN Yersinia ruckeri (ATCC 2947...	583	e-163
gi|8575701|gb|AF263561.1| Secondary endosymbiont of Glycasp...	579	e-162
gi|899430|emb|X89574.1|YS17B16S Yersinia sp. DNA for 16S ri...	579	e-162
gi|899429|emb|X89573.1|YS11A16S Yersinia sp. DNA for 16S ri...	579	e-162
gi|13991903|gb|AF366385.1|AF366385 Yersinia ruckeri 16S rib...	569	e-159
gi|11125694|dbj|AB038366.1| Arsenophonus endosymbiont of Di...	563	e-157
gi|8218294|emb|AJ289197.1|YRU289197 Yersinia ruckeri partia...	561	e-157
gi|515325|emb|X79937.1|RA287R R.aquatilis (2-87) 16S-rDNA	557	e-156
gi|173754|gb|M90801.1|ARSRR16S Arsenophonus nasoniae 16S ri...	557	e-156
gi|33392063|gb|AY345555.1| Bacterium H16 16S ribosomal RNA ...	555	e-155
gi|32478651|gb|AY264673.1| Arsenophonus endosymbiont of Aus...	555	e-155
gi|32478644|gb|AY264666.1| Arsenophonus endosymbiont of Ale...	555	e-155
gi|32478643|gb|AY264665.1| Arsenophonus endosymbiont of Ale...	555	e-155
gi|2290268|gb|U88434.1|RSU88434 Rahnella sp. 'CDC 1-576' 16...	553	e-154
gi|33087567|gb|AY332850.1| Yersinia ruckeri strain WS 20/94...	553	e-154
gi|20378118|gb|AF373200.1| Pectobacterium chrysanthemi stra...	551	e-154
gi|517364|emb|X79940.1|RA388R R.aquatilis (3-88) 16S-rDNA	551	e-154

Figure 3.12 The 16S ribosomal RNA gene fragment sequence from BR-DSMZ and BR-LM, (A) and SR-DSMZ and SR-LM, (B) isolated from 100 *S. feltiae/G. mellonella* 72hr natural infection. The first 20 BLASTn scores for each species are shown. The EcoR1 restriction site at the beginning of the construct is highlighted in yellow.

The number of SR, or *S. maltophilia* CFU does not increase until 24 hours after infection, here, the number of CFU increases to a peak of around 100 at 36 hours following infection, but has decreased back to zero by 48 hours following infection (Figure 3.8).

The number of BR, or *X. bovienii* CFU also remains undetectable until 24 hours following infection, here, the number of CFU displays a steady increase reaching a peak of nearly 500 at 72 hours following infection (Figure 3.8).

The population fluctuations are mirrored in the digital images of the NBTA agar plates (Figure 3.7), although in some cases the SR or *S. maltophilia* colonies may be too small to discern on the printed page.

The number of haemocytes/5 µl of haemolymph displays an almost exponential increase until it reaches a peak of around 140 haemocytes/5 µl of haemolymph at 24 hours following infection, at this point the number of haemocytes declines sharply until 36 hours following infection to around 30 haemocytes/5 µl of haemolymph. Following this, the number of haemocytes remains at an almost constant 15 haemocytes/5 µl of haemolymph until 72 hours following infection (Figure 3.9).

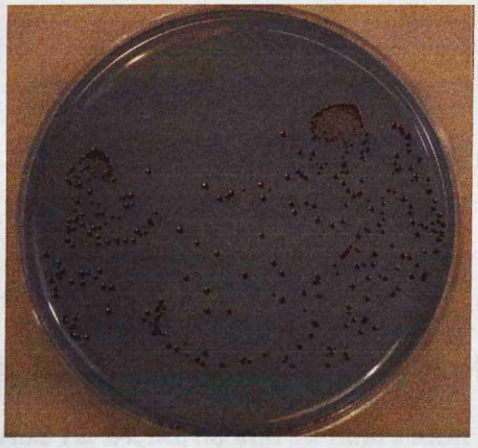
3.3.3 Natural infection time series with 1000 *S. feltiae*/caterpillar

G. mellonella were naturally infected with 1000 *S. feltiae*/caterpillar, as described in Section Two (Section 2.9.4). The caterpillars were treated as in Section 3.2.1.

An example of the NBTA agar plates from each time point was recorded as digital photographs (Figure 3.13) and a plot of the mean number of colony forming units (CFU) against time after infection was constructed (Figure 3.14).

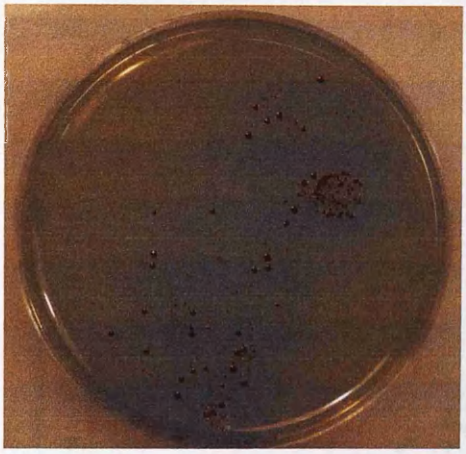
The number of haemocytes at each time point was recorded and a plot of the mean number of haemocytes against time after infection was constructed (Figure 3.15).

12 hours following infection

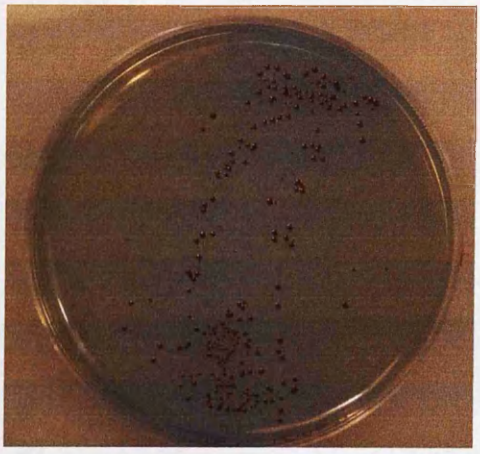


24 hours following infection

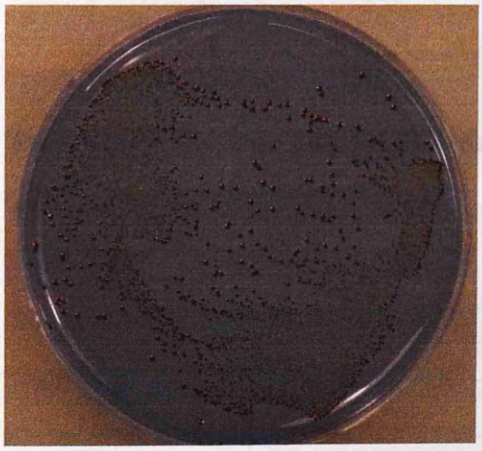
Figure 3.13 (continued)



36 hours following infection



48 hours following infection



72 hours following infection

Figure 3.13 NBTA agar plates after 48 hours of growth at 28°C showing the number of CFU from *G. mellonella* haemolymph following natural infection with 1000 *S. feltiae*/caterpillar over the time period 12, 24, 36, 48 and 72 hours after infection.

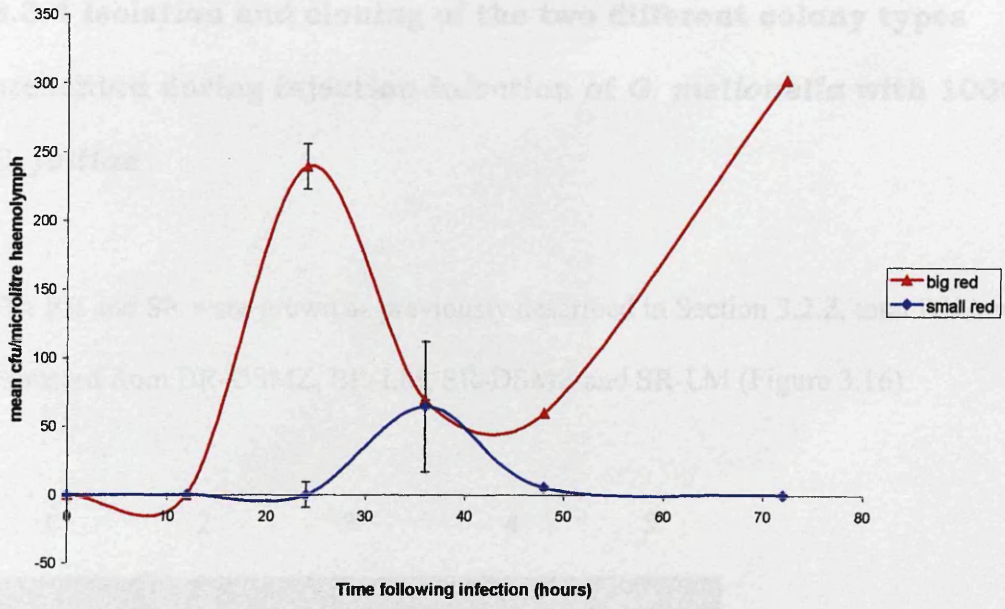


Figure 3.14 Mean number of BR and SR CFU from time series of 1000 *S. feltiae/G. mellonella* natural infection time series. Standard errors are shown. n = 10.

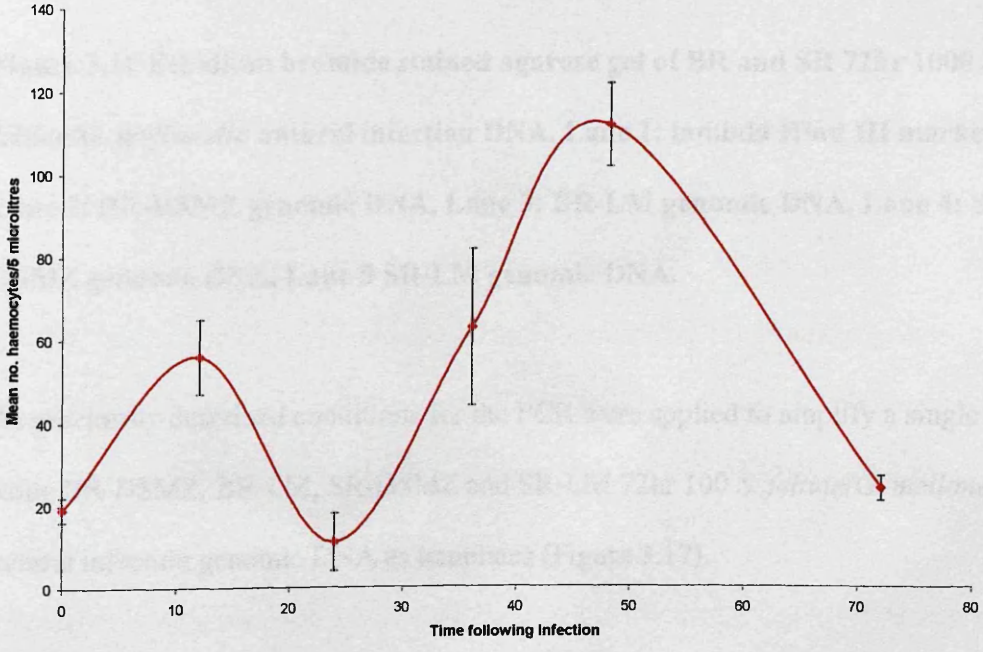


Figure 3.15 Mean number of haemocytes from time series of 100 *S. feltiae/G. mellonella* injection infection time series. Standard errors are shown. n = 10.

3.3.4 Isolation and cloning of the two different colony types presented during injection infection of *G. mellonella* with 1000 *S. feltiae*

The BR and SR were grown as previously described in Section 3.2.2, total DNA was extracted from BR-DSMZ, BR-LM, SR-DSMZ and SR-LM (Figure 3.16).

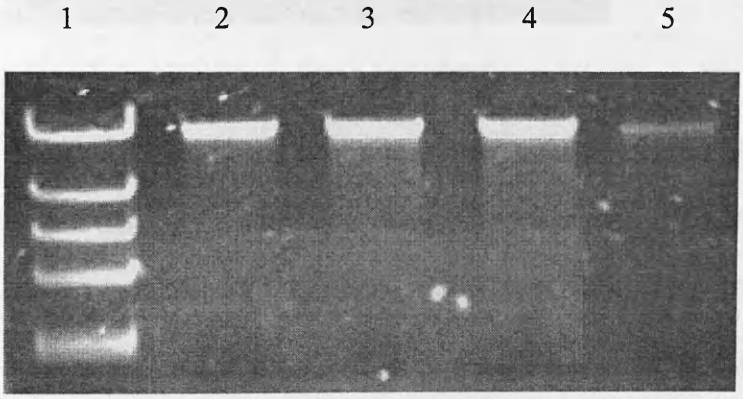


Figure 3.16 Ethidium bromide stained agarose gel of BR and SR 72hr 1000 *S. feltiae*/*G. mellonella* natural infection DNA. Lane 1: lambda Hind III markers. Lane 2: BR-DSMZ genomic DNA, Lane 3: BR-LM genomic DNA, Lane 4: SR-DSMZ genomic DNA, Lane 5 SR-LM genomic DNA.

As previously described conditions for the PCR were applied to amplify a single band using BR-DSMZ, BR-LM, SR-DSMZ and SR-LM 72hr 100 *S. feltiae*/*G. mellonella* natural infection genomic DNA as templates (Figure 3.17).

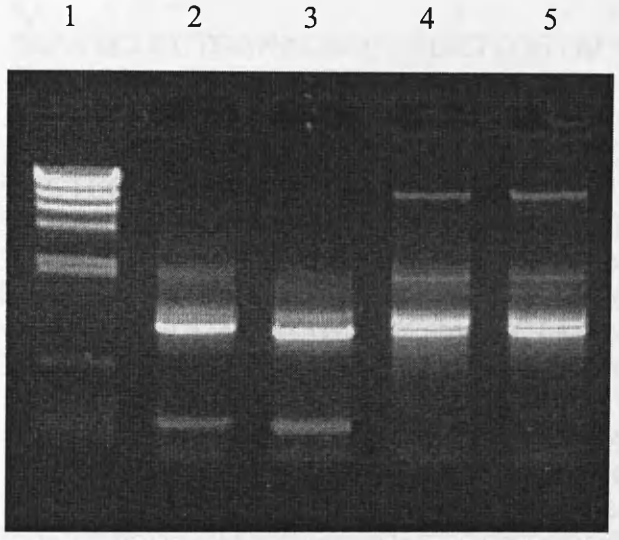


Figure 3.17 Ethidium bromide stained agarose gel of PCR products amplified with EUB 338 forward and reverse primers with BR-DSMZ (lane 2), BR-LM (lane 3), SR-DSMZ (lane 4) and SR-LM (lane 5), 72hr 1000 *S. feltiae/G. mellonella* natural infection, genomic DNA templates. Lane 1 contains lambda Hind III markers. All lanes amplified with 3.5mM Mg^{2+}.

The single band PCR product for all four isolates, shown in lanes one, two, three and four (Figure 3.17) were isolated and transformed into TOP10F' One Shot® (Invitrogen®). Sequencing of these plasmids, named pBR-DSMZ3, pBR-LM3, pSR-DSMZ3 and pSR-LM3 and a BLAST search revealed that the cloned fragment from SR-DSMZ and SR-LM showed the greatest homology with the 16S ribosomal RNA gene from the γ-proteobacterium *Stenotrophomonas maltophilia* (Figure 3.18 A)).

The cloned fragments from BR-DSMZ and BR-LM showed the greatest homology with the 16S ribosomal RNA gene from *X. nematophilus* (Figure 3.18 (B)).

A)

GAATTCCCCTGANACGCCAAGCTTGGTACCGAGCTCGGATCACTAGTAAC
GGCCGCCAGTGTGCTGGAATTCGCCCTTCAGCAGCCGCGGTAATACGAAG
GGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTT
GTTTAAGTCTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGA
TACTGGGCAACTAGAGTGTGGTAGAGGGTAGTGGAATTCCCGGTGTAGCA
GTGAAATGCGTAGAGATCGGGAGGAACATCCATGGCGAAGGCAGCTACC
TGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGT
GCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGCG
GAANTACGGTCGCAAGACTGAAACTCAAAGGGATTGACGGGGGCCCGCA
CAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAANAACCTTNCC
TGGTCTTNGACATGTCGAGAACTTTCCAAAATGGATTGGTGGCCTTCNGG
AACTCCAACACAGGTTGCTNGCATGGNTTGTCCCTCNAGNTCGTGGTTCC
TNAAGATNGTTNGGGTTAANTNCCGCAANCTAACGCAACCCCTTTGACCN
TNANTTGCCAANCCCCTTNATTGGTTGGGGAACTCTNNANGNAAACCNCC
NGGTTNNCAAAACCGCAANGAAAAGTNGNGGNATNACNTTCANATTCAT
CNTTGC

Sequences producing significant alignments:		Score (bits)	E Value
gi|4210842|emb|AJ131114.1|SMA131114	Stenotrophomonas maltop...	862	0.0
gi|8980468|emb|AJ293468.1|SMA293468	Stenotrophomonas maltop...	862	0.0
gi|4995826|emb|AJ131909.1|SMA131909	Stenotrophomonas maltop...	862	0.0
gi|15072519|gb|AY038626.1|	Uncultured eubacterium clone GL1...	862	0.0
gi|33943962|gb|AY360340.1|	Stenotrophomonas maltophilia 16S...	862	0.0
gi|33392078|gb|AY345544.1|	Unidentified bacterium clone LWS...	862	0.0
gi|1488281|gb|U62646.1|SAU62646	Stenotrophomonas africae 16...	862	0.0
gi|14091481|gb|AF368754.1|	Stenotrophomonas sp. 3A3C 16S ri...	858	0.0
gi|21901940|emb|AJ495804.1|STE495804	Stenotrophomonas sp. P...	858	0.0
gi|5359860|gb|AF142878.1|AF142878	Uncultured bacterium DEEP...	858	0.0
gi|7330813|gb|AF214139.1|AF214139	Stenotrophomonas sp. P916...	858	0.0
gi|15420788|gb|AF390081.1|	Stenotrophomonas maltophilia str...	856	0.0
gi|22135585|gb|AF529094.1|	Uncultured gamma proteobacterium...	856	0.0
gi|15420787|gb|AF390080.1|	Stenotrophomonas maltophilia str...	854	0.0
gi|22218228|gb|AF529349.1|	Uncultured gamma proteobacterium...	854	0.0
gi|8980467|emb|AJ293467.1|SMA293467	Stenotrophomonas maltop...	854	0.0
gi|4995833|emb|AJ131916.1|SMA131916	Stenotrophomonas maltop...	854	0.0
gi|4995824|emb|AJ131907.1|SMA131907	Stenotrophomonas maltop...	854	0.0
gi|16303665|gb|AY053488.1|	Uncultured bacterium AT425_EubD3...	854	0.0
gi|15072516|gb|AY038623.1|	Uncultured eubacterium clone GL1...	854	0.0

B)

GAATTCCCTTGCCCAACGACGGCCAGTGAATTGTAATACGACTCACTATA
GGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATG
GATATCTGCAGAATTCGGCTTAGAGTTTGATCCTGGCTCAGATTGAACGC
TGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGGGGGAAGCTT
GCTTCCCCGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGC
CCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACC
TCTGTGGAGTAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACC
CAGATGGGATTAGCTAGTANGTGGGGTAATGGCTCACCTAGGCGACGATC
CCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGC
CCANACTCCTACGGGANGCAACAGTGGGGAATATTGCACAATGGGCGCA
AGCCTGATGCANCATGCCCCCGTGTATTAAGAAGGCCTTCGGGTTGTAAA
GTACTTTCNACCGGGAGGAAGGCAACANCGTAAATAGCGCCGTTGATTGA
CNTTACCCGCANNAANAACCCCGGCTTAANNCTTNCGNGCNNNNNCCAT
NANTTGNANGGCGTTGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC
AGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTGGGAACG
GCATCTAAAACTGGTTGACTAGAGTCTCGTAGAGGGGGGTAGAATTCCAC
GTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGG
CGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGG
AGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACC
GCCTGGGGAGTACGGCCGCNAGGTTAAACTCAAATGAATTTGACGGGGG
CCCGCACANNCGGTGGAGCATGTGGTTTAATTCGATGCACNNNNNANAA
CCTTACCTACTCTTGACATCCTCANAATTTTGCTNGANACAGCNAAATTGC
CTTCNNGGGAACCTGAGAGACANTGTGCTTCATGGCTGNTTNTNAANTC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1754500|dbj|D78009.1| Xenorhabdus nematophilus DNA for 1...	751	0.0
gi|30842078|gb|AY278674.1| Xenorhabdus nematophila 16S ribo...	749	0.0
gi|21913297|gb|AF522294.1| Xenorhabdus nematophila 16S ribo...	749	0.0
gi|3172002|emb|Z76738.1|XNZ76738 X.nematophilus (strain RIO...	749	0.0
gi|3172001|emb|Z76737.1|XNZ76737 X.nematophilus (strain N2-...	749	0.0
gi|861162|emb|X82251.1|XN16RNA0 X.nematophilus 16S rRNA gen...	749	0.0
gi|1754499|dbj|D78008.1| Xenorhabdus japonicus DNA for 16S ...	749	0.0
gi|30842075|gb|AY278671.1| Photorhabdus sp. HIT 16S ribosom...	747	0.0
gi|30842074|gb|AY278670.1| Photorhabdus sp. JUN 16S ribosom...	747	0.0
gi|30842049|gb|AY278645.1| Photorhabdus luminescens subsp. ...	745	0.0
gi|30842046|gb|AY278642.1| Photorhabdus luminescens subsp. ...	745	0.0
gi|6456832|emb|AJ007359.1|PHO7359 Photorhabdus luminescens ...	745	0.0
gi|2182051|emb|Z76745.1|PLZ76745 Photorhabdus luminescens s...	745	0.0
gi|861163|emb|X82253.1|XP16RNA8 X.poinarii 16S rRNA gene (D...	745	0.0
gi|29164906|gb|AY216500.1| Photorhabdus sp. Q614 16S riboso...	741	0.0
gi|861085|emb|X82250.1|PLRNAHSH2 P.luminescens 16S rRNA gen...	741	0.0
gi|30842048|gb|AY278644.1| Photorhabdus luminescens subsp. ...	737	0.0
gi|30842047|gb|AY278643.1| Photorhabdus luminescens subsp. ...	737	0.0
gi|3256225|emb|Y17605.1|PLY17605 Photorhabdus luminescens 1...	737	0.0
gi|30842076|gb|AY278672.1| Photorhabdus asymbiotica 16S rib...	733	0.0

Figure 3.18 The 16S ribosomal RNA gene fragment sequence from BR-DSMZ and BR-LM, (A) and SR-DSMZ and SR-LM, (B) isolated from 1000 *S. feltiae/G. mellonella* 72hr natural infection. The first 20 BLASTn scores for each species

are shown. The EcoR1 restriction site at the beginning of the construct is highlighted in yellow.

The number of SR, or *S. maltophilia* CFU is virtually zero until 24 hours following infection, at this point the population increases to a peak of around 50 at 36 hours following infection. A decline in the population is seen until 48 hours following infection. After 48 hours the number of *S. maltophilia* CFU is zero until 72 hours (Figure 3.14)

The number of BR, or *X. bovienii* CFU increases sharply following 12 hours of infection to a peak of around 250 CFU at 24 hours. This is mirrored by an equally sharp decline to around 25 CFU at 48 hours following infection. A sharp increase in the number of *X. bovienii* CFU between 48 and 72 hours following infection results in a peak of around 300 CFU (Figure 3.14).

The population fluctuations are mirrored in the digital images of the NBTA agar plates (Figure 3.13), although in some cases the SR or *S. maltophilia* colonies may be too small to discern on the printed page.

The number of haemocytes follows an S shaped curve. Rising to a small peak of 60 haemocytes/5 µl of haemolymph at 12 hours following infection and declining to 15 haemocytes at 24 hours following infection. This rise and fall is repeated, with an increase to 120 haemocytes/5 µl of haemolymph at 48 hours following infection and a decrease to around 20 haemocytes at 72 hours following infection (Figure 3.15).

3.4 Discussion

In the field of insect nematology, the majority of research groups use the injection method detailed in this study as a means of infecting host species with entomopathogenic nematodes. Amongst others: Akhurst 1986, Bedding *et al* 1983, Ehlers *et al* 1997, Fischer-Le Saux, *et al* 1999, Glazer 1997, Grewal *et al* 1996, Jarosz 1998, Wang *et al* 1994, Wang & Gaugler, 1999, Yoko *et al* 1992, have all used the injection method when infecting host species. This study shows that the use of the injection method may result in different infection characteristics compared to an infection by natural means.

The different bacteria species isolated from injection infection *Galleria* compared to natural infection *Galleria* is a clear indication that the different methods of infection leads to the *in vivo* production of different bacterial populations. The bacterial species which were isolated from the NBTA agar plates may not be representative of the entire bacterial population from within the infected *Galleria*, as some bacterium will be unculturable on either liquid or solid medium. However, the large numbers which were present on the agar plates suggests that they are the most numerous and dominant culturable species in the population.

The injection infection method led to the production of two distinct colony types, *Stenotrophomonas maltophilia* and *Xanthomonas axonopidis*, known phenotypically as small red (SR) and big red (BR). *S. maltophilia* essentially had the largest population throughout almost the entire infection. Where a decline in *S. maltophilia* was observed it coincided with an increase in the *X. axonopidis* population. The

absence of *Xenorhabdus* spp from the injection infection bacterial populations is contradictory to the published literature, as an infection with *Xenorhabdus* carrying nematodes is thought to lead to a monoxenic population of *Xenorhabdus* within the host (Akhurst 1986).

In contrast, the 100 and 1000 nematode natural infection method led to the production of *Xenorhabdus bovienii,* BR and also *S. maltophilia*, SR. With the 100 nematode infection the *X. bovienii* population increased unchecked by the emergence of *S. maltophilia*. With the 1000 nematode infection, the emergence of *S. maltophilia* coincided with a decrease in the *X. bovienii* population. In both the 100 and 1000 nematode natural infections *X. bovienii* was the sole bacterium present towards the end of the infection, which resulted in a monoxenic population.

The number of haemocytes present during an injection infection and a natural infection with 1000 nematodes were almost the same (Figures 3.3 and 3.15), however the haemocyte numbers for the 100 nematode natural infection differed considerably (Figure 3.9). The decline in haemocyte numbers coincided with the rise in the *X. bovienii* population, as the number of bacterium began to increase, the number of haemocytes decreased and did not recover.

The natural infection with 100 nematodes/*Galleria* supports the reported theory that a monoxenic population of *Xenorhabdus* will become established, due to the secretion of several broad spectrum antibacterial and antifungal antibiotics (Akhurst & Boemare 1990; Forst & Nealson 1996) by the symbiotic bacterium which are produced in a high enough concentration to destroy any of the natural bacterial

populations which may already exist in reduced numbers within the host. The natural infection method allows *Xenorhabdus* to multiply within the confines of the gut and to destroy the majority of the natural gut flora before breaking through the gut membrane into the surrounding haemocoel.

One theory for the emergence of different bacterium species isolated during the injection infection is that injection of the nematodes directly into the insect haemocoel results in almost immediate disruption of the host gut prior to the establishment of a monoculture of *Xenorhabdus.* Thus, facilitating the release of the general gut flora into the haemocoel where the gut bacteria multiply and compete with *Xenorhadus* for resources.

Therefore, it is proposed that the injection infection of host insects with entomopathogenic nematodes should be reconsidered within the field of insect nematology and that the use of non-injection infection methods become more widespread in order to mimic the natural infection process.

CHAPTER FOUR

Time series of Injection and Natural infection of *G. mellonella* with *S. feltiae*, investigated using 16S rRNA oligonucleotide probes.

4.1 Introduction

In the previous chapter infection of *G. mellonella* with *S. feltiae* was investigated by examining populations of bacteria that can be cultured on nutrient agar plates. Differences in the infection characteristics, namely the different bacterial populations, were observed when comparing natural to injection infection methods. Here, the infection parameters are further explored by quantifying bacterial rRNA expression using Northern slot blots, during a time series of natural and injection infections.

The use of two different 16S ribosomal RNA probes, one which binds specifically to *X. bovienii* and a general probe that binds to *Eubacteriacea* allowing the fluctuating amounts of *X. bovienii* and general *Eubacteriacea* rRNA to be visualised, therefore further characterising the distinction between natural and injection infection time series.

4.2 Primer design and PCR amplification of the *X. bovienii* 16S rRNA gene

Specific primers were used to isolate the 16S rRNA gene from the total *X. bovienii* DNA.

The 16S ribosomal RNA sequence from *X. nematophilus* was aligned with other sequences from a number of other species using *ClustalX* software. Those sequences which displayed high homogeneity to the *X. nematophilus* sequence when performing a BLASTn search were chosen. This resulted in nine species of Enterobacteriaceae; *Rahnella* spp., *Serratia proteamaculans*, *Photorhabdus luminescens*, *Pectobacterium carotovorum*, *Erwinia carotovorum*, *Aranicola ptoteolyticus*, *Pantoea agglomerus*, *Salmonella bongori* and *Klebsiella pneumoniae* undergoing alignment with *X. nematophilus* (Figure 4.1). The conserved regions were investigated and the suitability of the 16S rRNA primers was considered (Figure 4.2).

* indicates an amino acid match across all sequences

```
salmonella      AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAAC
klebsiella      AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
pantoea         AAGAGTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCTGAC
Pectobacterium  AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
erwinia         AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
serratia        AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
aranicola       AGAGTTTGATCCTGGCTCAGATTGGACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
rahnella.spp    AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
X.nematophilus  AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
photorhabdus    AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGGGC
                ********************** ******************************   *

salmonella      GGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGG
klebsiella      GGTAGCACAGAG-AGCTTGCT-CTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
pantoea         GGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGTG-CGGACGGGTGAGTAATGTCTGG
Pectobacterium  GGTAGCACAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
erwinia         GGTAGCACAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
serratia        GGTAGCACAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
aranicola       G--TACACAGGAGAGCTTGCTC-CTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
rahnella.spp    GGCAGCGGGAAGTAGCTTGCTACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGG
X.nematophilus  GGCAGCGGGGGGAAGCTTGCTTCCCCGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGG
photorhabdus    GGTAACAGGAAAGCGCTTGCGCTTTTGCTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
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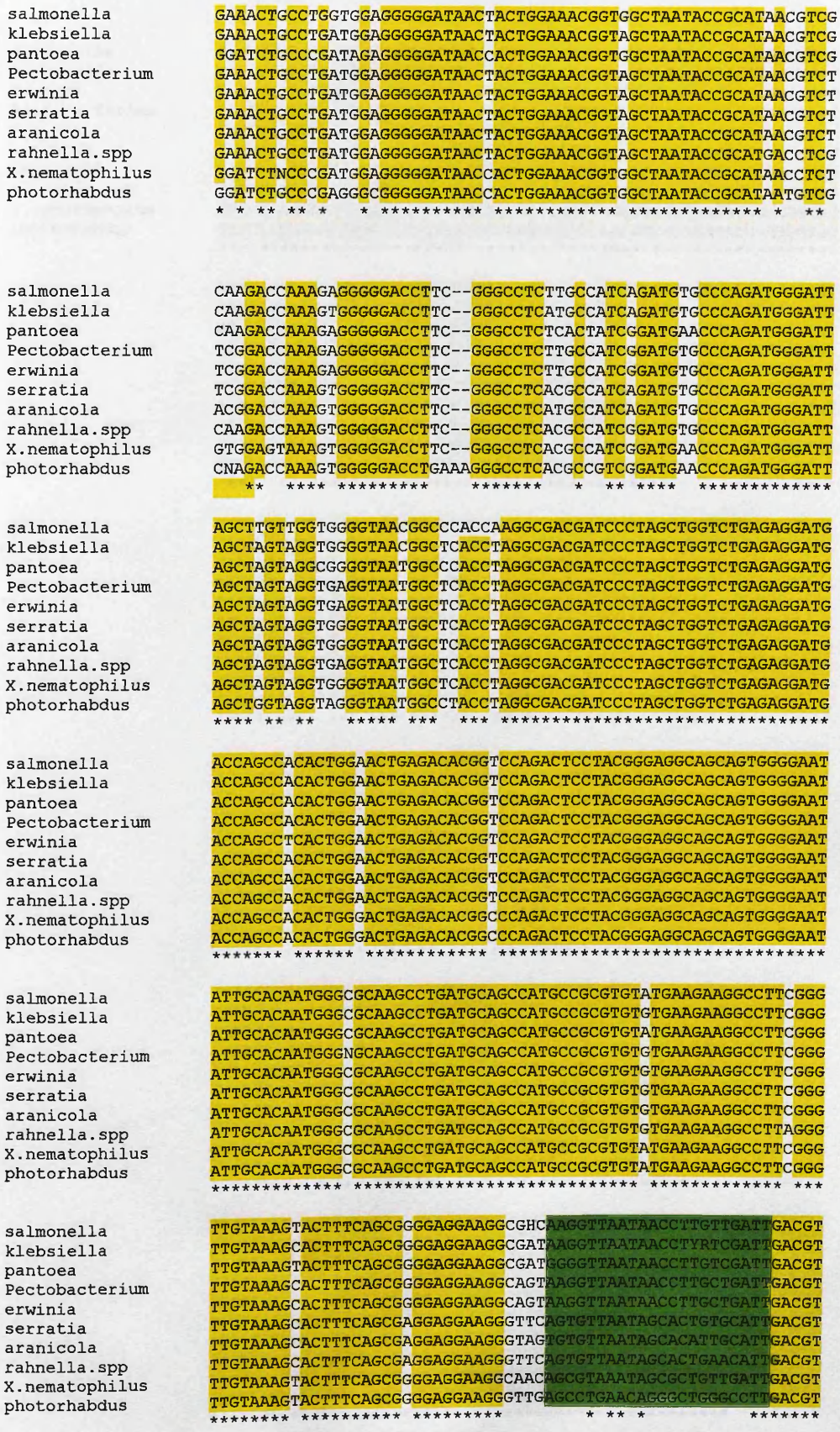
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klebsiella GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCG
pantoea GGATCTGCCCGATAGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACGTCG
Pectobacterium GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCT
erwinia GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCT
serratia GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCT
aranicola GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCT
rahnella.spp GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGACCTCG
X.nematophilus GGATCTNCCCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTCT
photorhabdus GGATCTGCCCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAATGTCG
* * ** ** * * ********** ************ ************ * **
salmonella CAAGACCAAAGAGGGGGACCTTC--GGGCCTCTTGCCATCAGATGTGCCCAGATGGGATT
klebsiella CAAGACCAAAGTGGGGGACCTTC--GGGCCTCATGCCATCAGATGTGCCCAGATGGGATT
pantoea CAAGACCAAAGAGGGGGACCTTC--GGGCCTCTCACTATCGGATGAACCCAGATGGGATT
Pectobacterium TCGGACCAAAGAGGGGGACCTTC--GGGCCTCTTGCCATCGGATGTGCCCAGATGGGATT
erwinia TCGGACCAAAGAGGGGGACCTTC--GGGCCTCTTGCCATCGGATGTGCCCAGATGGGATT
serratia TCGGACCAAAGTGGGGGACCTTC--GGGCCTCACGCCATCAGATGTGCCCAGATGGGATT
aranicola ACGGACCAAAGTGGGGGACCTTC--GGGCCTCATGCCATCAGATGTGCCCAGATGGGATT
rahnella.spp CAAGACCAAAGTGGGGGACCTTC--GGGCCTCACGCCATCGGATGTGCCCAGATGGGATT
X.nematophilus GTGGAGTAAAGTGGGGGACCTTC--GGGCCTCACGCCATCGGATGAACCCAGATGGGATT
photorhabdus CNAGACCAAAGTGGGGGACCTGAAAGGGCCTCACGCCGTCGGATGAACCCAGATGGGATT
** **** ********* ******* * ** **** **********
salmonella AGCTTGTTGGTGGGGTAACGGCCCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
klebsiella AGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
pantoea AGCTAGTAGGCGGGGTAATGGCCCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
Pectobacterium AGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
erwinia AGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
serratia AGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
aranicola AGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
rahnella.spp AGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
X.nematophilus AGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
photorhabdus AGCTGGTAGGTAGGGTAATGGCCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
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klebsiella ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
pantoea ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
Pectobacterium ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
erwinia ACCAGCCTCACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
serratia ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
aranicola ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
rahnella.spp ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
X.nematophilus ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
photorhabdus ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
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klebsiella ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
pantoea ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGG
Pectobacterium ATTGCACAATGGGNGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
erwinia ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
serratia ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
aranicola ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
rahnella.spp ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGGG
X.nematophilus ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGG
photorhabdus ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGG
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klebsiella TTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTYRTCGATTGACGT
pantoea TTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATGGGGTTAATAACCTTGTCGATTGACGT
Pectobacterium TTGTAAAGCACTTTCAGCGGGGAGGAAGGCAGTAAGGTTAATAACCTTGCTGATTGACGT
erwinia TTGTAAAGCACTTTCAGCGGGGAGGAAGGCAGTAAGGTTAATAACCTTGCTGATTGACGT
serratia TTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTGCATTGACGT
aranicola TTGTAAAGCACTTTCAGCGAGGAGGAAGGGTAGTGTGTTAATAGCACATTGCATTGACGT
rahnella.spp TTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGAACATTGACGT
X.nematophilus TTGTAAAGTACTTTCAGCGGGGAGGAAGGCAACAGCGTAAATAGCGCTGTTGATTGACGT
photorhabdus TTGTAAAGTACTTTCAGCGGGGAGGAAGGGTTGAGCCTGAACAGGGCTGGGCCTTGACGT
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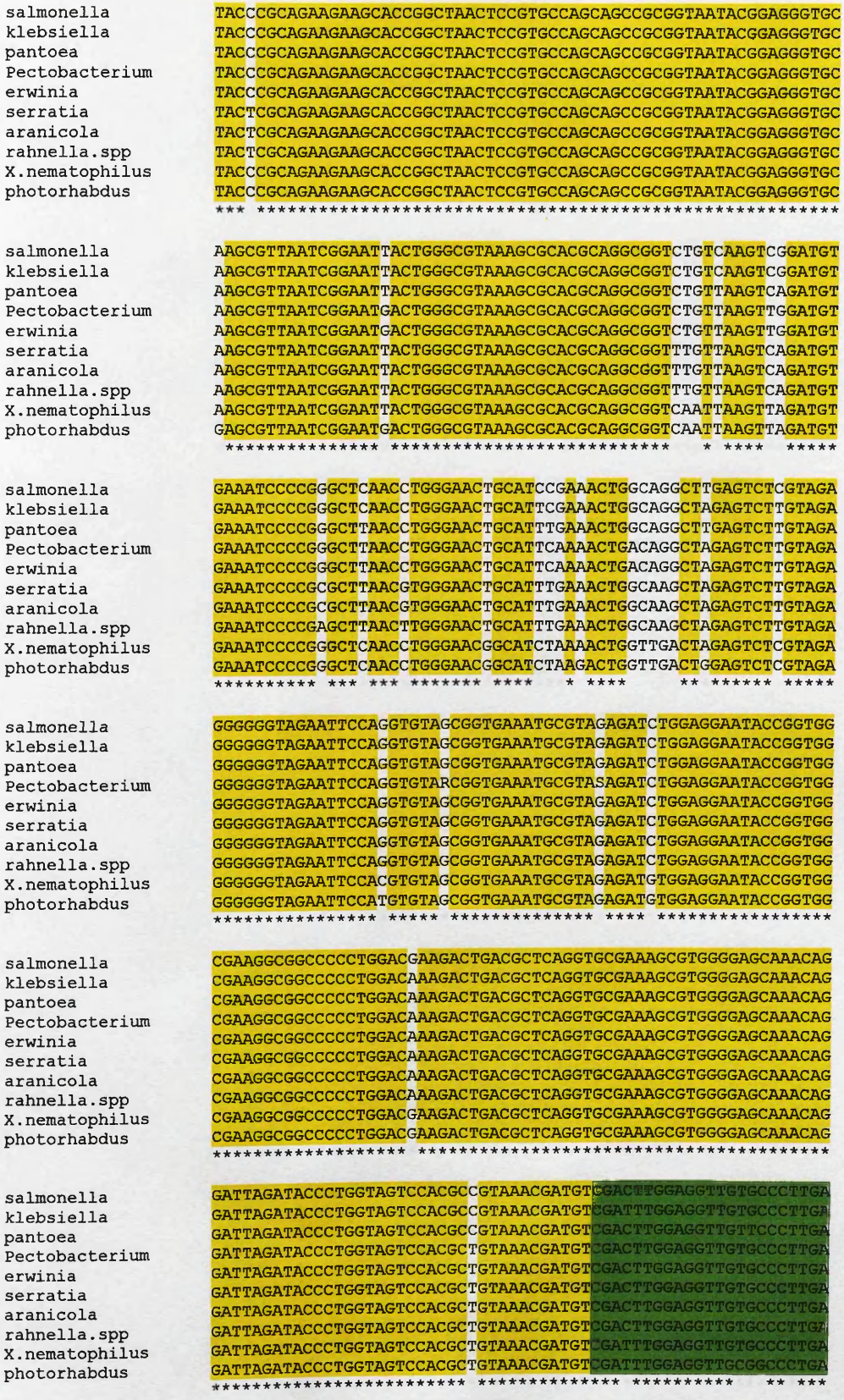
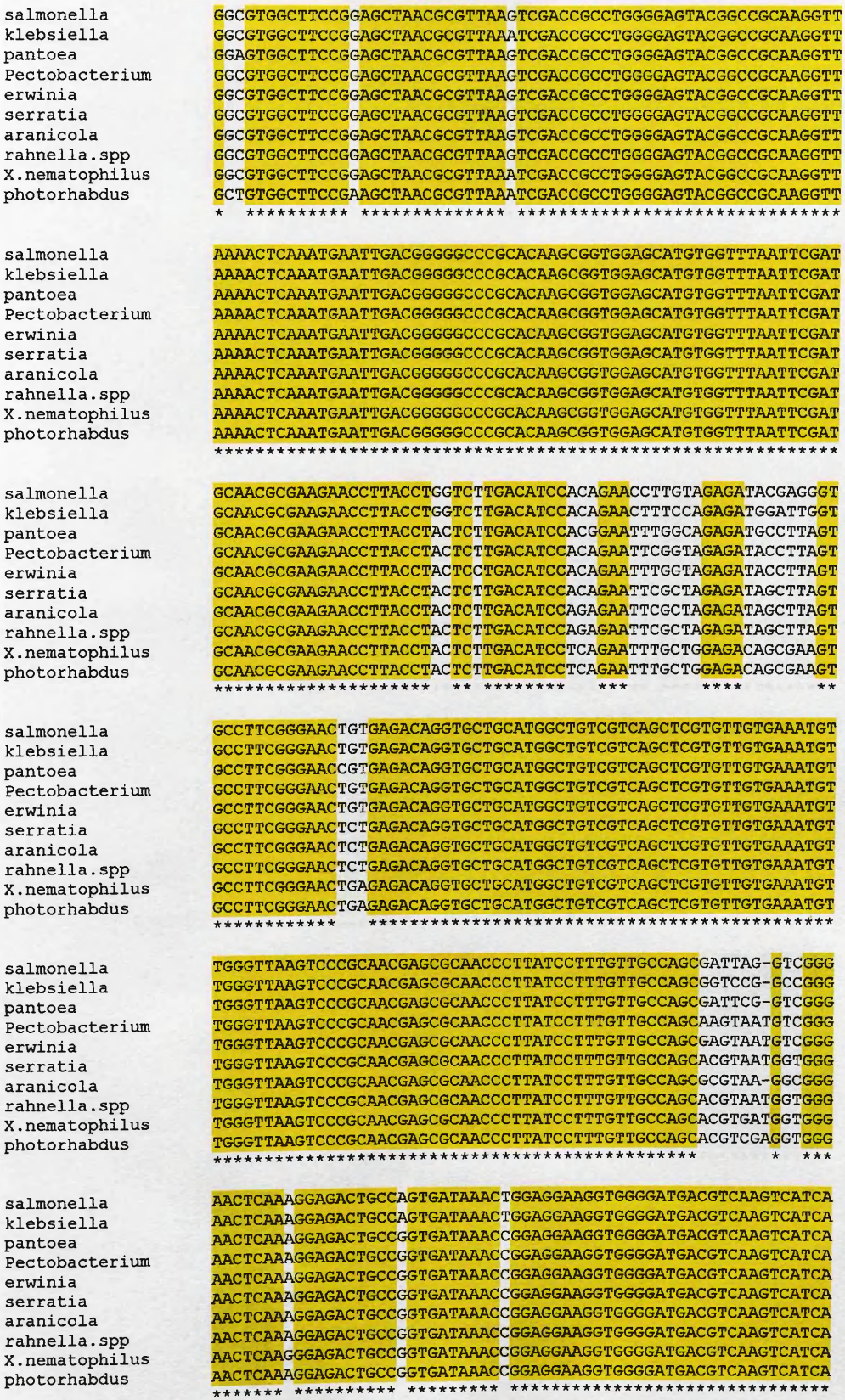

salmonella
klebsiella
pantoea
Pectobacterium
erwinia
serratia
aranicola
rahnella.spp
X.nematophilus
photorhabdus


salmonella
klebsiella
pantoea
Pectobacterium
erwinia
serratia
aranicola
rahnella.spp
X.nematophilus
photorhabdus

```
salmonella          TGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCT
klebsiella          TGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCT
pantoea             TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCT
Pectobacterium      TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCT
erwinia             TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCT
serratia            TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGAACT
aranicola           TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGAACT
rahnella.spp        TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCAAACT
X.nematophilus      TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCT
photorhabdus        TGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGGATACAAAGTGAAGCGACCT
                    ************  ************************  ******** ***** * **

salmonella          CGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
klebsiella          CGCGAGGGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
pantoea             CGCGAGAGCAAGCGGACCTCACAAAGTGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGA
Pectobacterium      CGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
erwinia             CGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
serratia            CGCGAGAGCCAGCGGACCTCATAAAGTACGTCGTAGTCCGGATCGGAGTCTGCAACTCGA
aranicola           CGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATCGGAGTCTGCAACTCGA
rahnella.spp        CGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
X.nematophilus      CGCGAGAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
photorhabdus        CGCGAGAGCAAGCGGAACACACAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
                    ****** ** ****** * ** *****  ************** ****************

salmonella          CTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCC
klebsiella          CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCC
pantoea             CTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCC
Pectobacterium      CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCC
erwinia             CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCC
serratia            CTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCC
aranicola           CTCCGTGAAGTCGGAATCGCTAGTAATCGTAGGTCAGAATGCTACGGTGAATACGTTCCC
rahnella.spp        CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCC
X.nematophilus      CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCC
photorhabdus        CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCC
                    **** ************************* * **** **** *****************

salmonella          GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
klebsiella          GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
pantoea             GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
Pectobacterium      GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
erwinia             GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
serratia            GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
aranicola           GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
rahnella.spp        GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
X.nematophilus      GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT
photorhabdus        GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTNGCAAAAGAAGTCGGTAGCT
                    ************************************** *********** *******

salmonella          TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
klebsiella          TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
pantoea             TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
Pectobacterium      TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
erwinia             TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACACGGGGTGAAGTCGTAACAA
serratia            TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
aranicola           TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
rahnella.spp        TAACCTTCG-GGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
X.nematophilus      TAACCTTCG-GGNGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
photorhabdus        TAACCTTTTTGGAGGGCGCTGACCACTTTGTGGCTCATGACTGGGGTGAAGTCGTAACAA
                    *******   ** ******* ***********  ******* ******************

salmonella          GGTAACC-------------------------------
klebsiella          GGTAACCGT-----------------------------
pantoea             GGTAACCGTAGGGG------------------------
Pectobacterium      GGTAACCGTAGGGGAACCTGC-----------------
erwinia             GGTAAC--------------------------------
serratia            GGTAACCGTAGGGGAAC---------------------
aranicola           GGTAACC-------------------------------
rahnella.spp        GGTAACCGTAGGGGAACCTGCGGCTGGATCACCTCC
X.nematophilus      GGTAACCGTA----------------------------
photorhabdus        GGTAACCGTAGGGGAACCT-------------------
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Figure 4.1 *ClustalX* alignment of 10 16S ribosomal RNA nucleotide sequences. The yellow highlighted area shows nucleotides conserved across all species. The blue highlighted areas are those used for primer design. The green highlighted areas are used for oligonucleotide probe design.

16S rRNA Forward primer:

5'-AGA GTT TGA TCC TGG CTC AG-3'

16S rRNA Reverse primer

5'- GGT TAG CTT GTT ACG ACT-3'

Figure 4.2 The forward and reverse 16S rRNA primers. Product size = 1465bp.

The *ClustalX* alignment (Figure 4.1) showed that 16S rRNA primer sites should be suitable for amplification of the *X. bovienii* 16 S rRNA gene.

To amplify a single band corresponding to the 16S rRNA gene, a number of different reaction conditions were tested using *X. bovienii* genomic DNA as a template. Using a hot start procedure and the PCR conditions described in Section Two (Section 2.3.4) an annealing temperature of 42°C was found to be optimal using a Mg^{2+} concentration of 1.5 mM (Figure 4.3).

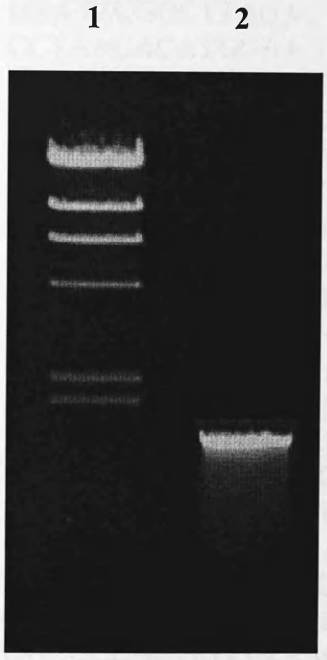

Figure 4.3 Ethidium bromide stained agarose gel of PCR products amplified with EUB338 forward and reverse primers with *X. bovienii* genomic DNA (lane 2) template. Lane 1 contains lambda Hind III markers. Lane 2 contains *X. bovienii* products amplified with 1.5 mM Mg^{2+}.

The single band PCR product for *X. bovienii* shown in lane two (Figure 4.3) was isolated from a LMP gel and was inserted in pCR®2.1 TOPO and transformed into TOP10F' One Shot® (Invitrogen®). Plasmid preparations and restriction digests with Eco R1 yielded the correct sized inserts, which were subsequently sequenced. Sequencing of the plasmid, named pxb16S and searching of the NCBI BLAST sequence similarity database revealed that the cloned fragment from *X. bovienii* was of the expected size, and showed the greatest homology with the 16S rRNA gene from *X. nematophilus* (Figure 4.4).

GAATTCGGCTTAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGG
CCTAACACATGCAAGTCGAGCGGCAGCGGGGGGAAGCTTGCTTCCCCGCC
GGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATCTGCCCGATGGAGG
GGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTCTGTGGAGTA
AAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGGGATT
AGCTAGTANGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTC
TGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCANACTCCTAC
GGGANGCAACAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAN
CATGCCCCCGTGTATTAAGAAGGCCTTCGGGTTGTAAAGTACTTTCNACCG
GGAGGAAGGCAACANCGTAAATAGCGCCGTTGATTGACNTTACCCGCAN
NAANAACCCCGGCTTAANNCTTNCGNGCNNNNNCCATNANTTGNANGGC
GTTGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATT
AAGTTAGATGTGAAATCCCCGGGCTCAACCTGGGAACGGCATCTAAAACT
GGTTGACTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
CGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTT
GAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTAC
GGCCGCNAGGTTAAACTCAAATGAATTTGACGGGGGCCCGCACANNCGGT
GGAGCATGTGGTTTAATTCGATGCAC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1754500|dbj|D78009.1| Xenorhabdus nematophilus DNA for 1...	751	0.0
gi|30842078|gb|AY278674.1| Xenorhabdus nematophila 16S ribo...	749	0.0
gi|21913297|gb|AF522294.1| Xenorhabdus nematophila 16S ribo...	749	0.0
gi|3172002|emb|Z76738.1|XNZ76738 X.nematophilus (strain RIO...	749	0.0
gi|3172001|emb|Z76737.1|XNZ76737 X.nematophilus (strain N2-...	749	0.0
gi|861162|emb|X82251.1|XN16RNA0 X.nematophilus 16S rRNA gen...	749	0.0
gi|1754499|dbj|D78008.1| Xenorhabdus japonicus DNA for 16S ...	749	0.0
gi|30842075|gb|AY278671.1| Photorhabdus sp. HIT 16S ribosom...	747	0.0
gi|30842074|gb|AY278670.1| Photorhabdus sp. JUN 16S ribosom...	747	0.0
gi|30842049|gb|AY278645.1| Photorhabdus luminescens subsp. ...	745	0.0
gi|30842046|gb|AY278642.1| Photorhabdus luminescens subsp. ...	745	0.0
gi|6456832|emb|AJ007359.1|PHO7359 Photorhabdus luminescens ...	745	0.0
gi|2182051|emb|Z76745.1|PLZ76745 Photorhabdus luminescens s...	745	0.0
gi|861163|emb|X82253.1|XP16RNA8 X.poinarii 16S rRNA gene (D...	745	0.0
gi|29164906|gb|AY216500.1| Photorhabdus sp. Q614 16S riboso...	741	0.0
gi|861085|emb|X82250.1|PLRNAHSH2 P.luminescens 16S rRNA gen...	741	0.0
gi|30842048|gb|AY278644.1| Photorhabdus luminescens subsp. ...	737	0.0
gi|30842047|gb|AY278643.1| Photorhabdus luminescens subsp. ...	737	0.0
gi|3256225|emb|Y17605.1|PLY17605 Photorhabdus luminescens 1...	737	0.0
gi|30842076|gb|AY278672.1| Photorhabdus asymbiotica 16S rib...	733	0.0

Figure 4.4 The 16S rRNA gene sequence from *X. bovienii*. The first 20 BLASTn scores are shown. The EcoR1 restriction site at the beginning of the construct is highlighted in yellow.

The 16S rRNA gene sequence from *X. bovienii* and *X. nematophilus* were aligned using *ClustalX* software (Figure 4.5). The unconserved regions' from the first *ClustalX* alignment (Figure 4.1), highlighted in green and blue, were considered for oligonucleotide probe design.

The oligonucleotide probes (Figure 4.6) designed from the green highlighted area (XEN1) and the blue highlighted area (XEN2) were manufactured by MWG-Biotech, purified to HPSF level and labelled with digoxygenin (DIG).

* indicates an amino acid match across all sequences

```
X.nematophilus  ------------------------------------------------------------
X.bovienii      CTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAGAGT

X.nematophilus  -----CCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAG
X.bovienii      TTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAG
                     ************************************************************

X.nematophilus  CGGGGGGAAGCTTGCTTCCCCGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATC
X.bovienii      CGGGGGGAAGCTTGCTTCCCCGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGGATC
                ************************************************************

X.nematophilus  TNCCCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTCTGTGGA
X.bovienii      TGCCCGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACCTCTGTGGA
                * ************************************************************

X.nematophilus  GTAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGGGATTAGCTAGT
X.bovienii      GTAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGAACCCAGATGGGATTAGCTAGT
                ************************************************************

X.nematophilus  AGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCC
X.bovienii      ANGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCC
                * ************************************************************

X.nematophilus  ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
X.bovienii      ACACTGGGACTGAGACACGGCCCANACTCCTACGGGANGCAACAGTGGGGAATATTGCAC
                ************************ ************ *** ******************

X.nematophilus  AATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAA
X.bovienii      AATGGGCGCAAGCCTGATGCANCATGCCCCCGTGTATTAAGAAGGCCTTCGGGTTGTAAA
                ********************** *    ** ******* ************************

X.nematophilus  GTACTTTCAGCGGGGAGGAAGGCAACAGCGTAAATAGCGCTGTTGATTGACGTTACCCGC
X.bovienii      GTACTTTCNACCGGGAGGAAGGCAACAGCGTAAATAGCGCCGTTGATTGACNTTACCCGC
                ******** * ************************** ********** ********

X.nematophilus  AGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT
X.bovienii      ANNAANAACCCCGGCTTAANNCTTNCGNGCNNNNNCCATNANTTGNANGGCGTT-GCGTT
                *  *  * * ****** *   * * *  **     *  * *   * * ** *   *****

X.nematophilus  AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCC
X.bovienii      AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTTAGATGTGAAATCC
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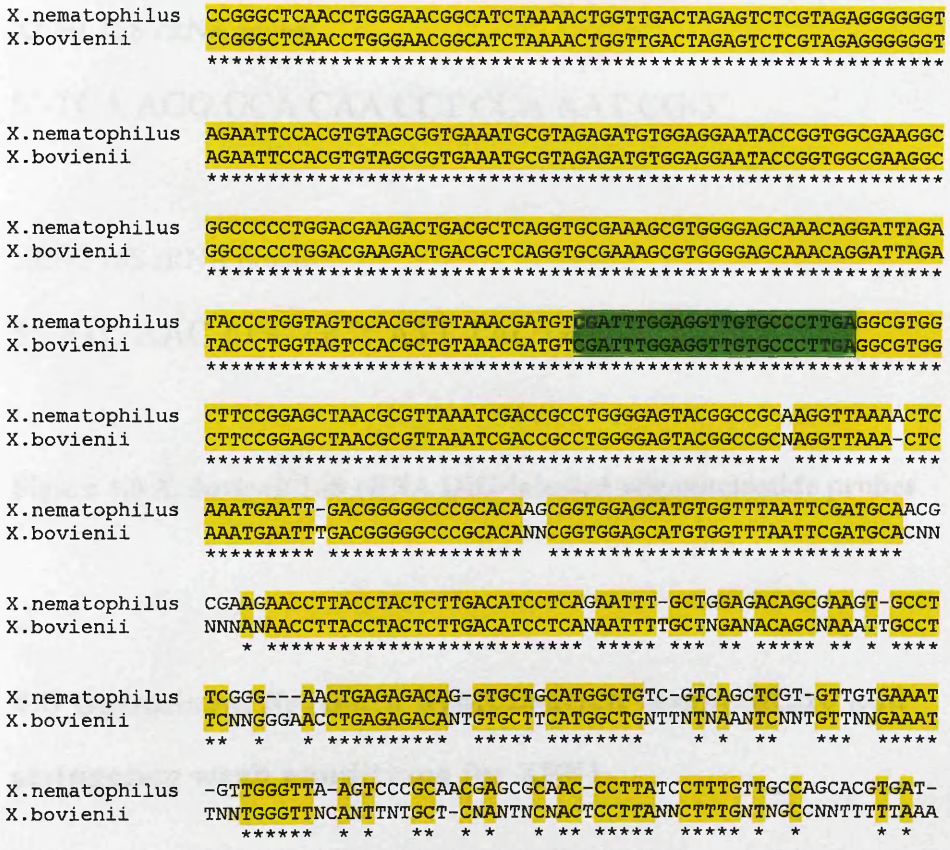


Figure 4.5 *ClustalX* alignment of *X. bovienii* and *X. nematophilus* nucleotide sequences. The yellow highlighted area are residues conserved across both species. The blue and green highlighted areas were those chosen for oligonucleotide probe design.

XEN1 16S rRNA probe

5'-TCA AGG GCA CAA CCT CCA AAT CG-3'

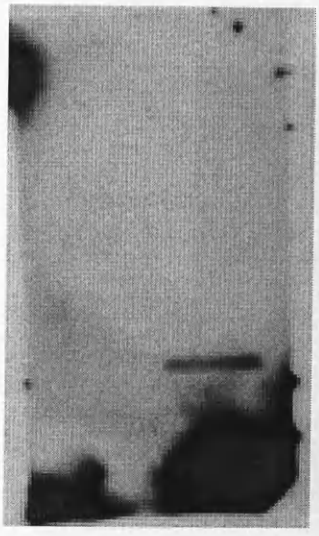
XEN2 16S rRNA probe

5'-ATC AAC AGC GCT ATT TAC GCT-3'

Figure 4.6 *X. bovienii* 16S rRNA DIG-labelled oligonucleotide probes

4.3 Optimising Northern hybridisation temperature and stringency wash conditions for XEN1

Northern slot blots of 1 μg and 0.1 μg total RNA isolated from *X. bovienii* and *E. coli* cultures grown until mid-log phase in DSMZ and LB medium, respectively were hybridised overnight in DIG Easyhyb™ (Roche) hybridisation solution at a range of temperatures from 50°C to 60°C. Stringency washes in 2x SSC/0.1% SDS, 1x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1% SDS at a range of temperatures from 50°C to 65°C were tested. A hybridisation temperature of 60°C with a stringency wash of 1x SSC/0.1% SDS at 65°C proved to be optimal for the XEN1 probe (Figure 4.7). The blot indicated that the XEN1 probe bound to 1 μg and 0.1 μg *X. bovienii* total RNA, but failed to detect 1 μg or 0.1 μg *E. coli* total RNA.



1 µg *E. coli*

0.1 µg *E.coli*

1 µg *X. bovienii*

0.1 µg *X. bovienii*

Figure 4.7 Northern slot blot of 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *X. bovienii* probe (XEN1). The 1 µg and 0.1 µg *E. coli* and the 0.1 µg *X. bovienii* loadings were not detectable.

The XEN1 probe was tested further with Northern slot blots of 20 µg, 2 µg, 0.2 µg and 0.02µg total RNA from injection infection and uninfected *G. mellonella* with 1 µg and 0.1 µg RNA from *X. bovienii* and *E. coli* as positive and negative controls. Blots were hybridised using the optimised conditions described above (Figure 4.8) Detection of *E. coli* remained negative, however the XEN1 probe hybridised to 20 µg of *G. mellonella* RNA for both uninfected and infected RNA. Detection of uninfected *G. mellonella* RNA showed that the XEN1 probe was not specific enough to distinguish between RNA from bacteria present in uninfected or infected *G. mellonella*. The alternative probe for detection of *X. bovienii* rRNA, XEN2, was optimised (Section 4.4).

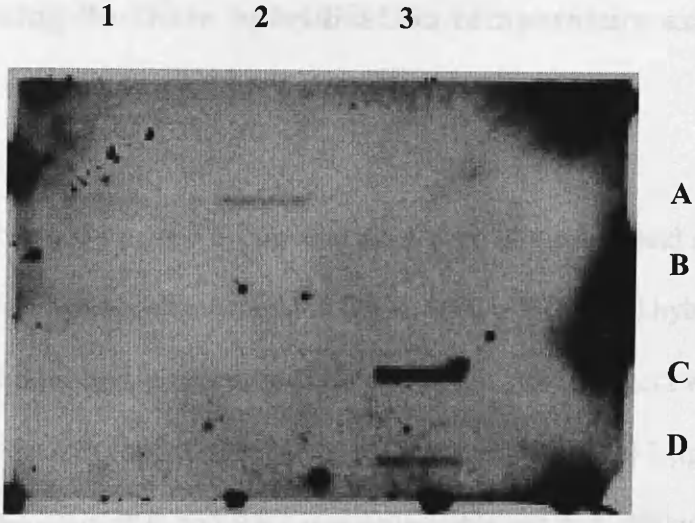


Figure 4.8 Northern slot blot of 20 µg, 2 µg, 0.2 µg and 0.02 µg total RNA from injection and uninfected *G. mellonella* and 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *X. bovienii* probe (XEN1). Lane 1 contains 20 µg (A) to 0.02 µg (D) of *G. mellonella* uninfected total RNA. Lane 2 contains 20 µg (A) to 0.02 µg (D) of *G. mellonella* natural infection total RNA. Lane 3 contains 1 ug (A) and 0.1ug (B) of *E. coli* RNA and 1 µg (C) and 0.1 µg (D) of *X. bovienii* RNA. The 1 µg and 0.1 µg *E. coli*, 0.1 µg *X. bovienii* and the 2 µg to 0.2 µg injection and natural infection *G. mellonella* loadings were not detectable.

4.4 Optimising Northern hybridisation temperature and stringency wash conditions for XEN2

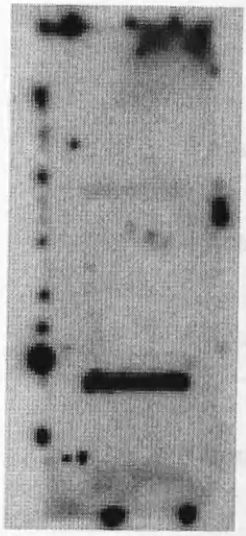
Northern slot blots of 1 μg and 0.1 μg total RNA from *X. bovienii* and *E. coli*, respectively were hybridised overnight in DIG Easyhyb™ (Roche) hybridisation solution. A hybridisation temperature of 60°C with the most stringent wash of 0.1x SSC/0.1% SDS at 65°C still resulted in detection of the 1 μg and 0.1 μg *E. coli* RNA (Figure 4.9). Detection of *E. coli* RNA was undesirable and was attributed to high cross-linkage of RNA at the site of the probe target resulting in high binding efficiency of the probe to the target RNA.

A new method of RNA denaturing prior to application to the membrane when loading Northern slot blots was applied. RNA was denatured by heating to 65°C for 15 minutes followed by cooling to below 0°C in an ethanol bath maintained at -20°C. A new loading buffer (500 μl formamide, 162 μl formaldehyde, 100 μl MOPS buffer (0.2 M MOPS, 0.5 M Na Acetate, 0.1 M EDTA, pH 7) 508 μl 20x SSC) was used. A hybridisation temperature of 55°C with a stringency wash of 1x SSC/0.1% SDS at 50°C proved to be optimal for distinction between *E. coli* and *X. bovienii* RNAs (Figure 4.10). The blot indicated that the XEN2 probe bound successfully to 1 μg and 0.1 μg *X. bovienii* total RNA, but failed to detect 1 μg or 0.1 μg *E. coli* total RNA.

The XEN2 probe was tested further with Northern slot blots of 20 μg, 2 μg and 0.2 μg total RNA from injection infected (Times 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, and 72 hours) and uninfected *G. mellonella* in combination with 1 μg and 0.1 μg RNA from *X. bovienii* and *E. coli* were hybridised using the optimised conditions mentioned

above. Positive signals were obtained post infection with loadings of 20 μg of *G. mellonella* RNA. The blot indicated that 1 μg and 0.1 μg of *E. coli* RNA and 20 μg of uninfected *G. mellonella* RNA remained undetectable, in comparison to 20 μg of *G. mellonella* RNA from two to 72 hours infection, which were all detected at variable signal strengths (Figure 4.11).

Northern slot blots of 20 μg total RNA from 100 *S. feltiae/G. mellonella* and 1000 *S. feltiae/G. mellonella* natural infection (Times 12, 24, 36, 48 and 72 hours) and uninfected *G. mellonella* in combination with 1 ug RNA from *E. coli* and 1 μg and 0.1 μg RNA from *X. bovienii* were hybridised with the optimised conditions mentioned above. 1 μg of *E. coli* RNA, 20 μg of 12 hour 100 *S. feltiae/G. mellonella* and 20 μg of uninfected *G. mellonella* RNA remained undetectable, in comparison to 20 μg of 24 to 72 hour 100 *S. feltiae/G. mellonella* and 20 μg of 12 to 72 hour 1000 *S. feltiae/G. mellonella* which were all detected at variable signal strengths (Figure 4.12).



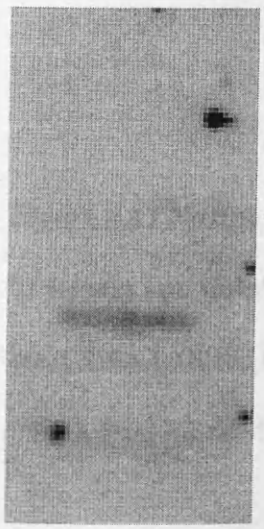
1 µg *E. coli*

0.1 µg *E. coli*

1 µg *X. bovienii*

0.1 µg *X. bovienii*

Figure 4.9 Northern slot blot of 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *X. bovienii* probe (XEN2).



1 µg *E. coli*

0.1 µg *E. coli*

1 µg *X. bovienii*

0.1 µg *X. bovienii*

Figure 4.10 Northern slot blot of 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *X. bovienii* probe (XEN2). The 1 µg and 0.1 µg *E. coli* loadings were not detectable.

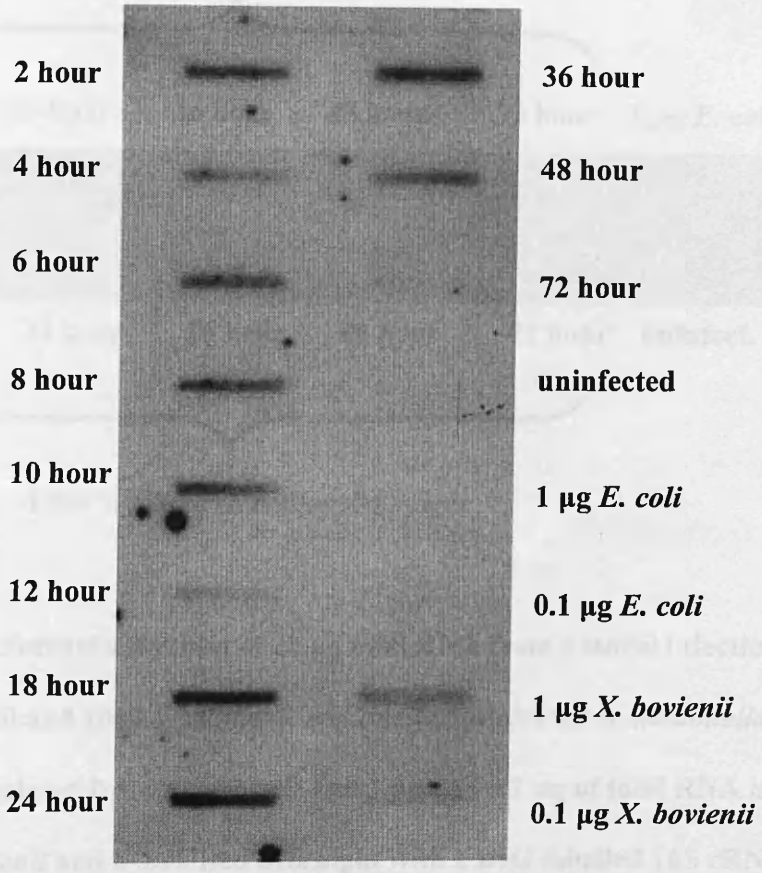


Figure 4.11 Northern slot blot of 20 µg total RNA from injection infection (2 to 72 hours) and uninfected *G. mellonella* and 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *X. bovienii* probe (XEN2).

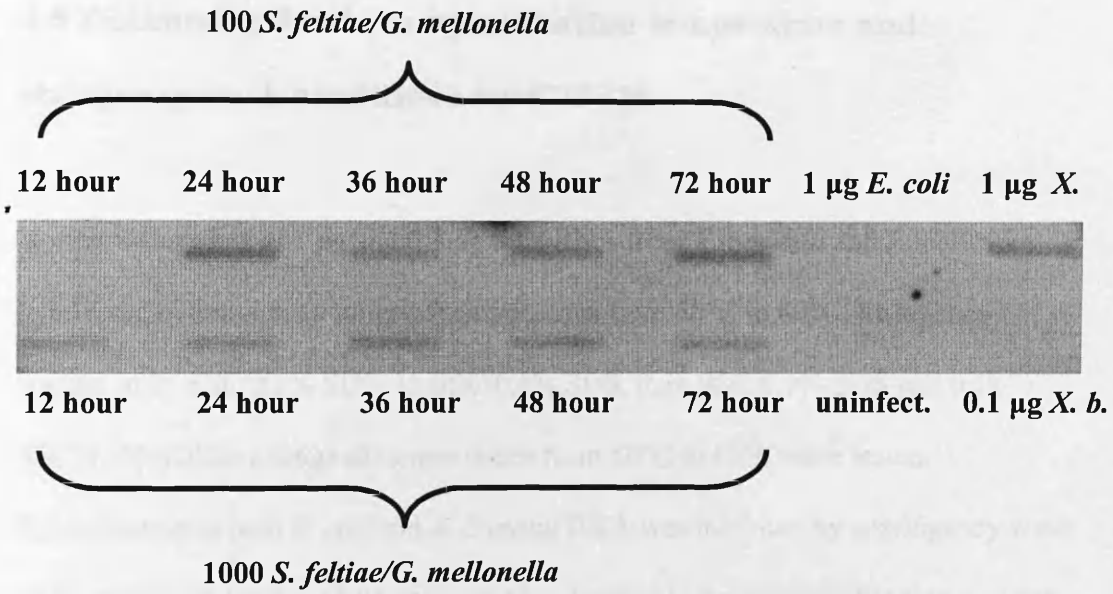


Figure 4.12 Northern slot blot of 20 µg total RNA from natural infection (12 to 72 hours) 100 and 1000 *S. feltiae/G. mellonella*, uninfected *G. mellonella*, 1 µg of total RNA isolated from *E. coli* cells and 1 µg and 0.1 µg of total RNA isolated from *X. bovienii* and hybridised overnight with a DIG labelled 16S rRNA *X. bovienii* probe (XEN2).

4.5 Optimising Northern hybridisation temperature and stringency wash conditions for EUB338

Northern slot blots of 1 μg and 0.1 μg total RNA from *X. bovienii* and *E. coli* were hybridised overnight at a range of temperatures from 50°C to 60°C. Stringency washes in 2x SSC/0.1% SDS, 1x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1% SDS at a range of temperatures from 50°C to 65°C were tested. Hybridisation to both *E. coli* and *X. boevinii* RNA was inhibited by a stringency wash of 1x SSC/0.1% SDS at 65°C, this was an indication that unspecific binding was not occurring, which would skew the results. With reference to this a hybridisation temperature of 60°C with a stringency wash of 1x SSC/0.1% SDS at 60°C proved to be optimal for the EUB338 probe (Figure 4.13).

The EUB338 probe was tested further with Northern slot blots of 1 μg and 0.1 μg total RNA from injection infection and uninfected *G. mellonella* in combination with 1 μg and 0.1 μg RNA from *X. bovienii* and *E. coli,* hybridised overnight at 60°C with stringency washes at 60°C and 1x SSC/0.1% SDS (Figure 4.14). The hybridisation signal from 1 μg and 0.1 μg *E. coli* and *X. bovienii* RNA was almost equal, whereas that from infected *G. mellonella* RNA was less so. This was to be expected, however, as a large percentage of the RNA present within the 1 μg and 0.1 μg total *G. mellonella* RNA loadings was caterpillar RNA, which would have been undetected by the EUB338 probe and would result in a weaker signal. RNA from uninfected *G. mellonella* did not hybridise, which was also predicted, as uninfected *G. mellonella* will carry only a few gut bacteria, the amount of bacterial rRNA in the total RNA preparation would therefore be minimal.

The EUB338 probe was tested further with Northern slot blots of 20 μg, 10 μg, 1 μg and 0.1 μg total RNA from injection infected (Times 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, and 72 hours) and uninfected *G. mellonella* in combination with 1 μg and 0.1 μg RNA from *X. bovienii* and *E. coli* hybridised overnight in DIG Easyhyb™ (Roche) hybridisation solution at 60°C with stringency washes at 60°C and 1x SSC/0.1% SDS. Positive signals were obtained post infection with loadings of 20 μg of *G. mellonella* RNA. 20 μg of uninfected *G. mellonella* RNA remained undetectable, as did 20 μg, two to 10 hours infection *G. mellonella* RNA, in comparison to 20 μg of *G. mellonella* RNA from 12 to 72 hours infection, which were all detected at variable signal strengths (Figure 4.15).

Northern slot blots of 20 μg total RNA from 100 *S. feltiae/G. mellonella* and 1000 *S. feltiae/G. mellonella* natural infection (Times 12, 24, 36, 48 and 72 hours) and uninfected *G. mellonella* in combination with 1ug RNA from *E. coli* and 1 μg and 0.1 μg RNA from *X. bovienii* hybridised overnight with DIG Easyhyb™ (Roche) hybridisation solution at 55°C with stringency washes at 50°C and 1x SSC/0.1% SDS. 20 μg of 12 hour 100 and 1000 *S. feltiae/G. mellonella* and 20 μg of uninfected *G. mellonella* RNA remained undetectable, in comparison to 20 μg of 24 to 72 hour 100 *S. feltiae/G. mellonella* and 20 μg of 12 to 72 hour 1000 *S. feltiae/G. mellonella* which were all detected at variable signal strengths (Figure 4.16).

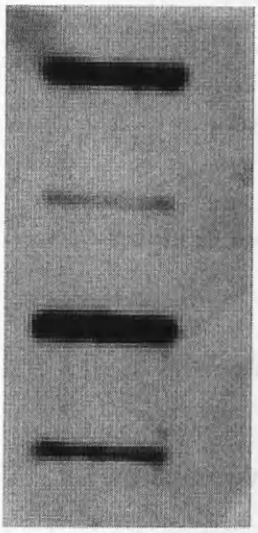


Figure 4.13 Northern slot blot of 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *Eubacteriaceae* probe (EUB338).

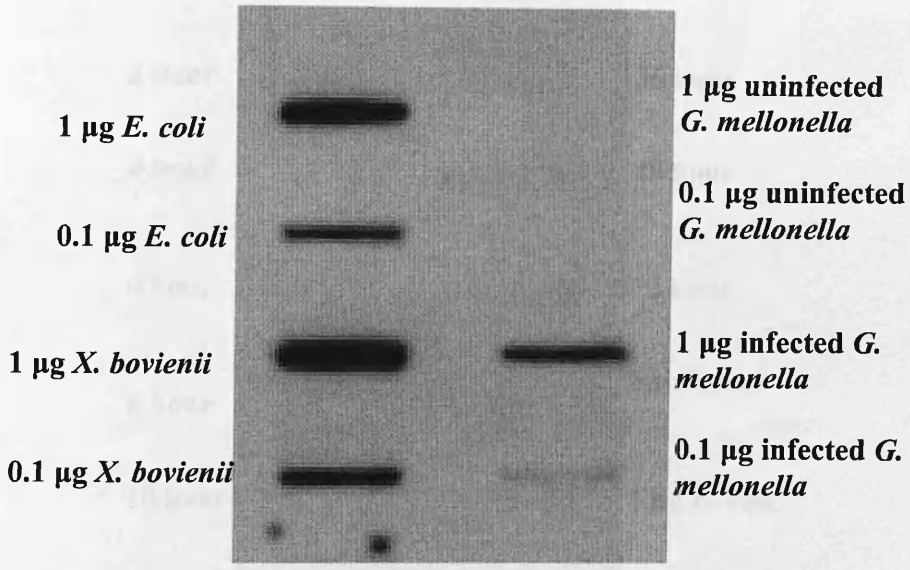


Figure 4.14 Northern slot blot of 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells; 1 µg and 0.1 µg of uninfected *G. mellonella* total RNA; 1 µg and 0.1 µg of *S. feltiae* infected *G. mellonella* total RNA, hybridised overnight with a DIG labelled 16S rRNA *Eubacteriaceae* probe. 1 µg and 0.1 µg uninfected *G. mellonella* RNA was undetectable.

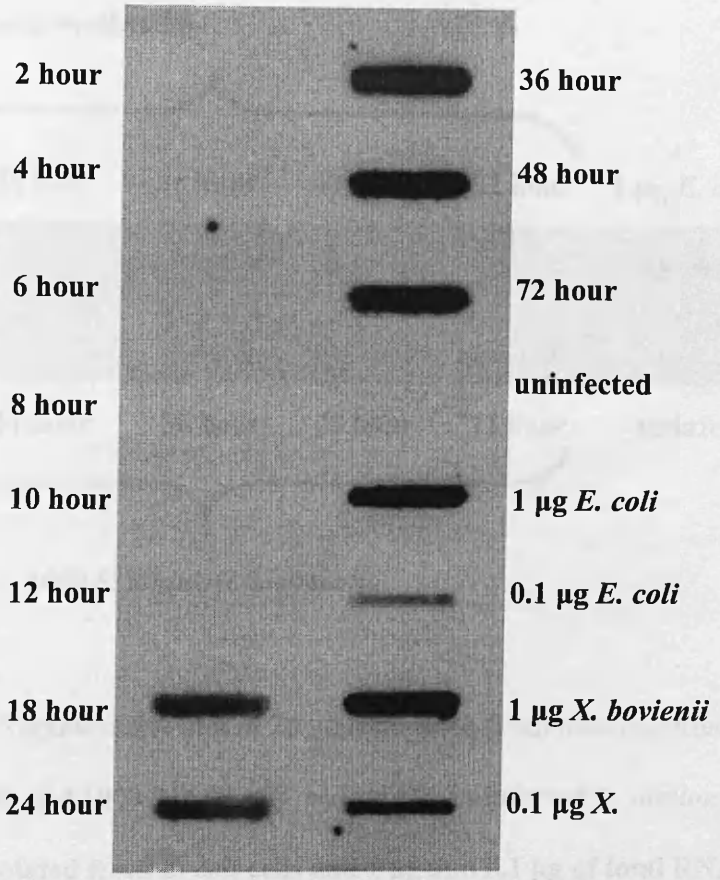


Figure 4.15 Northern slot blot of 20 µg total RNA from injection infection (2 to 72 hours) and uninfected *G. mellonella* and 1 µg and 0.1 µg concentrations of total RNA isolated from *X. bovienii* and *E. coli* cells and hybridised overnight with a DIG labelled 16S rRNA *Eubacteriaceae* probe (EUB338).

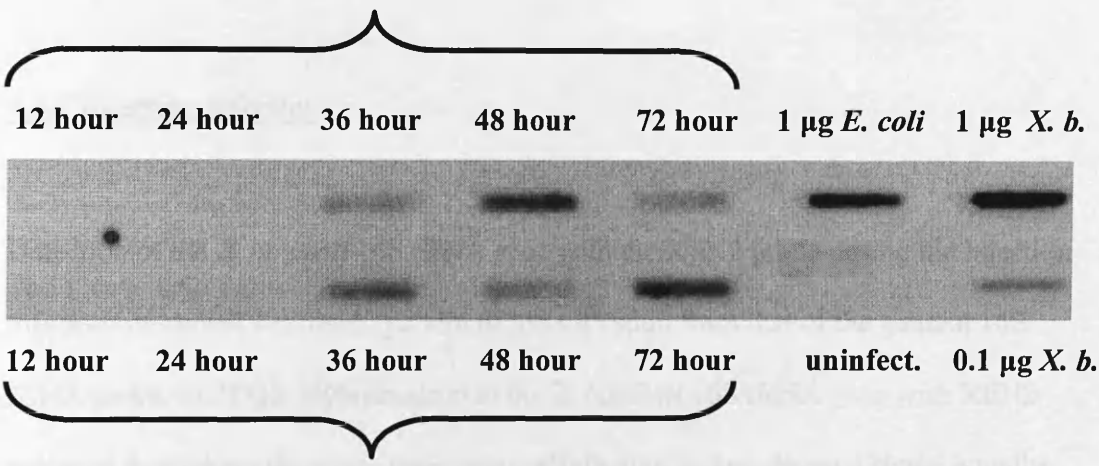


Figure 4.16 Northern slot blot of 20 µg total RNA from natural infection (12 to 72 hours) 100 and 1000 *S. feltiae/G. mellonella*, uninfected *G. mellonella*, 1 µg of total RNA isolated from *E. coli* cells and 1 µg and 0.1 µg of total RNA isolated from *X. bovienii* and hybridised overnight with a DIG labelled 16S rRNA *Eubacteriaceae* probe (EUB338).

4.6 Discussion

4.6.1 Injection infection

Detection of the *X. bovienii* 16S rRNA gene with the XEN2 probe during the injection infection presented a different pattern of hybridisation from that of the general 16S rRNA probe, EUB338. Hybridisation to the *X. bovienii* 16S rRNA gene with XEN2 occurred throughout the entire time series of infection and produced a signal equally as strong as that for 1 μg of *Xenorhabdus* RNA. On the other hand, hybridisation of the EUB338 probe was only detected after 18 hours of infection, this signal was also as strong as that for 1 μg of *Xenorhabdus* and *E. coli* RNA. The hybridisation signal detected for the EUB338 probe is an cumulative indication of the number of *Xenorhabdus* present at any given time point plus the number of general *Eubacteriaceae* present as well.

A decrease in the hybridisation signal for XEN2 was noted after 12 hours and again at 72 hours, suggesting that a decrease in the amount of *Xenorhabdus* rRNA and therefore, a decrease in the number or replication of *Xenorhabdus* occurred at these times. This can be correlated with the massive increase in the hybridisation signal for EUB338 at time 18 hours, that can be attributed to an increase in the number or replication rate of the general *Eubacteriaceae* bacterial populations. This increase could be attributed to *S. feltiae* breaking through the gut lumen of the host and releasing the general gut flora into the haemocoel where they could undergo rapid and reasonably unchecked division due to the availability of nutrients.

The rapid introduction of the gut flora to the haemocoel would have overwhelmed the *Galleria* immune system, allowing the *Xenorhabdus* population to increase with the general gut flora. At 72 hours, however, the number of active *Xenorhabdus* sharply declines yet the number of active *Enterobacteriaceae* remains constant. This suggests that the *Xenorhabdus* have been out competed for resources by the general gut flora. The literature states that an infection with *Xenorhabdus* will lead to a monoxenic population of the entomopathogenic bacterium (Akhurst 1988), this study shows that when the injection method of infecting host *Galleria* is employed this is not the case and the *Xenorhabdus* population is overwhelmed by the released gut bacteria.

4.6.2 Natural infection

Natural infection mimics the type of infection which would occur in the field, where the nematodes are allowed to enter the host by natural openings, such as the mouth, anus and spiracles. In the field, however, a single host *Galleria* is unlikely to encounter 1000 nematodes, as is often the case under laboratory conditions (Tachibana *et al* 1996, Saunders & Webster 1999, Bohan & Hominick 1995) therefore, a comparison of how the number of nematodes (1000 or 100 per host) during a natural infection effects the bacterial populations within *Galleria* was conducted.

A natural infection of 100 nematodes led to hybridisation of XEN2 at time 24 hours, which remained relatively constant until 72 hours. In contrast the infection of 1000 nematodes lead to hybridisation at 12 hours, which suggests that the increase in the number of nematodes overwhelmed the host immune system much earlier in the

infection, this suggests that the use of a large number of nematodes does not mimic the infection process as it would progress in the field where only a few nematodes at any one time would be in contact with the host.

However, hybridisation of the EUB338 probe shows that even natural infection of large numbers of nematodes results in a late emergence of the number of *Enterobacteriaceae* or a late up-regulation in their replication rate, as hybridisation does not occur until 36 hours of infection. Although both the 100 and 1000 nematode infections result in late replication of the general *Enterobacteriacea* the 100 nematode infection follows the more expected route, whereby the number of *Enterobacteriacea* decreases at 72 hours of infection. However, the 1000 nematode infection results in an increase in replication rate of the general *Enterobacteriaceae* from 36 to 72 hours following infection.

This study shows that the use of smaller numbers of nematodes and a natural infection process leads to infection parameters which mimic that which would be observed in the field situation.

4.6.3 Future work

Further analysis of natural infections with different numbers of infecting nematodes, and the densitometric analysis of the natural and injection infection Northern slot blots would allow quantitative analysis of the hybridisation of both the XEN2 and EUB338 probes to the *Galleria* total RNA.

The fixation of whole larvae and the subsequent use of *in-situ* hybridisation utilising the *X. bovienii* specific probe and the general *Eubacteriaceae* probe would ascertain the exact numbers of the different bacteria types within a single given host. This would improve upon the method employed in this study, where only the amount of bacterial RNA from a group of larvae is detectable. *In-situ* hybridisation would also allow the location of individual bacteria within the larvae to be detected, leading to further conclusions as to the preferred physiological environment of the different types of bacteria within an infected host.

CHAPTER FIVE

Results: Isolation and Characterisation an internal fragment of the rpoS and FliC gene from *Xenorhabdus bovienii* (strain UK76)

5.1 Introduction

The two different phases of *X. bovienii*, Phase I and Phase II are thought to express different genes depending on the environmental conditions to which they are exposed (Couche *et al*, 1987). *S. feltiae* are reported to carry Phase I *X. bovienii* within a pharyngeal pouch (Binnington & Brooks, 1993), although there is some contention about this as it is know that *Xenorhabdus* spp will revert to Phase II in the event of environmental stress, such as that experienced within the nematode (Krasomil-Osterfield 1993).

It is difficult to ascertain at what point during infection Phase change occurs as removing *Xenorhabdus* from the host results in a change in environmental conditions which could ultimately precipitate Phase change. Thus, the ability to monitor *in situ* Phase change through the expression of genes thought to be expressed solely in either Phase I or Phase II throughout an infection would be highly beneficial.

The FliC gene, along with FliA and FliD, encodes the structural components of the flagellum in many bacterial species including *E. coli* (Dybvig 1993), *Psueudomonas*

spp. (Brunham *et al* 1993), *Yersinia* spp. (Saunders 1986) and *Salmonella* spp. (Henderson *et al* 1999) and is thought to be a positive indicator of Phase I in *Xenorhabdus* spp. (Akhurst *et al* 1986).

Entry into stationary phase, or Phase II, is accompanied by the switch to a different sigma factor, σS, the product of the rpoS gene. The expression of the rpoS gene results in the activation of a number of different genes which are required for survival under conditions of environmental stress, such as a reduction in osmolarity (Yildiz & Schoolnik 1998).

5.2 Cloning and Sequencing of the rpoS fragment

5.2.1 Primer design and PCR amplification of a rpoS fragment

Specific primers were designed to isolate the gene fragment from the total DNA which was extracted from *X. bovienii* using the CSCl extraction method (Figure 5.1).

The rpoS peptide sequence of *X. nematophilus* was aligned with other sequences from a number of other species using *ClustalX* software. Those species which displayed the most similar rpoS sequence to the *X. nematophilus* sequence when performing a BLASTp search were chosen. This resulted in 11 species of Enterobacteriacea; *Escherichia coli*, *Yersinia pestis*, *Pantoea agglomeris*, *Enterobacter cloacae*, *Kluyvera cryocrescens*, *Shigella flexneri*, *Salmonella typhimurium*, *Pectobacterium enterica*, *Erwinia cartovorum* and *Erwinia amylovora* undergoing alignment with *X. bovienii* (Figure 5.2). The conserved regions were highlighted and investigated for

their suitability as primer design sites. Partially degenerate primers were designed for the sequences selected (Figure 5.3).

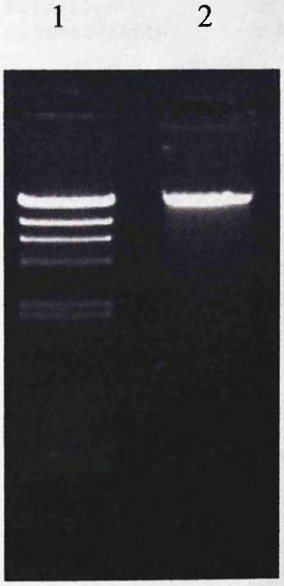


Figure 5.1 Ethidium Bromide stained agarose gel of *X. bovienii* Phase 1 DNA. Lane 1: lambda Hind III markers. Lane 2: *X. bovienii* DNA.

Figure 5.2 *ClustalX* alignment of 11 rpoS peptide sequences. The yellow highlighted area are residues conserved across all species.

* indicates an amino acid match across all sequences

: indicates highly conservative substitutions across the sequences

. indicates conservative substitutes across the sequences

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S.flexneri          ----MFRQGITGRSHLMSQNTLKVHDLNEDAEFDENG--VEVFDEKALVEEEPSDNDLAE
E.coli              ----MFRQGITGRSHLMSQNTLKVHDLNEDAEFDENG--VEVFDEKALVEEEPSDNDLAE
S.typhimurium       ----------------MSQNTLKVHDLNEDAEFDENG--VEAFDEKALSEDEPSDNDLAE
E.cloacae           MPARVSRPGITGRSHLMSQNTLKVHDLNEDAEFDENG--AEAFDEKALVEEEPSDNDLAE
K.cryocrescens      -MIRCIAWGSRVGATLMSQNTLKVHDLNEDAEFDENG--AETFDEKVLNEEEPSDSELAE
E.amylovora         ----------------MSQNTLKVNELNEDAEFDENG--AEVFDEKALVGNEPGDNELAE
P.agglomeris        ----------------MNQNTLKVNELQDDADFDENATEAESFDEKALVEEEASESDLA-
E.chrysanthemi      ----------------MSQNTLKVNELHEDAEFDENG--IDVFDDKALAEEETSDSDQLD
P.carotovorium      ----------------MSQSTLKVNELHEDTDFEENG--LDVFDDKALAEEDTNDNDSAE
Y.pestis            ----------------MSQSTLKVNELHEDADFDENSTETEIFDEKALVDDEPTESELAD
x.nematophilus      ----------------MSQSTLKVNELYDDADLDENSMEADDFDEALLKNGD-DVTNLDE
                                    *.*.****::* :*::::**.   : **:  *   :   .:
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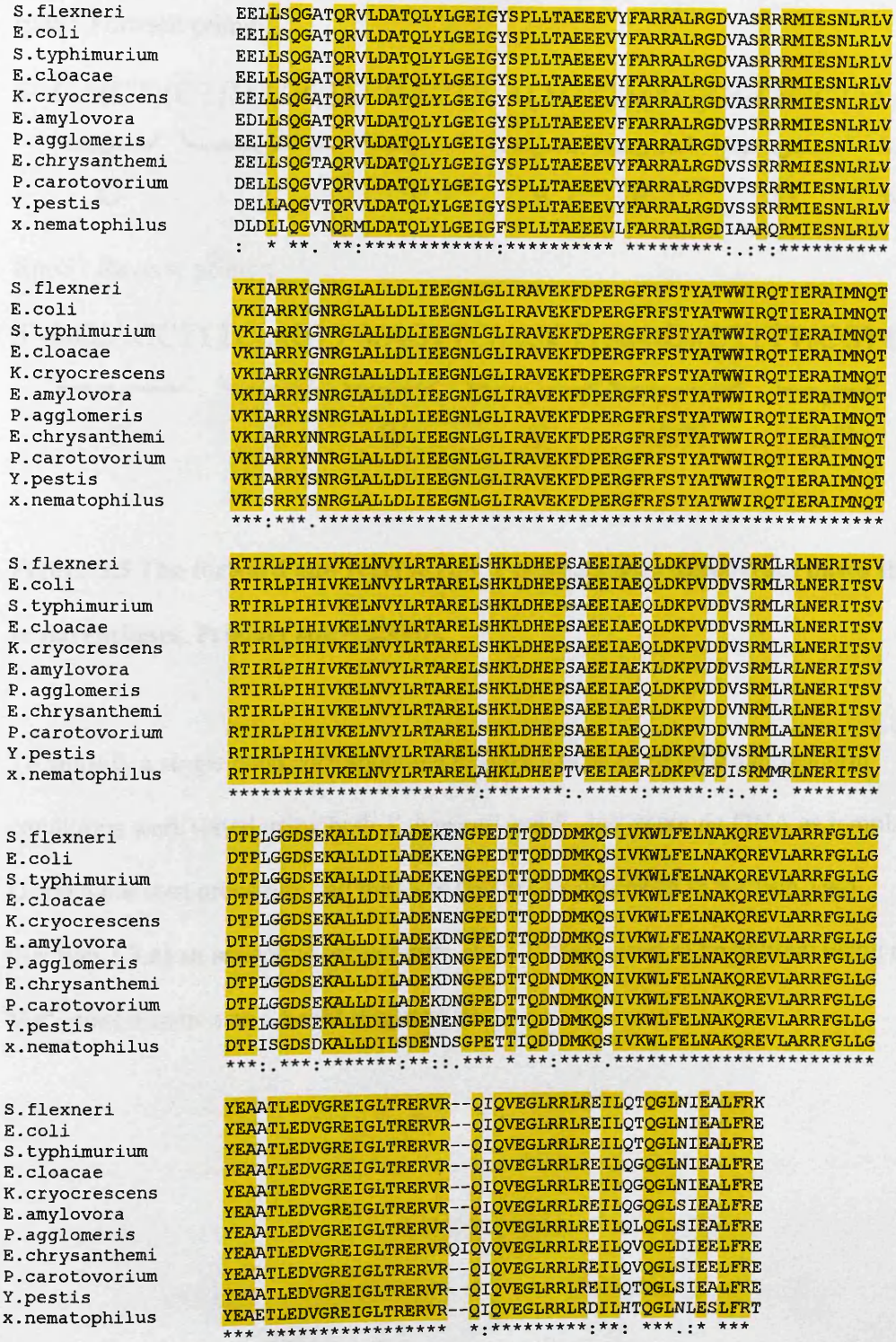
S.flexneri EELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLV
E.coli EELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLV
S.typhimurium EELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLV
E.cloacae EELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLV
K.cryocrescens EELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLV
E.amylovora EDLLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVFFARRALRGDVPSRRRMIESNLRLV
P.agglomeris EELLSQGVTQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVPSRRRMIESNLRLV
E.chrysanthemi EELLSQGTAQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVSSRRRMIESNLRLV
P.carotovorium DELLSQGVPQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVPSRRRMIESNLRLV
Y.pestis DELLAQGVTQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVSSRRRMIESNLRLV
x.nematophilus DLDLLQGVNQRMLDATQLYLGEIGFSPLLTAEEEVLFARRALRGDIAARQRMIESNLRLV
VKIARRYGNRGLALLDLIEEGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQT
RTIRLPIHIVKELNVYLRTARELSHKLDHEPSAEEIAEQLDKPVDDVSRMLRLNERITSV
DTPLGGDSEKALLDILADEKENGPEDTTQDDDMKQSIVKWLFELNAKQREVLARRFGLLG
YEAATLEDVGREIGLTRERVR--QIQVEGLRRLREILQTQGLNIEALFRK
S.flexneri
E.coli
S.typhimurium
E.cloacae
K.cryocrescens
E.amylovora
P.agglomeris
E.chrysanthemi
P.carotovorium
Y.pestis
x.nematophilus

rpoS1 Forward primer:

5'-GA(CT)(CT)T(AGCT)AT(ACT)GA(AG)GA(AG)GG(AGCT)AA-3'

D L I E E G N/K

RpoS1 Reverse primer:

5'-GG(AGCT) TC(AG) TG(AG) TC(AGCT) A(AG)(CT) TT(CG) TG-3'

G S W S S/N L/F C/W

Figure 5.3 The forward and reverse rpoS1 primers with degenerate bases show in parentheses. Product size = 250bp.

To amplify a single band corresponding to rpoS a number of different reaction conditions were tested using both *X. bovienii* and *E. coli* genomic DNA as templates. Using a hot start procedure and the PCR conditions described in Section Two (Section 2.3.4) an annealing temperature of 52°C was found to be optimal using a Mg^{2+} concentration of 3.5 mM (Figure 5.4).

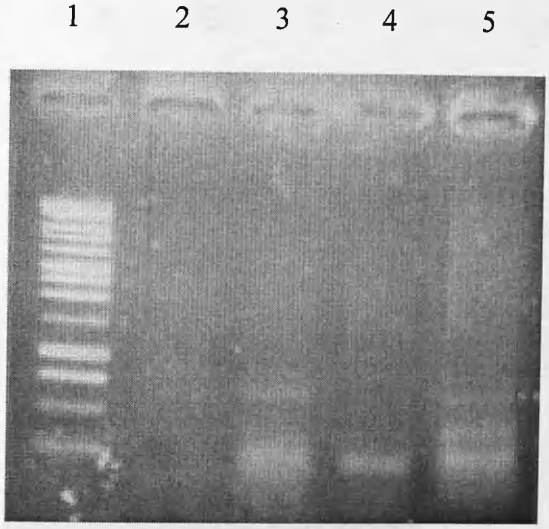

Figure 5.4 Ethidium bromide stained agarose gel of PCR products amplified with rpoS1 forward and reverse primers with *X. bovienii* genomic DNA (lanes 2 and 3) and *E. coli* genomic DNA (lanes 4 and 5) templates. Lane 1 contains 100kb markers. Lanes 2 and 3 contains *X. bovienii* products amplified with 1.55 mM $Mg^{2+}$and 3.5 mM Mg^{2+}respectively. Lanes 4 and 5 contains *E. coli* PCR products amplified with 1.55 mM $Mg^{2+}$and 3.5 mM Mg^{2+}respectively. The 250bp PCR product is highlighted in lane 5.

The single band PCR product for both species, shown in lanes three and four respectively (Figure 5.4) were isolated as previously described and were subjected to a further round of PCR utilising the same conditions as previously mentioned in order to obtain an increased concentration of product for subsequent cloning (Figure 5.5)

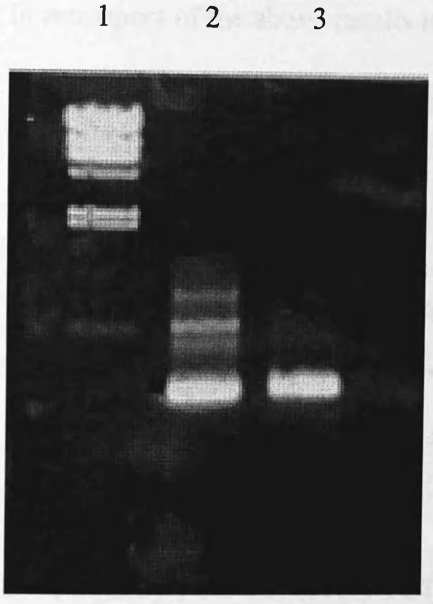

Figure 5.5 Ethidium bromide stained agarose gel of PCR products amplified with rpoS1 forward and reverse primers with rpoS1 amplified *X. bovienii* genomic DNA (lane 2) and rpoS1 amplified *E. coli* genomic DNA (lane 3) templates. Lane 1 contains lambda Hind III markers. Lane 2 contains rpoS1 amplified *X. bovienii* products amplified with 3.5 mM Mg^{2+}respectively. Lane 3 contains rpoS1 amplified *E. coli* PCR products amplified with 1.55 mM Mg^{2+}.

The single band PCR product for both species, shown in lanes two and three respectively (Figure 5.5) were isolated from a LMP gel and were inserted in pCR®2.1 TOPO and transformed into TOP10F' One Shot® (Invitrogen®). Plasmid preparations and restriction digests with Eco R1 yielded the correct sized inserts, which were subsequently sequenced. The NCBI BLAST sequence similarity database revealed that although the cloned fragment from both *X. bovienii* and *E. coli* were of the expected size, they showed greatest homology with unspecified regions of the entire genome of *Salmonella choleraesuis* and *Shigella flexinari* respectively.

In retrospect of the above results new primers were designed to isolate the fragment of the rpoS gene from *X. bovienii* (Figure 5.6)

rpoS2 Forward primer:

5'-AC(AGCT) TA(CT) GC(AGCT) AC(AGCT) TGG TGG AT-3'

D Y A T W W I/M

rpoS2 Reverse primer:

5'-TG(CT) TTC AT(AG) TC(AG) TC(AG) TC(CT) TG-3'

C F I/M S S S C/W

Figure 5.6 The forward and reverse rpoS primers with degenerate bases show in parentheses. Product size = 450bp.

To amplify a single band corresponding to rpoS, different reaction conditions were tested using both *X. bovienii* and *E. coli* genomic DNA as templates. An annealing temperature of 52°C was found to be optimal using a Mg^{2+} concentration of 2.5 mM and 1.5 mM respectively (Figure 5.7).

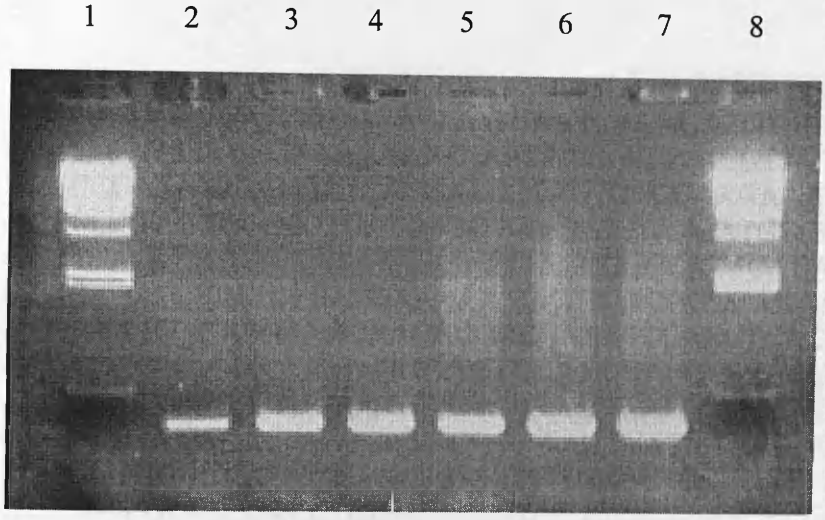

Figure 5.7 Ethidium bromide stained agarose gel of PCR products amplified with rpoS2 forward and reverse primers with *X. bovienii* genomic DNA (lanes 2, 3 and 4) and *E. coli* genomic DNA (lanes 5, 6 and 7) templates. Lanes 1 and 8 contains lambda Hind III markers. Lanes 2, 3 and 4 contains *X. bovienii* products amplified with 1.5 mM Mg^{2+}, 2.5 mM Mg^{2+} and 3.5 mM Mg^{2+}respectively. Lanes 5, 6 and 7 contains *E. coli* PCR products amplified with 1.5 mM Mg^{2+}, 2.5 mM Mg^{2+} and 3.5 mM Mg^{2+}respectively.

The single band PCR product for both species, shown in lanes three and five respectively (Figure 5.7) were isolated and transformed into TOP10F' One Shot® (Invitrogen®). Sequencing of the plasmids, named prpoS2xb and prpoS2ec and searching of the NCBI BLAST sequence similarity database revealed that the cloned fragment from both *X. bovienii* and *E. coli* were of the expected size, and showed the greatest homology with the rpoS gene fragment from *X. nematophilus* and *E. coli* respectively (Figure 5.8). A *ClustalX* alignment of the rpoS gene fragment from *X. bovienii* and the published *X. nematophilus* sequence was performed (Figure 5.9).

A)

TGATGGATATCTGCAGAATTCGCCCTTACGTATGCGACTTGGTGGATCCG
CCAGACAATTGAGCGGGCTATTATGAATCAGACACGTACGATCCGTCTGC
CTATTCATATTGTCAAAGAATTAAATGTTTATTTACGTACTGCAAGAGAGT
TAGCCCATAAATTGGATCATGAACCCAGTGTCGAAGAAATTGCGGAAAA
ACTGGATAGACCGGTTGATGATGTGAGCCGTATGATGCGGCTTAACGAAC
GCATTACTTCTGTTGATACACCAATAAGTGGGGATTCAGATAAAGCCTTA
CTGGATATTTTATCTGATGAAAAATGACTCAGGGNCANAAACAACCATTC
AGGATATGACATGAAACAACAGGGCGAATTTCANCTCACTGGCGGGCCC
GTTACTANTTGGATCCGAGCTTCGGGTACCCANGCTTGGCGTTAATCATG
GTCCATTAGCTTGTTTCCTGTNGTTGAAANTTGTTTATCNGGTCACCCATT
CCCACACAAANATTACGGANNCGGNAANCNATAAANGNGTTNAAGCCNT
GGNGGTGGCCTAAATGGANTNGAANCTNNACTCCCCATTTNAAATTTGGG
GTTTGCCGCCTCCACCTGC

B)

Sequences producing significant alignments:	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	289	3e-75
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	172	5e-40
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	172	5e-40
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	161	2e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	129	6e-27
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	98	2e-17
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	1e-15
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	76	8e-11
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	76	8e-11
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	76	8e-11
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	76	8e-11
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	76	8e-11
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	76	8e-11
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	68	2e-08
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	68	2e-08
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	68	2e-08
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	68	2e-08
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	68	2e-08
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	68	2e-08
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	68	2e-08

C)

GTGCAAGGAATTCCGGTAGAGAAGTTTGACCCGGAACGTGGTTTCCGCTT
CTCAACATACGCAACCTGGTGGATTCGCCAGACGATTGAACGGGCGATTA
TGAACCAAACCCGTACATTCGTTTGCCGATTCACATCGTAAAGGAGCTGA
ACGTTTACCTGCGAACAGCACGTGAGTTGTCCCATAAGCTGGACCATGAA
CCAAGTGCGGAAGAGATCGCAGAGCAACTGGATAAGCCAGTTGATGACG
TCAGCCGTATGCTTCGTCTTAACGAGCGCATTACCTCGGTAGACACCCCG
CTGGGTGGTGATTCCGAAAAAGCGTTGCTGGACANCCTGGCCGATGAAAA
AGAGAACGGTCCGNAAGATACCACGCAAGATGACGATATGAAGCAGAGC
ATCGTCAAATGGCTGTTCGAGCTGAACGCCAAACAGCGTGAAGTACTGGC
ACGTCGATTCGGTTTGCTGGGGTACGAAGCGGCAACACTGGAAGATGTAG
GTCGTGAAATTGGCCTCACCCGTGAACGTGTTCGCNNGATTCAGGTTGAA
GGCCTGCGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGCTGAATATCGA
AGCGCTGTTCC

D)

Sequences producing significant alignments:		Score (bits)	E Value
gi|5804830|emb|AJ245980.1|ECO245980	Escherichia coli O157:H...	1144	0.0
gi|4887553|emb|AJ006210.1|ECAJ6210	Escherichia coli O157:H7...	1144	0.0
gi|4100841|gb|AF002209.1|AF002209	Escherichia coli RpoS (rp...	1144	0.0
gi|4100839|gb|AF002208.1|AF002208	Escherichia coli RpoS (rp...	1144	0.0
gi|4100837|gb|AF002207.1|AF002207	Escherichia coli RpoS (rp...	1144	0.0
gi|13362858|dbj|AP002562.1|	Escherichia coli O157:H7 DNA, c...	1144	0.0
gi|12517193|gb|AE005502.1|AE005502	Escherichia coli O157:H7...	1140	0.0
gi|24053148|gb|AE015290.1|	Shigella flexneri 2a str. 301 se...	1128	0.0
gi|23306711|gb|AY142208.1|	Escherichia coli strain W3110D R...	1128	0.0
gi|23306709|gb|AY142207.1|	Escherichia coli strain W3110C N...	1128	0.0
gi|23306707|gb|AY142205.1|	Escherichia coli strain W3350 N-...	1128	0.0
gi|23306705|gb|AY142209.1|	Escherichia coli strain W3110E N...	1128	0.0
gi|23306701|gb|AY142204.1|	Escherichia coli strain W3110A R...	1128	0.0
gi|23306699|gb|AY142202.1|	Escherichia coli strain ZK 126 R...	1128	0.0
gi|23306697|gb|AY142203.1|	Escherichia coli strain JM 109 R...	1128	0.0
gi|41860|emb|Z14969.1|ECKATFWT	E.coli katF gene (WT)	1128	0.0
gi|41859|emb|Z14968.1|ECKATFUM	E.coli katF gene (UM122)	1128	0.0
gi|41858|emb|Z14967.1|ECKATFRH	E.coli katF gene (RH90)	1128	0.0
gi|41856|emb|Z14965.1|ECKATFJF	E.coli katF gene (JF618)	1128	0.0
gi|18073319|emb|AJ270954.1|ECO270954	Escherichia coli rpoS ...	1128	0.0

Figure 5.8 The rpoS gene fragment sequence from *X. bovienii* (A) and *E. coli* (C) with the first 20 BLAST scores for both *X. bovienii* (B) and *E.coli* (D). The sites chosen for RT-PCR primer design are highlighted in blue. The EcoR1 restriction site at the beginning of the construct is highlighted in yellow.

Figure 5.9 *ClustalX* alignment the rpoS gene fragment from *X. bovienii* and the published *X. nematophilus* rpoS sequence. The yellow highlighted area are residues conserved across all species.

* indicates an amino acid match across all sequences

```
x.bov.rpoS                ----------------------TGATGGATATCTGCAGAATTCGCCCTT--ACGTATGCGA
x.nematophilus.rpoS       TATTCGTGCGGTGGAAAAATTTGATCC-TGAAAGGGGGTTTCGTTTTTCTACTTATGCGA
                                                ****   *    *  *  ****   **  ** *******

x.bov.rpoS                CTTGGTGGATCCGCCAGACAATTGAGCGGGCTATTATGAATCAGACACGTACGATCCGTC
x.nematophilus.rpoS       CATGGTGGATCCGTCAAACAATTGAACGTGCCATTATGAATCAAACCCGCACAATTCGTC
                          * *********** ** ******** ** ** *********** ** ** ** ** ****

x.bov.rpoS                TGCCTATTCATATTGTCAAAGAATTAAATGTTTATTTACGTACTGCAAGAGAGTTAGCCC
x.nematophilus.rpoS       TGCCTATTCACATAGTCAAAGAACTCAATGTCTATTTACGTACTGCAAGAGAGCTGGCAC
                          ********** ** ********* * ***** ********************** * ** *

x.bov.rpoS                ATAAATTGGATCATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTG
x.nematophilus.rpoS       ACAAATTGGATCATGAACCCACGGTTGAAGAAATTGCGGAAAGGTTGGATAAACCTGTTG
                          * ******************  ** ****************   ****** *** ****

x.bov.rpoS                ATGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATACACCAATAA
x.nematophilus.rpoS       AAGATATCAGCCGTATGATGCGGCTGAACGAACGCATAACTTCTGTTGATACGCCAATTA
                          * *** * ***************** *********** ************** ***** *

x.bov.rpoS                GTGGGGATTCAGATAAAGCCTTACTGGATATTTTATCTGATGAAAAATGACTCAGGGNCA
x.nematophilus.rpoS       GTGGTGATTCAGATAAGGCATTATTGGATATTTTGTCTGATGAAAA-TGATTCTGGGCCA
                          **** *********** ** *** ********** *********** *** ** *** **

x.bov.rpoS                NAAACAACCATTCAGGAT-ATGACATGAAACAA--CAGGGCGAATTTCANCTCACTGGCG
x.nematophilus.rpoS       GAAACAACCATTCAGGATGATGATATGAAACAAAGCATTGTCAAATGGTTGTTTGAATTG
                           ***************** **** *********  **  *  ** *     *       *

x.bov.rpoS                GGCCCGTTACTANTTGGA-TCCGAGCTTCGGGTACCCAN--GCTTGGCGTTAATCATGGT
x.nematophilus.rpoS       AACGCGAAACAGCGTGAAGTTTTAGCTCGTCGTTTCGGTTTACTTGGATACGAAGCAGAA
                            * **  **    ** * *   ****    **  *     *****     *    *

x.bov.rpoS                CCATTAGCTTGTTTCCTGTNGTTGAAANTTGTTTATCNGGTCACCCATTCCCACACAAAN
x.nematophilus.rpoS       ACATTAGAAGATGTTGGACGG--GAAATAGGATTAACAAGAGAGAGAGTGCGTCAGATTC
                           ******    * *      *  ****   * *** *  *  *   * * *  ** *

x.bov.rpoS                ATTACGGANNCGGNAANCNATAAANGNGTTNAAGCCNTGGNGGTGGCCTAAATGGANTNG
x.nematophilus.rpoS       AAGTTGAAGGGTTGCGTCGTCTCAGAGATATATTGCATACCCAAGGTCTGAAT---TTAG
                          *    * *          *    *   *  *  * *       ** ** ***    * *

x.bov.rpoS                AANCTNNACTCCCCATTTNAAATTTGGGGTTTGCC--GCCTCCACCTGC----------
x.nematophilus.rpoS       AATCATTATTCCGGACTTAATTATTTATATCCGTCCAGAAAGAACACGTTATATTGGGTG
                          ** *   * ***  * ** *   **    *  * *  *     **  *
```

5.2.2 RT-PCR with rpoS3 forward and reverse primers

To characterise the regulation of the rpoS gene during the infection of *G. mellonella* with *S. feltiae* RT-PCR primers were designed (Figure 5.10) from the *X. bovienii* sequence (Figure 5.8). Total RNA from the *G. mellonella* time series of infection, as described in Chapter 3 (Section 3.1) was subjected to RT-PCR to ascertain at what time points during infection up-regulation of the rpoS gene occurred.

Forward primer:

5'-TAT GCG ACA TGG TGG ATC-3'

rpoS3 reverse primer

5'-ATT GGC GTA TCA ACA GAA G-3'

Figure 5.10 The forward and reverse rpoS3 RT-PCR primers. Product size = 245bp.

G. mellonella total RNA was checked for DNA contamination. A PCR reaction using the rpoS3 forward and reverse primers was performed on all the RNA samples at the optimum temperature of 60°C with a concentration of 1.5 Mg^{2+} (Figure 5.11).

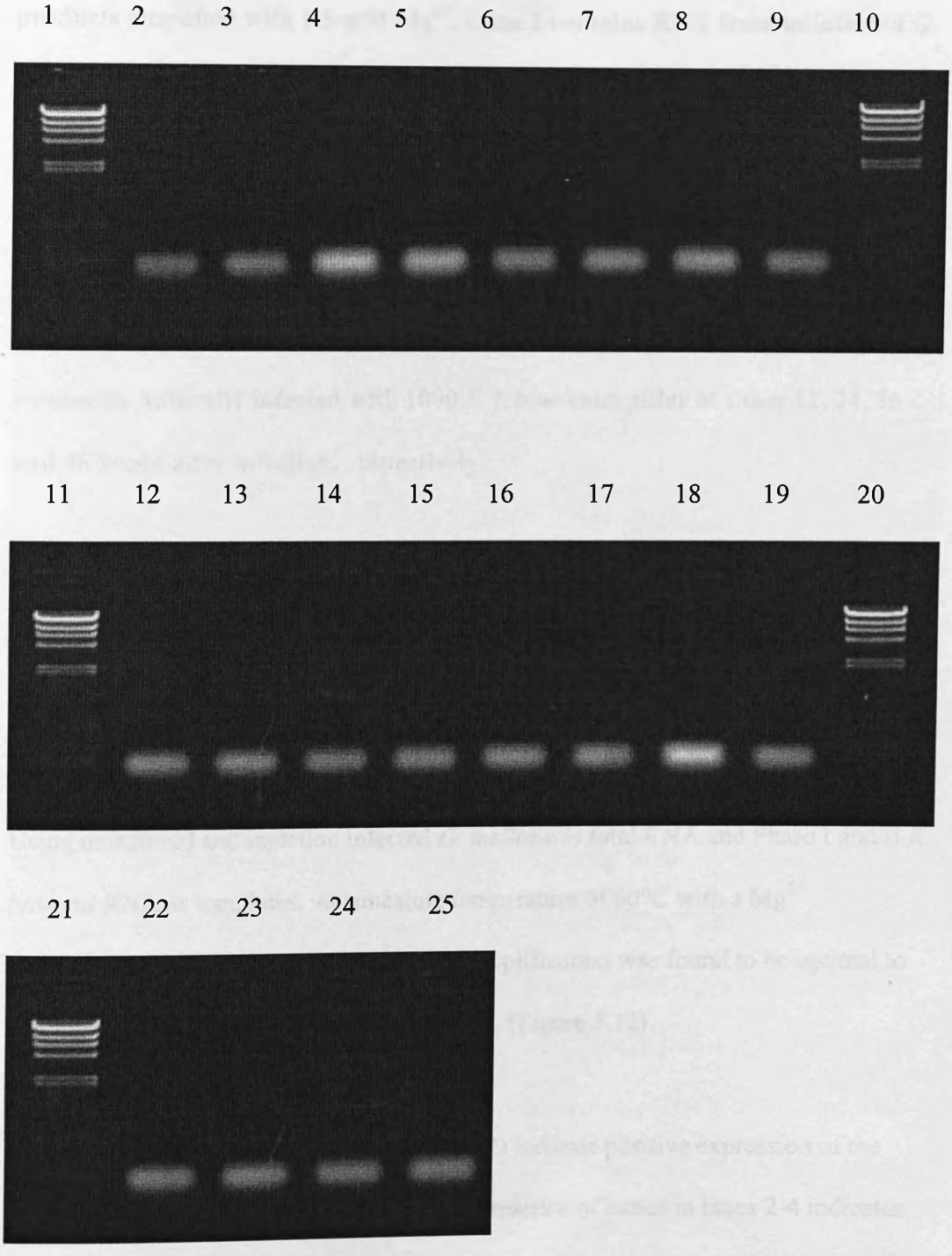

Figure 5.11 Ethidium bromide stained agarose gel of PCR products amplified with rpoS3 forward and reverse primers with *G. mellonella* total RNA (lanes 2-9 and 12-19) template. Lanes 1, 10, 11, 20 and 21 contains lambda Hind III markers. Lanes 2-9 and 12-19 and 22-25 contains *S. feltiae* infected *G. mellonella*

products amplified with 1.5 mM Mg^{2+}. Lane 2 contains RNA from uninfected *G. mellonella*. Lanes 3-9 and 12-15 contains RNA from *G. mellonella* injection infected with 100 *S. feltiae*/caterpillar sampled at times 2, 4, 6, 8, 10, 12, 18, 24, 36, 48 and 72 hours after infection, respectively. Lanes 16-19 contains RNA from *G. mellonella* naturally infected with 100 *S. feltiae*/caterpillar at times 12, 24, 36 ande 48 hours after infection, respectively. Lanes 22-25 contains RNA from *G. mellonella* naturally infected with 1000 *S. feltiae*/caterpillar at times 12, 24, 36 and 48 hours after infection, respectively.

The absence of an rpoS amplified PCR product in lanes 2-9, 11-19 and 22-25 (Figure 5.11) was a positive indication that the *G. mellonella* total RNA samples were free from DNA contamination.

Using uninfected and injection infected *G. mellonella* total RNA and Phase I and II *X. bovienii* RNA as templates, an annealing temperature of 60°C with a Mg^{2+} concentration of 1.5 mM with 40 cycles of amplification was found to be optimal to amplify a single band corresponding to rpoS3. (Figure 5.12)

The single bands in lanes 5 and 6 (Figure 5.12) indicate positive expression of the rpoS gene in Phase I and II *X. bovienii*. The presence of bands in lanes 2-4 indicates positive expression of the rpoS gene in the 4 hour and 72 hour injection infection *G. mellonella* and in uninfected *G. mellonella*.

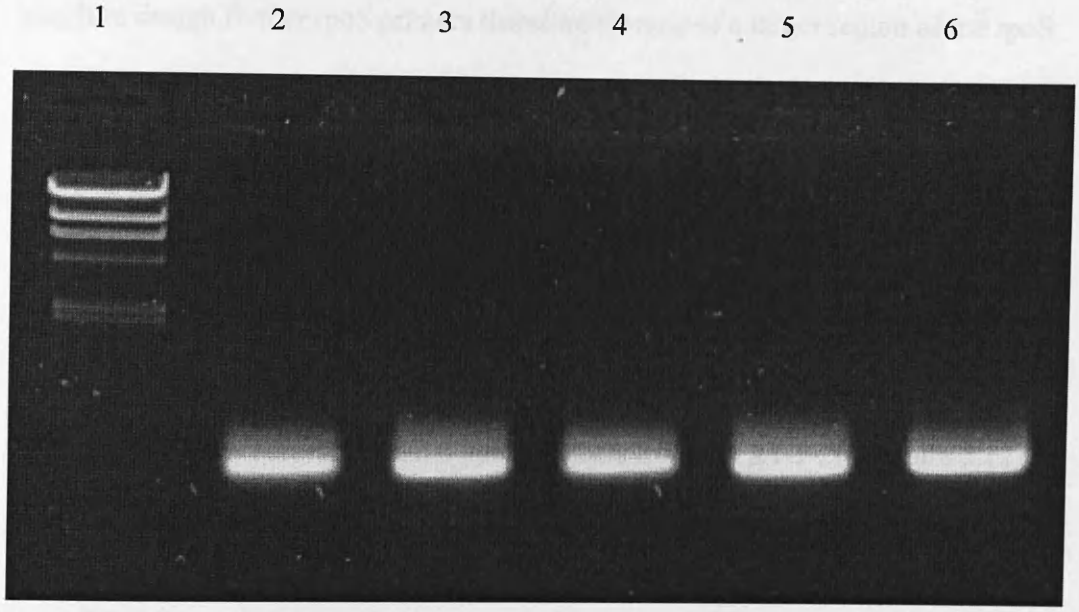

Figure 5.12 Ethidium bromide stained agarose gel of RT-PCR products amplified with rpoS3 forward and reverse primers with *G. mellonella* total RNA (lanes 2-4) and *X. bovienii* total RNA (lanes 5-6) template. Lane 1 contains lambda Hind III markers. Lanes 2-4 and 5-6 contains *S. feltiae* infected *G. mellonella* and *X. bovienii* products, respectively, amplified with 1.5 mM Mg^{2+}. Lane 2 contains RNA from uninfected *G. mellonella*. Lane 3 contains RNA from *G. mellonella* injection infected with 100 *S. feltiae*/caterpillar sampled at 4 hours after infection. Lane 4 contains RNA from *G. mellonella* injection infected with 100 *S. feltiae*/caterpillar sampled at 72 hours after infection. Lane 5 contains *X. bovienii* Phase I RNA. Lane 6 contains *X. bovienii* Phase II RNA.

The amplification of the *X. bovienii* rpoS gene fragment from the uninfected caterpillar was unexpected and indicated the unsuitability of the rpoS3 primers for quantitative RT-PCR. The NCBi database did not contain sequences of suitable

length to design further rpoS primers therefore cloning of a larger region of the rpoS gene was undertaken (Section 5.2.3)

Meanwhile the rpoS RT-PCR amplified region from the uninfected and 4 hour injection infected caterpillar total RNA was isolated, cloned and sequenced in order to ascertain which type of organism was present in both the uninfected and infected *G. mellonella*. The uninfected and 4 hour products from the RT-PCR (Figure 5.12), lanes 2 and 4, respectively, were separated on a 2% LMP agarose gel (Figure 5.13).

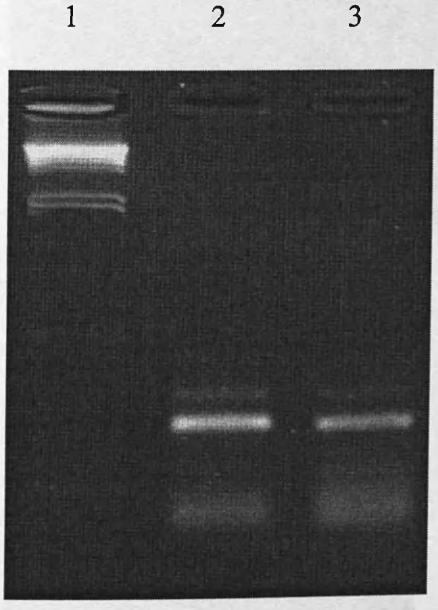

Figure 5.13 Ethidium bromide stained agarose gel of RT-PCR products amplified with rpoS3 forward and reverse primers with *G. mellonella* total RNA (lanes 2 and 3) template. Lane 1 contains lambda Hind III markers. Lane 2 contains 4 hour *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}. Lane 3 contains uninfected *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}.

The products were isolated and were and transformed into TOP10F' One Shot® (Invitrogen®). The cloned TOP10F' colonies containing the putative rpoS uninfected and rpoS 4hour inserts were screened by PCR, utilising the optimum annealing temperature of 60°C with a Mg^{2+} concentration of 1.5 mM (Figure 5.14).

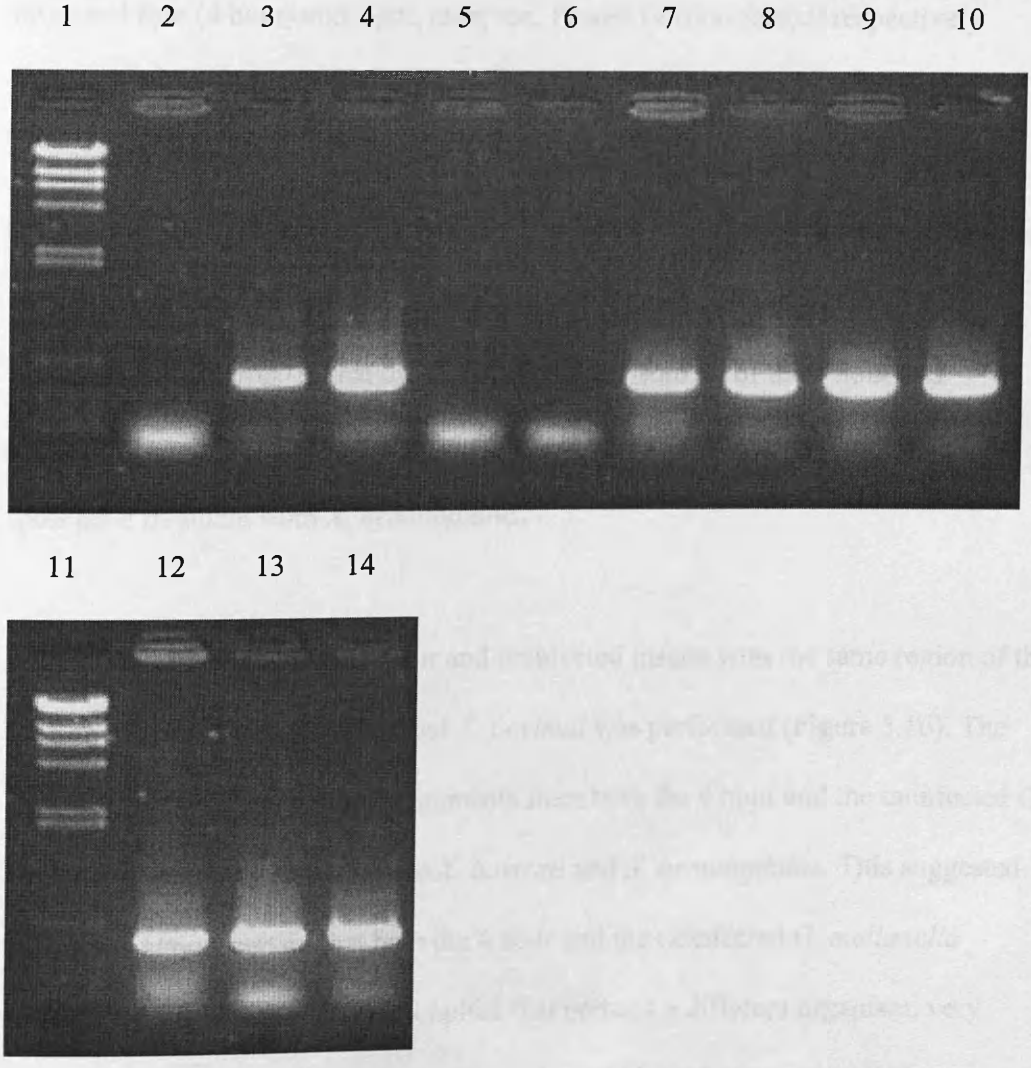

Figure 5.14 Ethidium bromide stained agarose gel of RT-PCR products amplified with rpoS3 forward and reverse primers with TOP10F' One Shot® *G. mellonella* total RNA (lanes 2-10 and 12-14) templates. Lanes 1 and 11 contains lambda Hind III markers. Lanes 2-6 contains TOP10F' One Shot® 4 hour

infected *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}. Lanes 7-10 and 12-14 contains TOP10F' One Shot® uninfected *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}.

The single band PCR product for both 4 hour infected and uninfected, shown in lanes three and four (4 hour) and eight, nine, ten, 12 and 14 (uninfected) respectively (Figure 5.14) were isolated and transformed into TOP10F' One Shot® (Invitrogen®). The plasmids were named prpoS4.1 and prpoS4.2, for the plasmids with the 4 hour insert, and prpoSui.1, prpoSui.2, prpoSui.3, prpoSui.4 and prpoSui.5 for the plasmids with the uninfected insert. Sequencing of these plasmids (Figure 5.15) and a BLASTn search revealed that the cloned fragment from all of the 4 hour and uninfected were of the expected size, and showed the greatest homology with the rpoS gene fragment from *X. nematophilus*.

A ClustalX alignment of the 4 hour and uninfected inserts with the same region of the rpoS from both *X. nematophilus* and *X. bovienii* was performed (Figure 5.16). The alignment showed that the rpoS fragments from both the 4 hour and the uninfected *G. mellonella* were very similar to the *X. bovienii* and *X. nematophilus.* This suggested that is was almost certain that both the 4 hour and the uninfected *G. mellonella* contain *X. bovienii* bacteria. This implied that perhaps a different organism, very similar to *X. bovienii* and as yet unsequenced, could have been present in the uninfected *G. mellonella.*

prpoSui.1

GAATTCGCCCTTTATGCGACATGGTGGATCCGCCAGACAATTGAGCGGGC
TATTATGGATCAGACACGTACGATCCGTCTGCCTATTCATATTGTCAAAGA
ATTAAATGTTTATTTACGTACTGCAAGAGAGCTAGCCCATAAATTGGATC
ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
TGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATA
CGCCAATAAGGGC

Sequences producing significant alignments:

	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	123	2e-25
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	6e-16
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	82	5e-13
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	66	3e-08
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	66	3e-08
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	66	3e-08
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	66	3e-08
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	66	3e-08
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	66	3e-08
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	66	3e-08
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	66	3e-08
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	66	3e-08
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	66	3e-08
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	66	3e-08
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	66	3e-08
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	66	3e-08

prpoSui.2

GAATTCGCCCTTTATGCGACATGGTGGATCCGCCAGACAATTGAGCGGGC
TATTATGGATCAGACACGTACGATCCGTCTGCCTATTCATATTGTCAAAGA
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ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
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CGCCAATAAGGGC

Sequences producing significant alignments:

	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	123	2e-25
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	6e-16
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	82	5e-13
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	66	3e-08
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	66	3e-08
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	66	3e-08
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	66	3e-08
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	66	3e-08
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	66	3e-08
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	66	3e-08
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	66	3e-08
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	66	3e-08
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	66	3e-08
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	66	3e-08
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	66	3e-08
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	66	3e-08

prpoSui.3

GAATTCGCCCTTTATGCGACATGGTGGATCCGCCAGACAATTGAGCGGGC
TATTATGGATCAGACACGTACGATCCGTCTGCCTATTCATATTGTCAAAGA
ATTAAATGTTTATTTACGTACTGCAAGAGAGCTAGCCCATAAATTGGATC
ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
TGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATA
CGCCAATAAGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	123	2e-25
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	6e-16
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	82	5e-13
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	66	3e-08
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	66	3e-08
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	66	3e-08
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	66	3e-08
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	66	3e-08
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	66	3e-08
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	66	3e-08
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	66	3e-08
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	66	3e-08
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	66	3e-08
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	66	3e-08
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	66	3e-08
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	66	3e-08

prpoSui.4

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ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
TGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATA
CGCCAATAAGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	123	2e-25
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	6e-16
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	82	5e-13
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	66	3e-08
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	66	3e-08
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	66	3e-08
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	66	3e-08
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	66	3e-08
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	66	3e-08
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	66	3e-08
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	66	3e-08
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	66	3e-08
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	66	3e-08
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	66	3e-08
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	66	3e-08
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	66	3e-08

prpoSui.5

GAATTCGCCCTTTATGCGACATGGTGGATCCGCCAGACAATTGAGCGGGC
TATTATGGATCAGACACGTACGATCCGTCTGCCTATTCATATTGTCAAAGA
ATTAAATGTTTATTTACGTACTGCAAGAGAGCTAGCCCATAAATTGGATC
ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
TGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATA
CGCCAATAAGGGC

Sequences producing significant alignments:

	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	123	2e-25
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	98	9e-18
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	6e-16
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	76	3e-11
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	76	3e-11
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	76	3e-11
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	76	3e-11
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	76	3e-11
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	76	3e-11
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	68	8e-09
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	68	8e-09
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	68	8e-09
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	68	8e-09
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	68	8e-09
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	68	8e-09
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	68	8e-09

prpoS4.1

GAATTCGCCCTTTATGCGACATGGTGGATCCGCCAGACAATTGAGCGGGC
TATTATGGATCAGACACGTACGATCCGTCTGCCTATTCATATTGTCAAAGA
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ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
TGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATA
CGCCAATAAGGGC

Sequences producing significant alignments:

	Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628 Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1| Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1| Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152 Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043 Yersinia enterocolitica rpoS...	123	2e-25
gi|3183702|emb|AJ222716.1|ERA222716 Erwinia amylovora rpoS ...	92	6e-16
gi|2961259|emb|Y13230.1|ECRPOS Enterobacter cloacae rpoS gene	82	5e-13
gi|16421460|gb|AE008833.1| Salmonella typhimurium LT2, sect...	66	3e-08
gi|29138627|gb|AE016843.1| Salmonella enterica subsp. enter...	66	3e-08
gi|3820467|emb|Y17610.1|STY17610 Salmonella typhi rpoS gene...	66	3e-08
gi|602086|emb|X77752.1|STKATFR S.typhimurium katF; rpoS gene	66	3e-08
gi|695757|emb|X81641.1|STRPOS S.typhi nlpD and rpoS genes	66	3e-08
gi|558643|emb|X82129.1|SEKATF S.enterica katF (rpoS) gene	66	3e-08
gi|16503805|emb|AL627276.1| Salmonella enterica serovar Typ...	66	3e-08
gi|6010737|gb|AF184104.1|AF184104 Salmonella typhimurium LT...	66	3e-08
gi|6010735|gb|AF184103.1|AF184103 Salmonella typhimurium LT...	66	3e-08
gi|6010733|gb|AF184102.1|AF184102 Salmonella typhimurium LT...	66	3e-08
gi|6010731|gb|AF184101.1|AF184101 Salmonella typhimurium LT...	66	3e-08
gi|6010727|gb|AF184099.1|AF184099 Salmonella typhimurium LT...	66	3e-08
gi|6010725|gb|AF184098.1|AF184098 Salmonella typhimurium LT...	66	3e-08

prpoS4.2

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TATTATGAATCAGACACGTACGATCCGTCTGCCTATTCATATTGTCAAAGA
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ATGAACCCAGTGTCGAAGAAATTGCGGAAAAACTGGATAGACCGGTTGA
TGATGTGAGCCGTATGATGCGGCTTAACGAACGCATTACTTCTGTTGATA
CGCCAATAAGGGC

Sequences producing significant alignments:		Score (bits)	E Value
gi|6643918|gb|AF198628.1|AF198628	Xenorhabdus nematophilus ...	234	6e-59
gi|21957562|gb|AE013686.1|	Yersinia pestis KIM section 86 o...	159	3e-36
gi|15981150|emb|AJ414156.1|	Yersinia pestis strain CO92 com...	159	3e-36
gi|687581|gb|U16152.1|YEU16152	Yersinia enterocolitica sigm...	159	3e-36
gi|726191|gb|U22043.1|YEU22043	Yersinia enterocolitica rpoS...	123	2e-25
gi|2961259|emb|Y13230.1|ECRPOS	Enterobacter cloacae rpoS gene	98	9e-18
gi|3183702|emb|AJ222716.1|ERA222716	Erwinia amylovora rpoS ...	92	6e-16
gi|29138627|gb|AE016843.1|	Salmonella enterica subsp. enter...	76	3e-11
gi|3820467|emb|Y17610.1|STY17610	Salmonella typhi rpoS gene...	76	3e-11
gi|695757|emb|X81641.1|STRPOS	S.typhi nlpD and rpoS genes	76	3e-11
gi|558643|emb|X82129.1|SEKATF	S.enterica katF (rpoS) gene	76	3e-11
gi|16503805|emb|AL627276.1|	Salmonella enterica serovar Typ...	76	3e-11
gi|6010737|gb|AF184104.1|AF184104	Salmonella typhimurium LT...	76	3e-11
gi|16421460|gb|AE008833.1|	Salmonella typhimurium LT2, sect...	68	8e-09
gi|602086|emb|X77752.1|STKATFR	S.typhimurium katF; rpoS gene	68	8e-09
gi|6010735|gb|AF184103.1|AF184103	Salmonella typhimurium LT...	68	8e-09
gi|6010733|gb|AF184102.1|AF184102	Salmonella typhimurium LT...	68	8e-09
gi|6010731|gb|AF184101.1|AF184101	Salmonella typhimurium LT...	68	8e-09
gi|6010727|gb|AF184099.1|AF184099	Salmonella typhimurium LT...	68	8e-09
gi|6010725|gb|AF184098.1|AF184098	Salmonella typhimurium LT...	68	8e-09

Figure 5.15 The RT-PCR amplified rpoS gene fragment sequence from uninfected (ui) and 4 hour infected (4) *G.mellonella* total RNA with the first 20 BLAST scores for each plasmid. The restriction site at the beginning of the construct is highlighted.

* indicates an amino acid match across all sequences

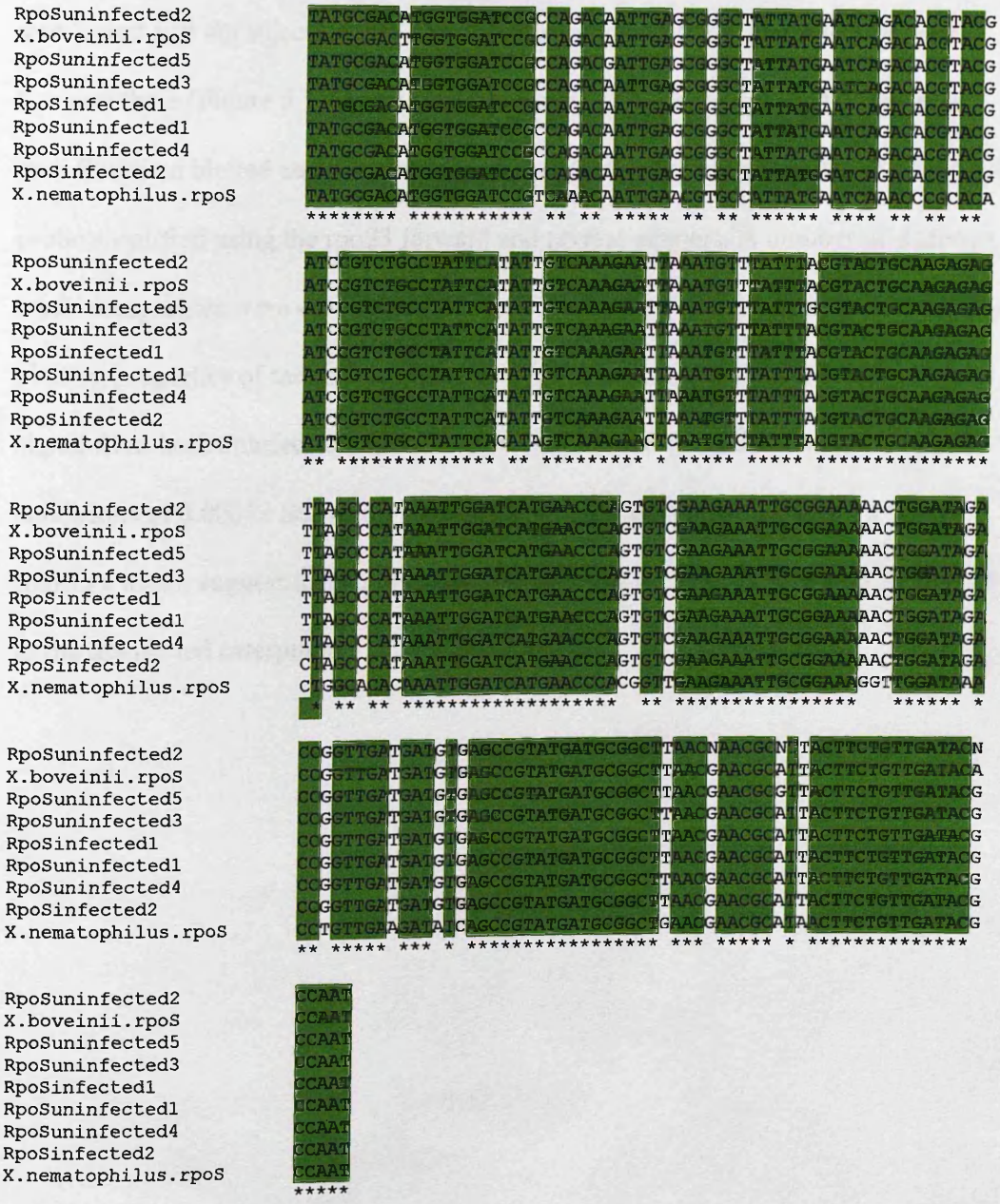
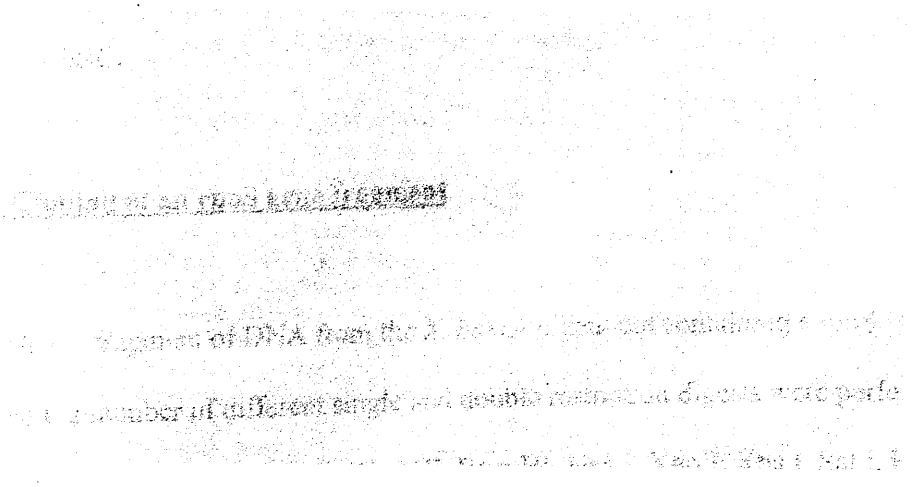


Figure 5.16 *ClustalX* alignment of 9 rpoS nucleotide sequences. The green highlights are where all the residues are identical.

In order to further investigate the possible presence of a *Xenorhabdus*-like organism in uninfected *G. mellonella* a Southern blot of uninfected and 4 hour infected *G. mellonella* total RNA was performed. The rpoS3 PCR amplified products from the uninfected and 4hr injection infected *G. mellonella* total RNA samples from lanes two and three (Figure 5.11) were electrophoresed on a 1% agarose gel which was then Southern blotted and the membrane hybridised with the PCR-DIG labelled rpoS probe amplified using the rpoS3 forward and reverse primers. A number of different wash stringencies were used from 1x to 0.0001x SSC at 68°C in order to ascertain the binding properties of the probe (Figure 5.17). The rpoS probe resulted in a positive signal from both uninfected and 4 hour infected RNA even under the most stringent conditions of 0.0001x SSC/0.1% SDS at 68°C. This supports this study's previous findings which suggest that an organism very similar to *Xenorhabdus* may be present in the uninfected caterpillar.



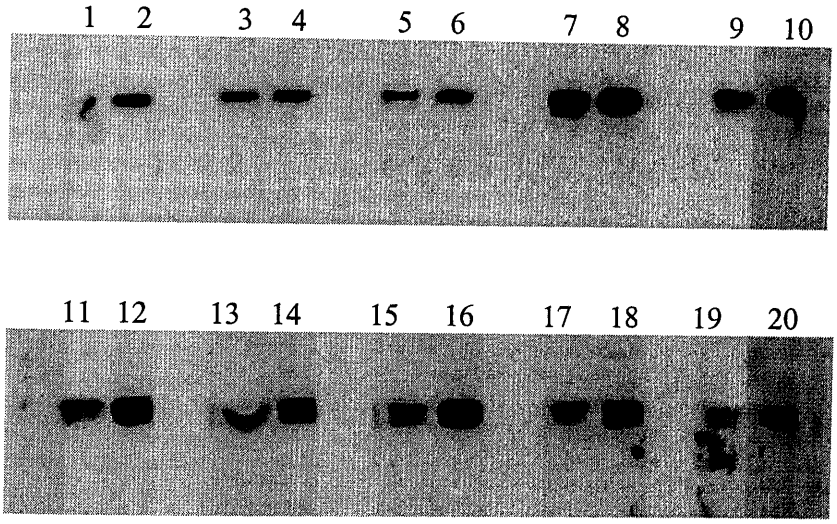


Figure 5.17 Southern blots with the rpoS probe of the 4hr injection infection and uninfected *G. mellonella* total RNA. Lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17 contain 4hr injection infection RNA. Lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 contain the uninfected RNA. All lanes were hybridized and washed at 68°C. Lanes 1 and 2 were washed in 1 x SSC; lanes 3 and 4: 0.5 x SSC; lanes 5 and 6: 0.1 x SSC; lanes 7 and 8: 0.05 x SSC; lanes 9 and 10: 0.01 x SSC; lanes 11 and 12: 0.005 x SSC; lanes 13 and 14: 0.001 x SSC; lanes 15 and 16: 0.0005 x SSC; lanes 17 and 18: 0.0001 x SSC.

5.2.3 Cloning of an rpoS gene fragment

To isolate a fragment of DNA from the *X. bovienii* genome containing a rpoS fragment, a number of different single and double restriction digests were performed. Restriction digests using Eco RI, Hind III, Bam HI, Nco I, Xho I, Xba I, Sal I, Pst I and Kpn I, were electrophoresed on a 1% agarose gel which was then Southern

blotted and the membrane hybridised with the PCR DIG-labelled rpoS probe (Figure 5.18)

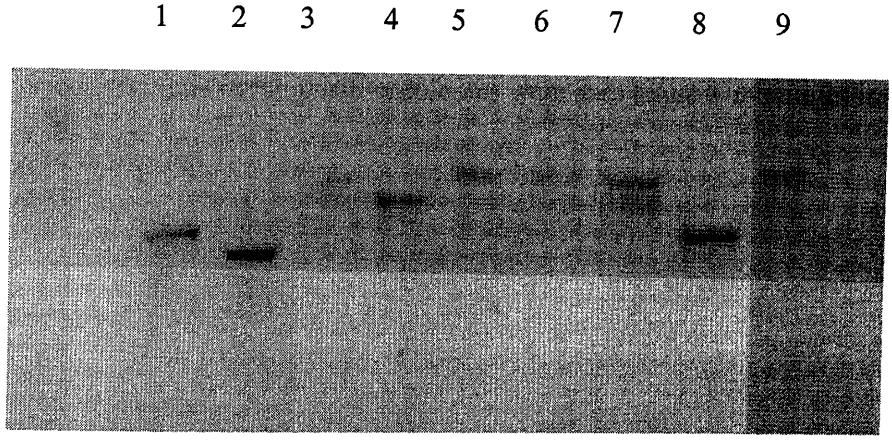


Figure 5.18 Southern blots with the rpoS probe of restriction digests of *X. bovienii* genomic DNA. Lane 1: Eco RI, lane 2: Hind III, Lane 3: Bam HI, lane 4: Nco I, lane 5: Xho I, lane 6: Xba I, lane 7: Sal I, lane 8: Pst I, lane 9: Kpn I.

As digestion with Eco RI, Hind III, Nco I and Pst I provided the smallest fragments a series of double restriction digests were performed. Restriction digests using Hind III/Eco RI, Eco RI/Nco I, Eco RI/Pst I, Hind III/Nco I, Hind III/Pst I and Nco I/Pst I were electrophoresed on a 1% agarose gel which was then Southern blotted with the FliC DIG-labelled probe (Figure 5.19)

A hybridising fragment of approximately 3kb produced using the restriction enzymes Hind III and Eco RI was chosen for isolation from the genomic *X. bovienii* DNA. A partial genomic library in TOP10F' One ShotPUC® was constructed. PUC 18 and *X. bovienii* genomic DNA were both cut with the restriction enzymes Hind III and Eco RI were electrophoresed on a 1% gel (Figure 5.20), the 2.5-3.5kb region from *X.*

bovienii and the PUC 18 vector were excised and purified. The 3.5kb putative rpoS fragment was ligated into the PUC vector transformed into TOP10F' One Shot®. The library was screened using the rpoS probe and putitive positive colonies were picked and grown overnight in LB + amplicillin broth. Unfortunately, despite frequent repetition, none of the positive colonies were able to grow overnight in the selective growth media.

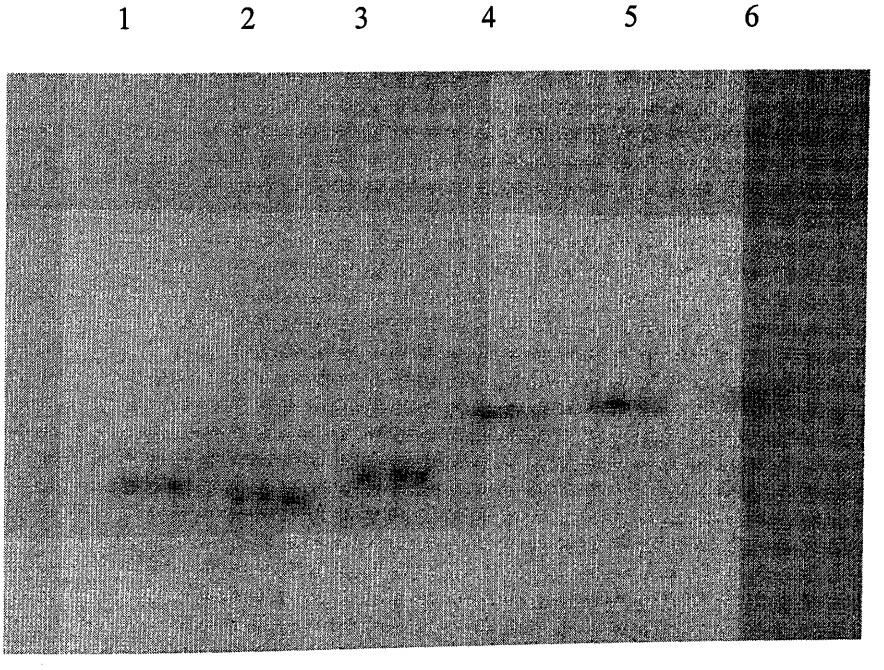


Figure 5.19 Southern blots with the rpoS probe of restriction digests of *X. bovienii* genomic DNA. Lane 1: Hind III/Eco RI, lane 2: Eco RI/Nco I, lane 3: Eco RI/Pst I, lane 4: Hind III/Nco I, lane 5: Hind III/Pst I, lane 6: Nco I/Pst I,.

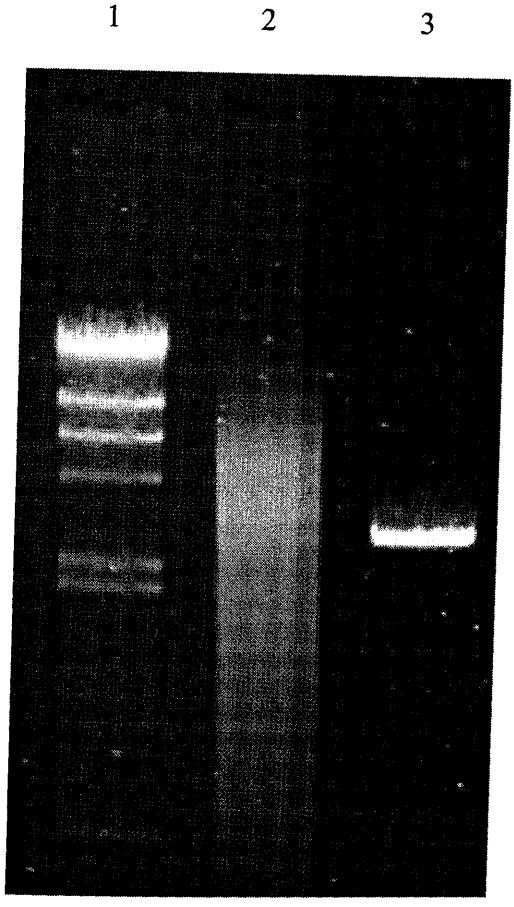


Figure 5.20 Ethidium bromide stained agarose gel with Eco RI/Hind III digested *X. bovienii* template DNA lane 2 and Eco RI/Hind III digested PUC18 template DNA, lane 3. Lane 1 shows lambda Hind III markers.

5.3 Cloning and Sequencing of the FliC fragment

5.3.1 Primer design and PCR amplification of a FliC fragment

Specific probes were designed to isolate the FliC gene fragment from the *X. bovienii* total DNA (Figure 5.1).

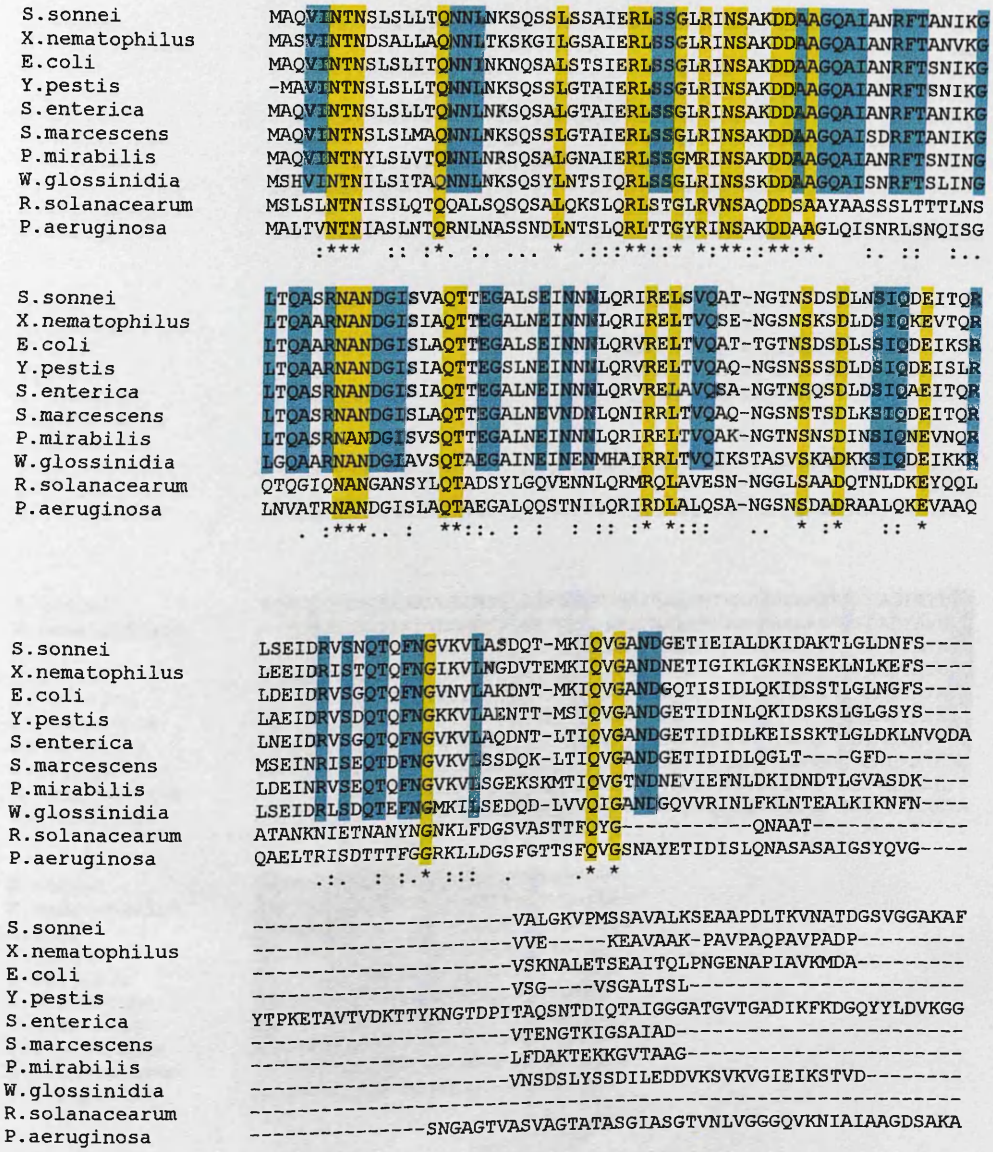
The FliC peptide sequence of *X. nematophilus* was aligned with other sequences from a number of other species using *ClustalX* software. Those species which displayed the most similar FliC sequence to the *X. bovienii* sequence when performing a BLASTp search were chosen. This resulted in seven species of Enterobacteriacea; *Escherichia coli*, *Yersinia pestis*, *Shigella sonneii*, *Salmonella enterica*, *Wigglesworthia glossinidia* and *Serratia marscescens*, one species of Burkholderiaceae; *Ralstonia solanacearum*, and one species of Pseudomonadaceae; *Pseudomonas aeruginosa* undergoing alignment with *X. bovienii* (Figure 5.21) The conserved regions were highlighted and investigated for their suitability as primer design sites. Partially degenerate primers were designed for the sequences selected (Figure 5.22).

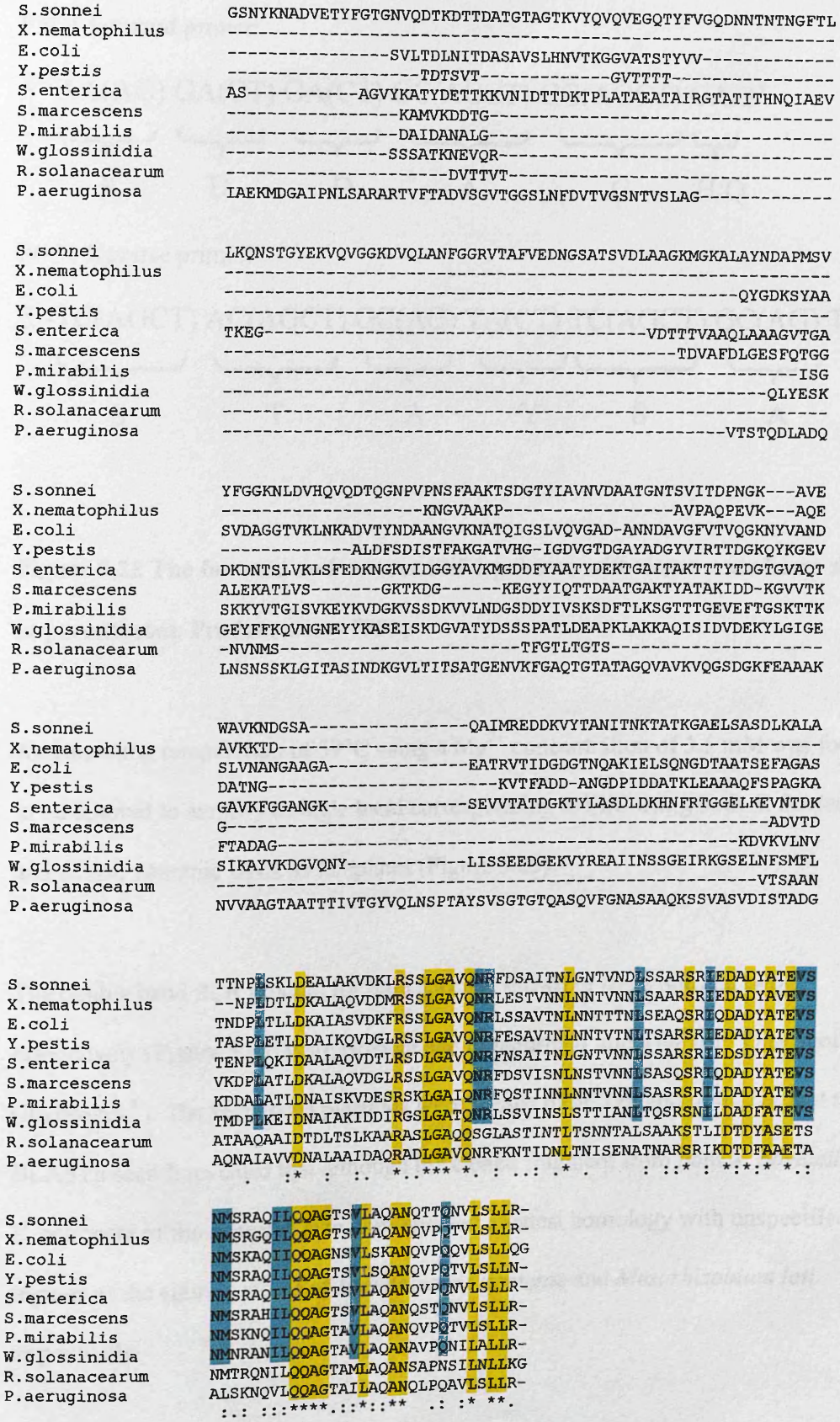
Figure 5.21 *ClustalX* alignment of 10 FliC peptide sequences. The yellow highlighted areas are residues conserved across all species. The blue residues are areas where the eight Enterobacteriaceae, including *X. nematophilus* contain identical residues.

* indicates an amino acid match across all sequences

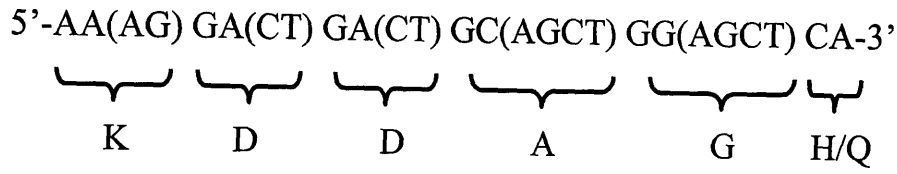
: indicates highly conservative substitutions across the sequences

. indicates conservative substitutes across the sequences




S.sonnei
X.nematophilus
E.coli
Y.pestis
S.enterica
S.marcescens
P.mirabilis
W.glossinidia
R.solanacearum
P.aeruginosa

FliC1 Forward primer:



FliC1 Reverse primer:

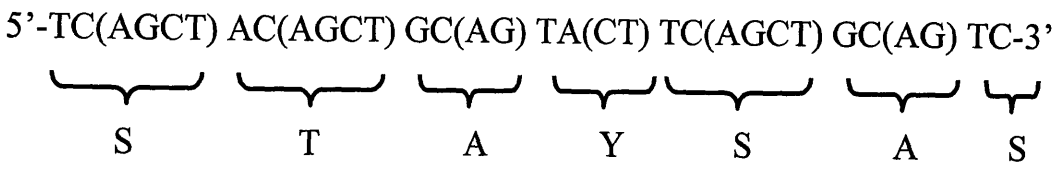


Figure 5.22 The forward and reverse FliC1 primers with degenerate bases show in parentheses. Product size = 725bp

An annealing temperature of 59°C using a Mg^{2+} concentration of 3.5 mM was found to be optimal to amplify a single band corresponding to FliC using both *X. bovienii* and *E. coli* genomic DNA as templates (Figure 5.23).

The double band PCR product for both species, shown in lanes one and two respectively (Figure 5.23) were isolated and transformed into TOP10F' One Shot® (Invitrogen®). The sequenced plasmids were named pFliC1xb and pFliC1ec and the BLASTn search revealed that although the cloned fragment from both *X. bovienii* and *E. coli* were of the expected size, they showed greatest homology with unspecified regions of the entire genome of *Pseudomonas syringae* and *Mesorhizobium loti* respectively.

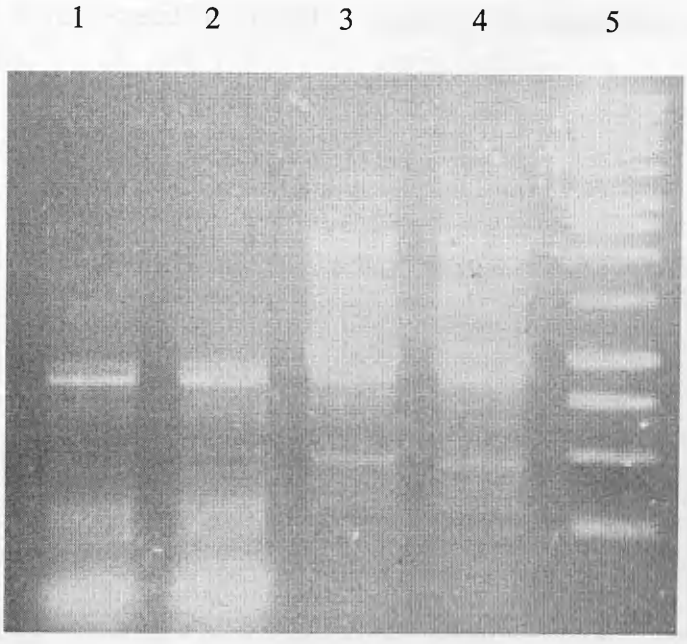


Figure 5.23 Ethidium bromide stained agarose gel of PCR products amplified with FliC1 forward and reverse primers with *X. bovienii* genomic DNA (lane1) and *E. coli* genomic DNA (lane 2) templates. Lane 5 contains 100kb markers. Lanes 1 contains *X. bovienii* products amplified with 3.5 mM Mg^{2+}. Lane 2 contains *E. coli* PCR products amplified with 3.5 mM Mg^{2+}.

In light of the above results it was decided to screen the TOP10F' One Shot® (Invitrogen®) cells containing the FliC1 amplified insert using PCR. Using the optimised conditions described above, eight colonies carrying the *Xenorhabdus* construct and eight containing the *E. coli* construct were screened (Figure 5.24).

No bands of a suitable size could be isolated from the gel (Figure 5.24). It was decided to return to the original PCR (Figure 5.23) which was subjected to a further round of PCR using the FliC1 forward and reverse primers under the same conditions

as previously mentioned in order to obtain an increased concentration of product for subsequent cloning (Figure 5.25)

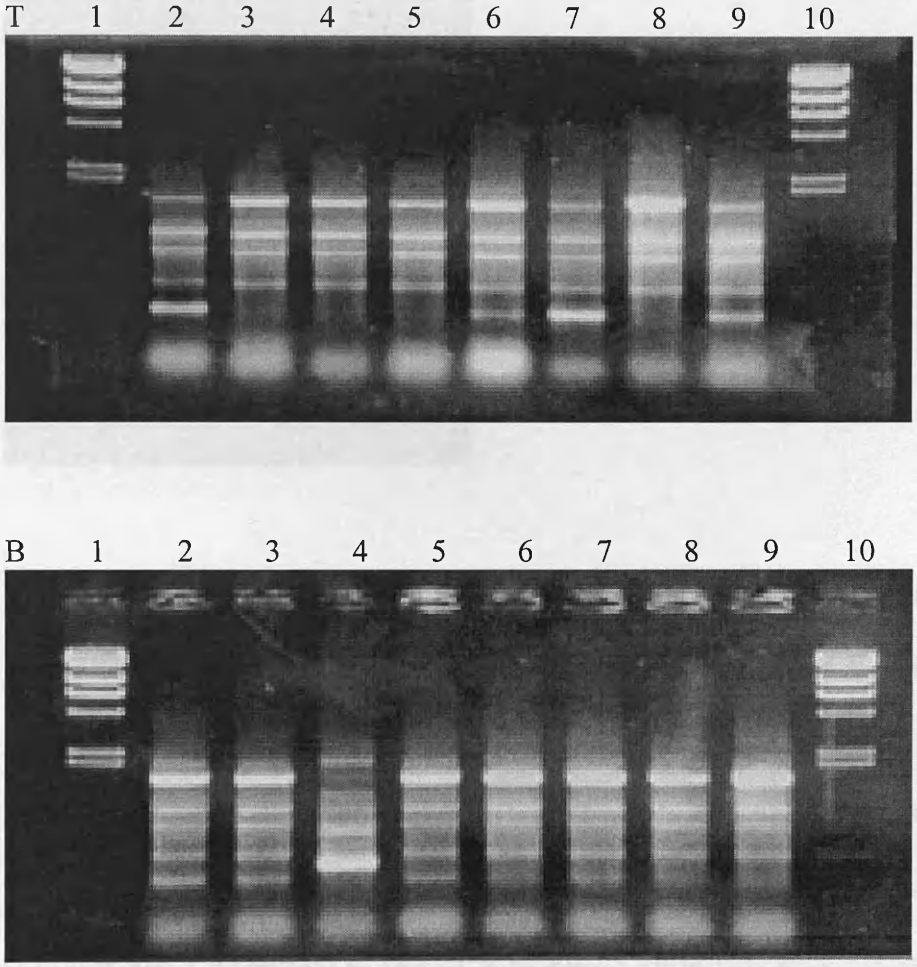


Figure 5.24 Ethidium bromide stained agarose gel of PCR products amplified with FliC1 forward and reverse primers with TOP10F' One Shot® *X. bovienii* (top row (T) lanes 2-9) and TOP10F' One Shot® *E. coli* (bottom row (B) lanes 2-9) templates. Lanes 1 and 10, top and bottom rows, contains lambda Hind III markers. Lanes T2-9 contains TOP10F' One Shot® *X. bovienii* products amplified with 3.5 mM Mg^{2+}. Lane 2 contains TOP10F' One Shot® *E. coli* PCR products amplified with 3.5 mM Mg^{2+}.

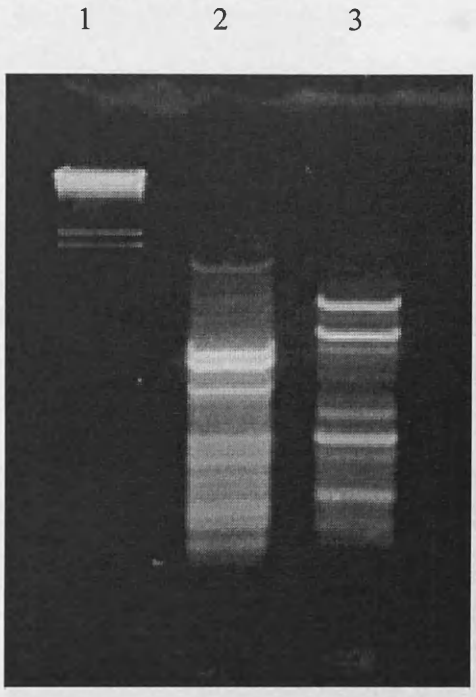


Figure 5.25 Ethidium bromide stained agarose gel of PCR products amplified with FliC1 forward and reverse primers with FliC1 amplified *X. bovienii* genomic DNA (lane 2) and FliC1 amplified *E. coli* genomic DNA (lane 3) templates. Lane 1 contains lambda Hind III markers. Lane 2 contains FliC1 amplified *X. bovienii* products amplified with 3.5 mM Mg^{2+}respectively. Lane 3 contains FliC1 amplified *E. coli* PCR products amplified with 1.55mM Mg^{2+}.

The triple band PCR product for *X. bovienii* shown in lanes two (Figure 5.25) were isolated individually to give three bands: Top, T, Middle, M, and Bottom, B (Figure 5.26). The three isolates were transformed into TOP10F' One Shot® (Invitrogen®). The sequenced plasmids were named pFliC1xbT, pFliC1xbM and pFliC1xbB and the BLASTn search revealed that although the cloned fragments from *X. bovienii* were of the expected size, they showed greatest homology with the β-galactosidase (lac Z) gene from *Yersinia pestis*.

1 2 3 4

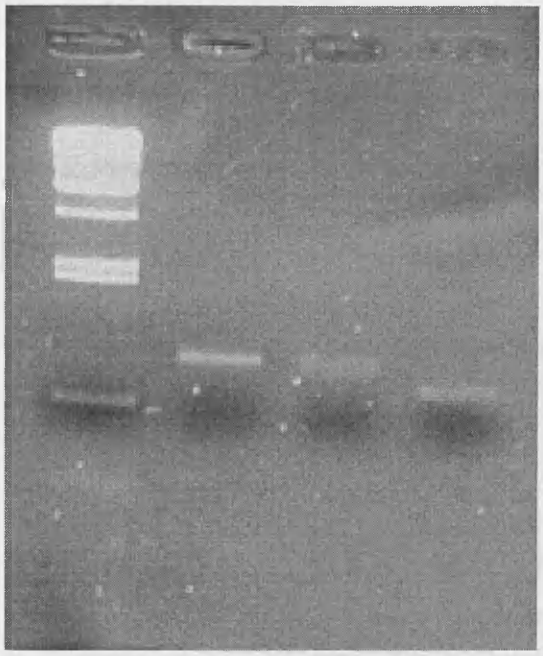


Figure 5.26 Ethidium bromide stained agarose gel of PCR products amplified with FliC1 forward and reverse primers with FliC1 amplified *X. bovienii* genomic DNA (lanes 2, 3 and 4) and purified using Hybaid gel purification kit as in Section Two (Section 2.3.3). Lane 1 contains lambda Hind III markers. Lane 2 contains the top band (T) of the FliC1 amplified *X. bovienii* gel purification products. Lane 3 contains the middle band (M) of the FliC1 amplified *X. bovienii* gel purification products. Lane 4 contains the bottom band (B) of the FliC1 amplified *X. bovienii* gel purification products.

New primers were designed to isolate the fragment of the FliC gene from *X. bovienii* (Figure 5.27)

FliC2 forward primer

5'-GA(CT) GA(CT) GC(AGCT) GC(AGCT) GG(AGCT) CA(AG)GC-3'

D D A A G Q A

FliC2 reverse primer

5'-GC(AGCT) CC(AGCT) AC(CT) TG(AGT) AT(CT) TTC AT-3'

A P T V I F I

Figure 5.27 The forward and reverse FliC2 primers with degenerate bases show in parentheses. Product size = 324bp

PCR using using *X. bovienii* genomic DNA as a template and an optimised annealing temperature of 66°C with a Mg^{2+} concentration of 2.5 mM resulted in a multi-banded PCR product (Figure 5.28). The region of interest (324kb) was excised and purified from the gel and subjected to a further round of PCR with the FliC2 forward and reverse primers, using the optimised conditions described above (Figure 5.29).

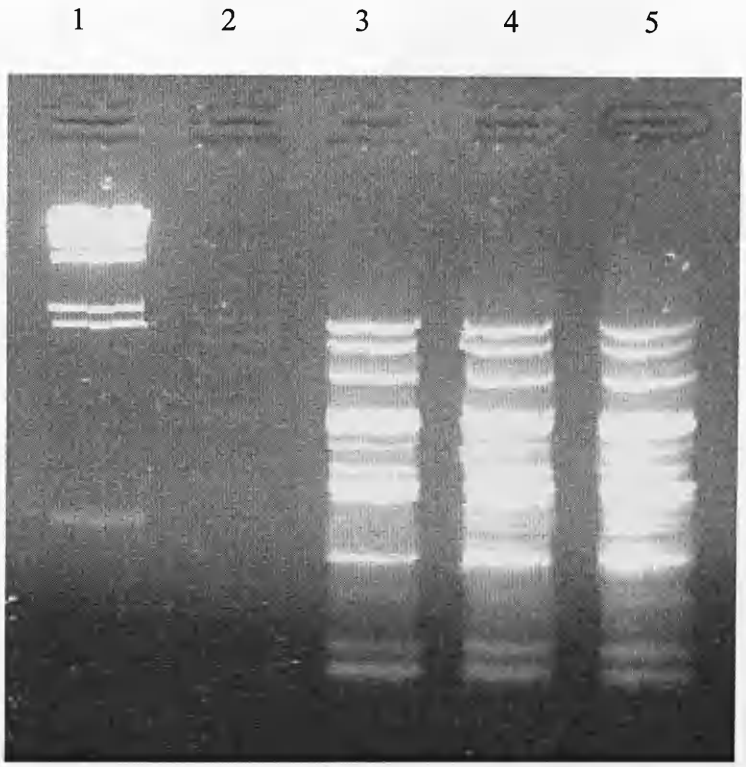


Figure 5.28 Ethidium bromide stained agarose gel of PCR products amplified with FliC2 forward and reverse primers with *X. bovienii* genomic DNA (lanes 2, 3, 4 and 5) templates. Lane 1 contains lambda Hind III markers. Lane 2, 3, 4 and 5 contains *X. bovienii* products amplified with 1.5 mM Mg^{2+} , 2.5 mM Mg^{2+}, 3.5 mM $Mg^{2+}$and 4.5mM Mg^{2+} respectively.

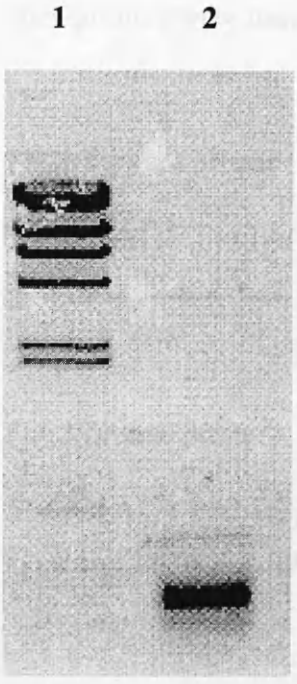
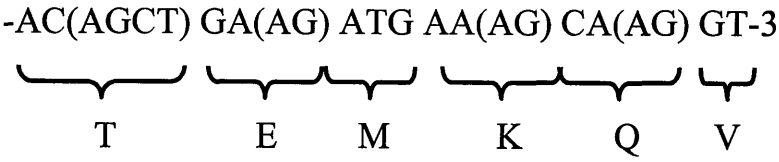


Figure 5.29 Ethidium bromide stained agarose gel of PCR products amplified with FliC2 forward and reverse primers with FliC2 amplified *X. bovienii* genomic DNA (lane 2) template. Lane 1 contains lambda Hind III markers. Lane 2 contains FliC1 amplified *X. bovienii* products amplified with 2.5 mM Mg^{2+}.

The single band PCR product, shown in lane two (Figure 5.29) was isolated and transformed into TOP10F' One Shot® (Invitrogen®). The sequenced plasmid was named pFliC2xb2 and the BLASTn search revealed that although the cloned fragment from *X. bovienii* was of the expected size, it showed greatest homology with an unspecified region from *Vibrio vulnificus*.

New primers were designed to isolate FliC from *X. bovienii* (Figure 5.30)

FliC3 forward primer



FliC3 reverse primer

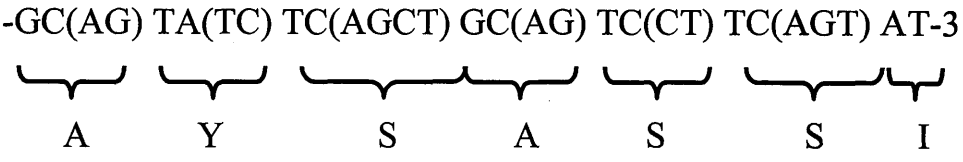


Figure 5.30 The forward and reverse FliC3 primers with degenerate bases show in parentheses. Product size = 400bp

To amplify a single band corresponding to FliC3 a number of different reaction conditions were tested using *X. bovienii* genomic DNA as a template. An optimal annealing temperature of 42°C and a Mg^{2+} concentration of 2.5 mM failed to amplify a region of sufficient brightness to continue with the isolation of the FliC gene fragment.

A new FliC forward and reverse primer was designed to amplify FliC from *X. bovienii* (Figure 5.31)

FliC4 forward primer

5'-AT(CT) GAG CGT CTG TCT TC(CT) GG-3'

A E R L S S G

FliC4 reverse primer

5'-CG(CT) TG(CT) A(AG)G TTG TT(AG) TTG ATT TC-3'

R T K/R L L L I S

Figure 5.31 The forward and reverse FliC4 primers with degenerate bases shown in parentheses. Product size = 180bp

To amplify a single band corresponding to FliC4 an optimised annealing temperature of 55°C and a Mg^{2+} concentration of 1.5 mM were used with *X. bovienii* genomic DNA as a template (Figure 5.32).

The single band PCR product shown in lane two (Figure 5.32) was isolated and transformed into TOP10F' One Shot® (Invitrogen®). The BLASTn search of the sequenced plasmid revealed that the cloned fragment from *X. bovienii* was of the expected size and showed greatest homology with the FliC region from *X. nematophilus* (Figure 5.33). A *ClustalX* alignment of the FliC fragment from *X. bovienii* and the published sequence from *X. nematophilus* was performed (Figure 5.34).

Figure 5.32 Ethidium bromide stained agarose gel of PCR products amplified with FliC4 forward and reverse primers with *X. bovienii* genomic DNA (lane 2) template. Lane 1 contains lambda Hind III markers. Lane 2 contains *X. bovienii* products amplified with 1.5 mM Mg^{2+}.

GAATTCGCCCTTCGCTGCAAGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCAATA
AGGGC

Figure 5.33 continued

Sequences producing significant alignments:		Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD	X.nematophilus fliC and fli...	297	4e-78
gi|16903563|gb|AF425736.1|AF425736	Salmonella enterica subs...	208	3e-51
gi|882142|gb|U17176.1|SCU17176	Salmonella choleraesuis ATCC...	208	3e-51
gi|882138|gb|U17174.1|SCU17174	Salmonella choleraesuis ATCC...	208	3e-51
gi|882132|gb|U17171.1|SCU17171	Salmonella choleraesuis ATCC...	208	3e-51
gi|30041418|gb|AE016984.1|	Shigella flexneri 2a str. 2457T ...	200	6e-49
gi|16420488|gb|AE008787.1|	Salmonella typhimurium LT2, sect...	200	6e-49
gi|16421311|gb|AE008826.1|	Salmonella typhimurium LT2, sect...	200	6e-49
gi|30059927|gb|AY250028.1|	Escherichia coli strain C2187-69...	200	6e-49
gi|30059917|gb|AY250023.1|	Escherichia coli strain 4106-54 ...	200	6e-49
gi|30059893|gb|AY250011.1|	Escherichia coli strain HW32 Fli...	200	6e-49
gi|30059887|gb|AY250008.1|	Escherichia coli strain HW27 Fli...	200	6e-49
gi|30059869|gb|AY249999.1|	Escherichia coli strain E 39a Fl...	200	6e-49
gi|26108544|gb|AE016762.1|	Escherichia coli CFT073 section ...	200	6e-49
gi|24052307|gb|AE015215.1|	Shigella flexneri 2a str. 301 se...	200	6e-49
gi|47470|emb|X04505.1|SRH1R	S. rubislaw H-1(r) gene for pha...	200	6e-49
gi|8071787|gb|AF228494.1|AF228494	Escherichia coli strain A...	200	6e-49
gi|8071785|gb|AF228493.1|AF228493	Escherichia coli strain D...	200	6e-49
gi|8071781|gb|AF228491.1|AF228491	Escherichia coli strain A...	200	6e-49
gi|8071779|gb|AF228490.1|AF228490	Escherichia coli strain A...	200	6e-49

Figure 5.33 The FliC gene fragment sequence from *X. bovienii* with the first 20 BLAST scores. The EcoR1 restriction site at the beginning of the construct is highlighted.

Figure 5.34 *ClustalX* alignment the fliC gene fragment from *X. bovienii* and the published *X. nematophilus* fliC sequence. The yellow highlighted areas are residues conserved across all species.

* indicates an amino acid match across all sequences

```
X.bovienii      ----------GAATTCGCCCTT-CGCTGCAAGTTGTTGTTGATTTCGTTCAGAGCG
X.nematophilus  TCAGATTGAACAGTCAGTTCACGAATACGTTGCAGGTTGTTATTGATTTCATTCAGAGCA
                          * *** *   * ** **** ****** ******** ********

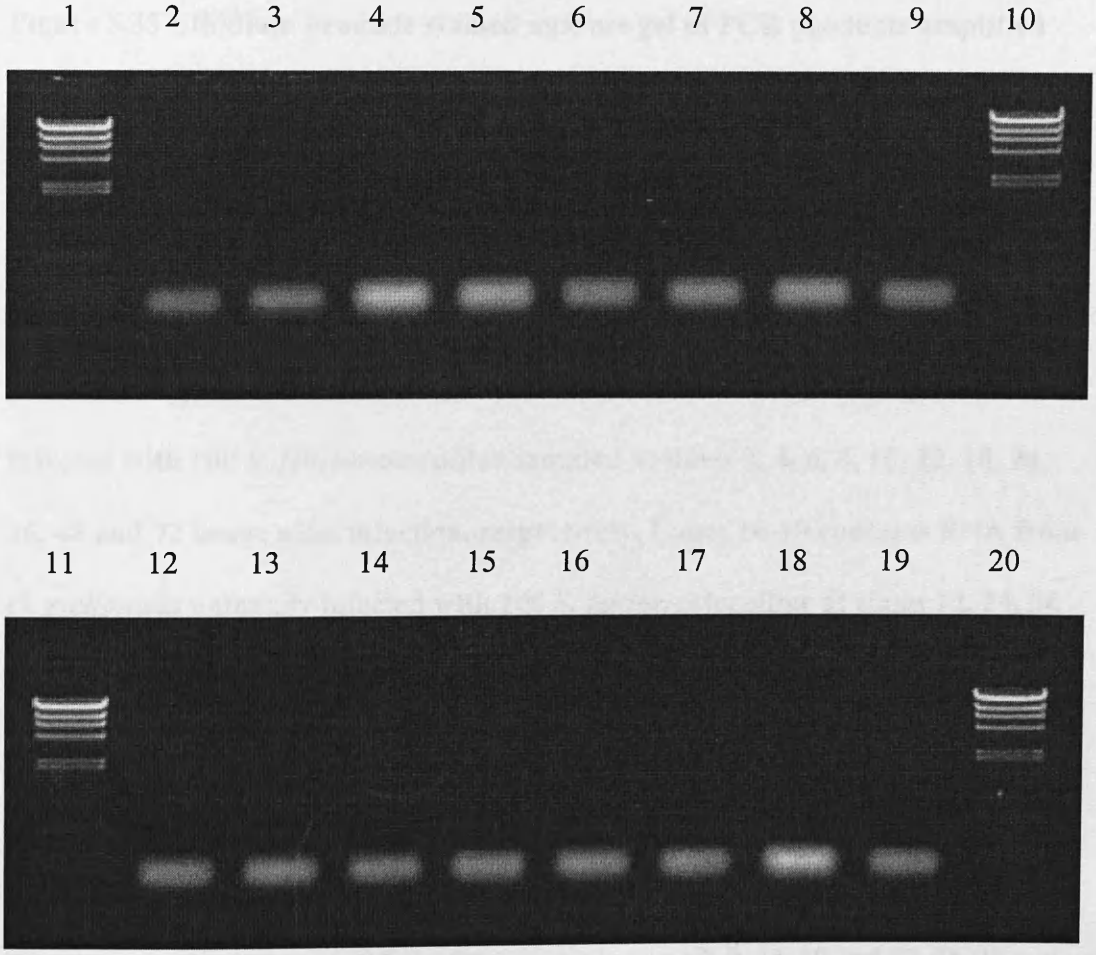
X.bovienii      CCTTCAGTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGTCAGA
X.nematophilus  CCTTCGGTAGTCTGAGCAATGGAGATACCGTCGTTTGCGTTACGTGCAGCCTGAGTCAGG
                ***** ********** ********************** * ***********

X.bovienii      CCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAGCGTCATCCTTCGCG
X.nematophilus  CCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAGCGTCATCCTTCGCG
                ************************************************************

X.bovienii      CTGTTAATACGTAAACCAGAAGACAGACGCTCAATAAGGGC------------------
X.nematophilus  CTGTTAATACGTAAACCAGAAGACAGACGCTCAATAGCAGAACCTAAAATGCCTTTAGAT
                ***********************************   *
```

5.3.2 RT-PCR with FliC4 forward and reverse primers

Total RNA isolated from *G. mellonella* during the time series, as described in Chapter Three, was checked for DNA contamination. A PCR reaction using the FliC4 forward and reverse primers was performed on all the RNA samples at the optimum temperature of 55°C with a concentration of 1.5 Mg^{2+} (Figure 5.35).



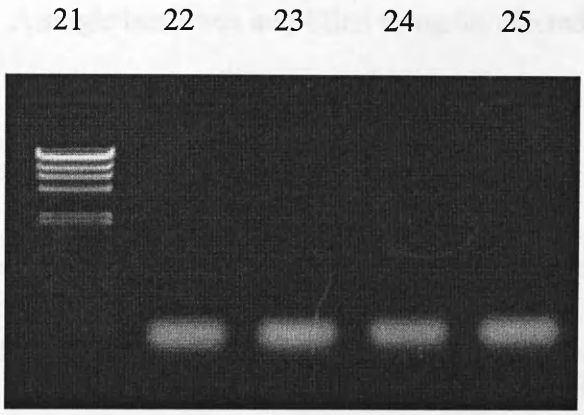


Figure 5.35 Ethidium bromide stained agarose gel of PCR products amplified with FliC4 forward and reverse primers with *G. mellonella* total RNA (lanes 2-9 and 12-19) template. Lanes 1, 10, 11, 20 and 21 contains lambda Hind III markers. Lanes 2-9 and 12-19 and 22-25 contains *S. feltiae* infected *G. mellonella* products amplified with 1.5 mM Mg^{2+}. Lane 2 contains RNA from uninfected *G. mellonella*. Lanes 3-9 and 12-15 contains RNA from *G. mellonella* injection infected with 100 *S. feltiae*/caterpillar sampled at times 2, 4, 6, 8, 10, 12, 18, 24, 36, 48 and 72 hours after infection, respectively. Lanes 16-19 contains RNA from *G. mellonella* naturally infected with 100 *S. feltiae*/caterpillar at times 12, 24, 36 and 48 hours after infection, respectively. Lanes 22-25 contains RNA from *G. mellonella* naturally infected with 1000 *S. feltiae*/caterpillar at times 12, 24, 36 and 48 hours after infection, respectively.

The absence of a FliC4 amplified PCR product in lanes 2-9, 11-19 and 22-25 (Figure 5.35) was a positive indication that the *G. mellonella* total RNA samples were free from DNA contamination.

A single band was amplified using uninfected and injection infected *G. mellonella* total RNA and Phase I and II *X. bovienii* as templates, an annealing temperature of 60°C with a Mg^{2+} concentration of 2mM and 30 cycles of amplification was found to be optimum (Figure 5.36).

The single bands in lanes 5 and 6 (Figure 5.36) indicate positive expression of the FliC gene in Phase I and II *X. bovienii*. The presence of bands in lanes 2-4 (Figure 5.34) indicates positive expression of the FliC gene in the 4 hour and 72 hour injection infection *G. mellonella* and in uninfected *G. mellonella*.

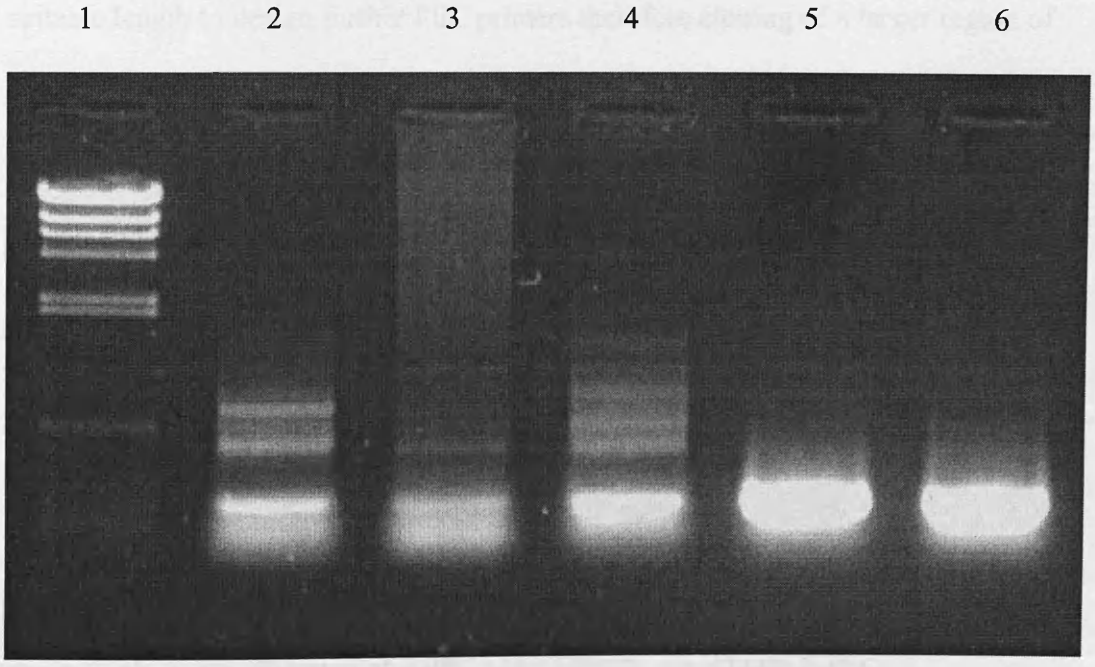


Figure 5.36 Ethidium bromide stained agarose gel of RT-PCR products amplified with FliC4 forward and reverse primers with *G. mellonella* total RNA (lanes 2-4) and *X. bovienii* total RNA (lanes 5-6) template. Lane 1 contains lambda Hind III markers. Lanes 2-4 and 5-6 contains *S. feltiae* infected *G.*

***mellonella* and *X. bovienii* products, respectively, amplified with 1.5 mM Mg^{2+}. Lane 2 contains RNA from uninfected *G. mellonella*. Lane 3 contains RNA from *G. mellonella* injection infected with 100 *S. feltiae*/caterpillar sampled at 4 hours after infection. Lane 4 contains RNA from *G. mellonella* injection infected with 100 *S. feltiae*/caterpillar sampled at 72 hours after infection. Lane 5 contains *X. bovienii* Phase I RNA. Lane 6 contains *X. bovienii* Phase II RNA.**

The amplification of the *X. bovienii* FliC gene fragment from the uninfected caterpillar (Figure 5.36) was unexpected and indicated the unsuitability of the FliC4 primers for quantitative RT-PCR. The NCBI database did not contain sequences of suitable length to design further FliC primers therefore cloning of a larger region of the FliC gene was undertaken (Section 5.3.4). The FliC RT-PCR amplified region from the uninfected and 4hr injection infected caterpillar total RNA was isolated, cloned and sequenced, to assess the similarity of these regions to another organism.

5.3.3 Isolation and cloning of the FliC RT-PCR product

The uninfected and 4 hour products from the RT-PCR (Figure 5.36), lanes 2 and 4, respectively, were separated on a 2% LMP agarose gel (Figure 5.37).

The products were isolated from a LMP gel and were inserted in pCR®2.1 TOPO and transformed into TOP10F' One Shot® (Invitrogen®). The cloned TOP10F' colonies containing the putative FliC uninfected and FliC 4hour inserts were screened by PCR,

utilising the optimum annealing temperature of 55°C with a Mg^{2+} concentration of 1.5 mM (Figure 5.38).

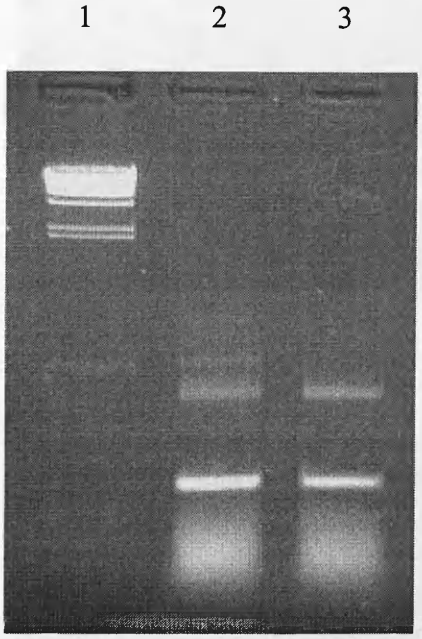


Figure 5.37 Ethidium bromide stained agarose gel of RT-PCR products amplified with FliC4 forward and reverse primers with *G. mellonella* total RNA (lanes 2 and 3) template. Lane 1 contains lambda Hind III markers. Lane 2 contains 4 hour *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}. Lane 3 contains uninfected *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}.

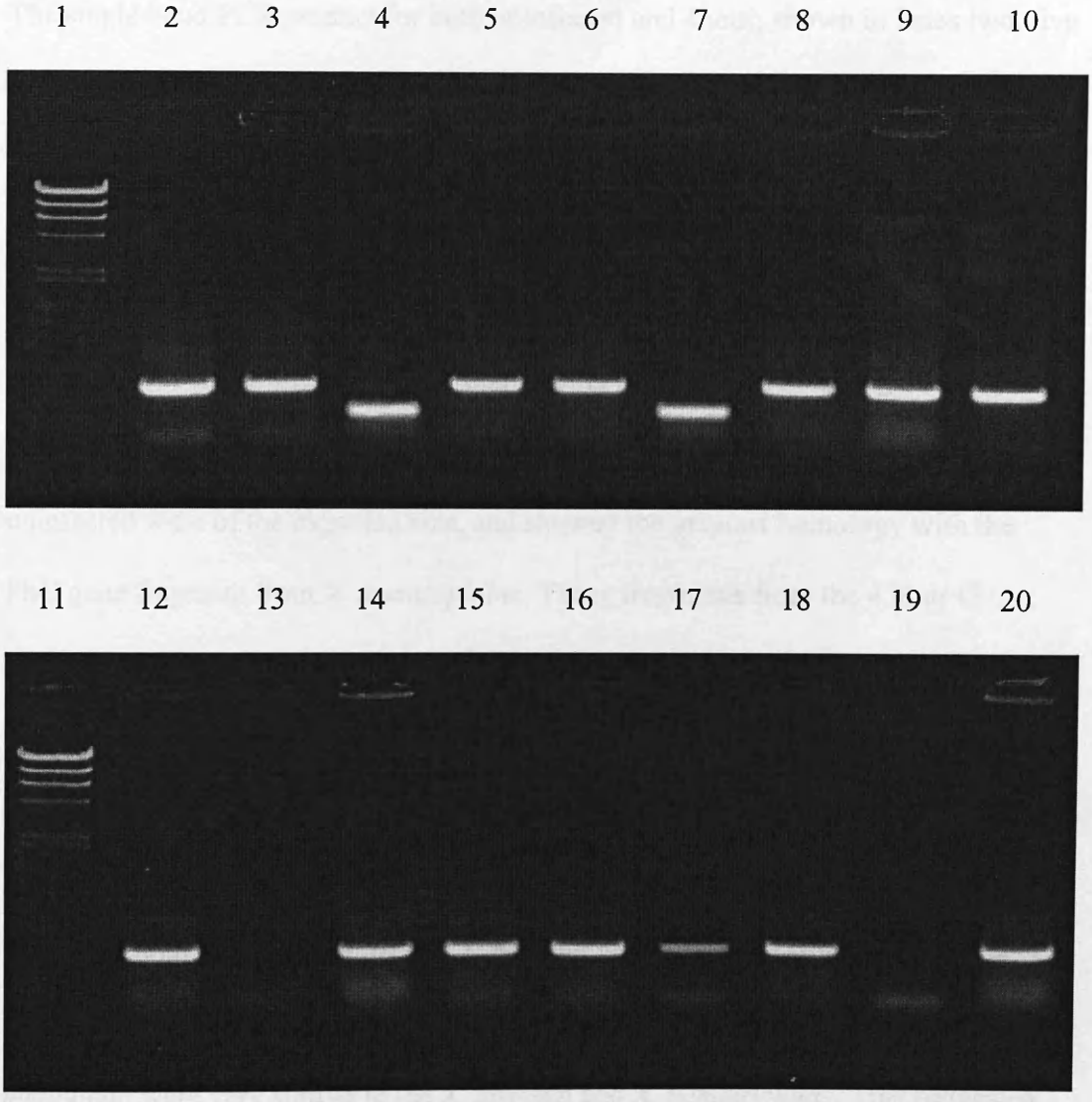


Figure 5.38 Ethidium bromide stained agarose gel of RT-PCR products amplified with FliC4 forward and reverse primers with TOP10F' One Shot® *G. mellonella* total RNA (rows 2-10 and 12-20) templates. Lanes 1 and 11 contains lambda Hind III markers. Lanes 2-10 contains TOP10F' One Shot® 4 hour *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}. Lanes 12-20 contains TOP10F' One Shot® uninfected *G. mellonella* total RNA RT-PCR products amplified with 1.5 mM Mg^{2+}.

The single band PCR product for both uninfected and 4hour, shown in lanes two, five eight and ten (4hour) and 12, 15, 18 and 20 (uninfected) respectively (Figure 5.38) were isolated and transformed into TOP10F' One Shot® (Invitrogen®). The sequenced plasmids were named pFliC4.1, pFliC4.2, pFliC4.3 and pFliC4.4 for the plasmids with the 4 hour insert, and pFliCui.1, pFliCui.2, pFliCui.3 and pfliCui.4 for the plasmids with the uninfected insert. Sequencing of these plasmids (Figure 5.39) and a BLASTn search revealed that the cloned fragment from all of the 4 hour and uninfected were of the expected size, and showed the greatest homology with the FliC gene fragment from *X. nematophilus*. Those fragments from the 4 hour *G. mellonella* tended to display a lower score value for *X. nematophilus* than the uninfected values.

A ClustalX alignment of the 4 hour and uninfected inserts with the same region of the FliC from both *X. nematophilus* and *X. bovienii* was performed (Figure 5.40). The alignment showed that the FliC fragments from the 4 hour and the uninfected *G. mellonella* were very similar to the *X. bovienii* and *X. nematophilus*. This suggested that is was almost certain that the 4 hour and the uninfected *G. mellonella* contain *X. bovienii* bacteria. This implied that perhaps a different organism, similar to *X. bovienii* but as yet unsequenced could have been present in the uninfected *G. mellonella*.

pFliCui.1

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:		Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD	X.nematophilus fliC and fli...	287	4e-75
gi|16903563|gb|AF425736.1|AF425736	Salmonella enterica subs...	188	2e-45
gi|882142|gb|U17176.1|SCU17176	Salmonella choleraesuis ATCC...	188	2e-45
gi|882138|gb|U17174.1|SCU17174	Salmonella choleraesuis ATCC...	188	2e-45
gi|882132|gb|U17171.1|SCU17171	Salmonella choleraesuis ATCC...	188	2e-45
gi|16420488|gb|AE008787.1|	Salmonella typhimurium LT2, sect...	180	6e-43
gi|16421311|gb|AE008826.1|	Salmonella typhimurium LT2, sect...	180	6e-43
gi|47470|emb|X04505.1|SRH1R	S. rubislaw H-1(r) gene for pha...	180	6e-43
gi|4001806|gb|AF045151.1|AF045151	Salmonella typhimurium ph...	180	6e-43
gi|882144|gb|U17177.1|STU17177	Salmonella typhimurium phase...	180	6e-43
gi|882140|gb|U17175.1|SCU17175	Salmonella choleraesuis ATCC...	180	6e-43
gi|882136|gb|U17173.1|SCU17173	Salmonella choleraesuis ATCC...	180	6e-43
gi|217062|dbj|D13689.1|STYFLICS	S.typhimurium gene for phas...	180	6e-43
gi|153978|gb|M11332.1|STYFLGH1I	Salmonella typhimurium H-1-...	180	6e-43
gi|30041418|gb|AE016984.1|	Shigella flexneri 2a str. 2457T ...	178	2e-42
gi|30059927|gb|AY250028.1|	Escherichia coli strain C2187-69...	178	2e-42
gi|30059917|gb|AY250023.1|	Escherichia coli strain 4106-54 ...	178	2e-42
gi|30059893|gb|AY250011.1|	Escherichia coli strain HW32 Fli...	178	2e-42
gi|30059887|gb|AY250008.1|	Escherichia coli strain HW27 Fli...	178	2e-42
gi|30059869|gb|AY249999.1|	Escherichia coli strain E 39a Fl...	178	2e-42

pFliCui.2

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:		Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD	X.nematophilus fliC and fli...	291	2e-76
gi|16903563|gb|AF425736.1|AF425736	Salmonella enterica subs...	216	1e-53
gi|882142|gb|U17176.1|SCU17176	Salmonella choleraesuis ATCC...	216	1e-53
gi|882138|gb|U17174.1|SCU17174	Salmonella choleraesuis ATCC...	216	1e-53
gi|882132|gb|U17171.1|SCU17171	Salmonella choleraesuis ATCC...	216	1e-53
gi|30041418|gb|AE016984.1|	Shigella flexneri 2a str. 2457T ...	208	3e-51
gi|16420488|gb|AE008787.1|	Salmonella typhimurium LT2, sect...	208	3e-51
gi|16421311|gb|AE008826.1|	Salmonella typhimurium LT2, sect...	208	3e-51
gi|30059927|gb|AY250028.1|	Escherichia coli strain C2187-69...	208	3e-51
gi|30059917|gb|AY250023.1|	Escherichia coli strain 4106-54 ...	208	3e-51
gi|30059893|gb|AY250011.1|	Escherichia coli strain HW32 Fli...	208	3e-51
gi|30059887|gb|AY250008.1|	Escherichia coli strain HW27 Fli...	208	3e-51
gi|30059869|gb|AY249999.1|	Escherichia coli strain E 39a Fl...	208	3e-51
gi|26108544|gb|AE016762.1|	Escherichia coli CFT073 section ...	208	3e-51
gi|24052307|gb|AE015215.1|	Shigella flexneri 2a str. 301 se...	208	3e-51
gi|47470|emb|X04505.1|SRH1R	S. rubislaw H-1(r) gene for pha...	208	3e-51
gi|8071787|gb|AF228494.1|AF228494	Escherichia coli strain A...	208	3e-51
gi|8071785|gb|AF228493.1|AF228493	Escherichia coli strain D...	208	3e-51
gi|8071781|gb|AF228491.1|AF228491	Escherichia coli strain A...	208	3e-51
gi|8071779|gb|AF228490.1|AF228490	Escherichia coli strain A...	208	3e-51

pFliCui.3

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD X.nematophilus fliC and fli...	280	9e-73
gi|16903563|gb|AF425736.1|AF425736 Salmonella enterica subs...	218	3e-54
gi|882142|gb|U17176.1|SCU17176 Salmonella choleraesuis ATCC...	218	3e-54
gi|882138|gb|U17174.1|SCU17174 Salmonella choleraesuis ATCC...	218	3e-54
gi|882132|gb|U17171.1|SCU17171 Salmonella choleraesuis ATCC...	218	3e-54
gi|16420488|gb|AE008787.1| Salmonella typhimurium LT2, sect...	210	7e-52
gi|16421311|gb|AE008826.1| Salmonella typhimurium LT2, sect...	210	7e-52
gi|47470|emb|X04505.1|SRH1R S. rubislaw H-1(r) gene for pha...	210	7e-52
gi|4001806|gb|AF045151.1|AF045151 Salmonella typhimurium ph...	210	7e-52
gi|882144|gb|U17177.1|STU17177 Salmonella typhimurium phase...	210	7e-52
gi|882140|gb|U17175.1|SCU17175 Salmonella choleraesuis ATCC...	210	7e-52
gi|882136|gb|U17173.1|SCU17173 Salmonella choleraesuis ATCC...	210	7e-52
gi|217062|dbj|D13689.1|STYFLICS S.typhimurium gene for phas...	210	7e-52
gi|153978|gb|M11332.1|STYFLGH1I Salmonella typhimurium H-1-...	210	7e-52
gi|27448112|gb|AF336929.1| Salmonella enterica subsp. enter...	202	2e-49
gi|16226012|gb|AF420426.1|AF420426 Salmonella enterica subs...	202	2e-49
gi|8895078|gb|AF159459.1|AF159459 Salmonella choleraesuis f...	202	2e-49
gi|882134|gb|U17172.1|SCU17172 Salmonella choleraesuis ATCC...	202	2e-49
gi|476237|gb|U06199.1|SNU06199 Salmonella newmexico S5323 p...	202	2e-49
gi|47233|emb|X03395.1|SMH1D Salmonella muenchen H1-d gene f...	202	2e-49

pFliCui.4

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD X.nematophilus fliC and fli...	274	5e-71
gi|16420488|gb|AE008787.1| Salmonella typhimurium LT2, sect...	182	2e-43
gi|882140|gb|U17175.1|SCU17175 Salmonella choleraesuis ATCC...	182	2e-43
gi|217062|dbj|D13689.1|STYFLICS S.typhimurium gene for phas...	182	2e-43
gi|153978|gb|M11332.1|STYFLGH1I Salmonella typhimurium H-1-...	182	2e-43
gi|27448112|gb|AF336929.1| Salmonella enterica subsp. enter...	174	4e-41
gi|16903563|gb|AF425736.1|AF425736 Salmonella enterica subs...	174	4e-41
gi|16226012|gb|AF420426.1|AF420426 Salmonella enterica subs...	174	4e-41
gi|8895078|gb|AF159459.1|AF159459 Salmonella choleraesuis f...	174	4e-41
gi|882142|gb|U17176.1|SCU17176 Salmonella choleraesuis ATCC...	174	4e-41
gi|882138|gb|U17174.1|SCU17174 Salmonella choleraesuis ATCC...	174	4e-41
gi|882132|gb|U17171.1|SCU17171 Salmonella choleraesuis ATCC...	174	4e-41
gi|47233|emb|X03395.1|SMH1D Salmonella muenchen H1-d gene f...	174	4e-41
gi|46855|emb|X03394.1|SCH1C Salmonella cholerae-suis H-1c g...	174	4e-41
gi|30041418|gb|AE016984.1| Shigella flexneri 2a str. 2457T ...	167	9e-39
gi|16421311|gb|AE008826.1| Salmonella typhimurium LT2, sect...	167	9e-39
gi|30059927|gb|AY250028.1| Escherichia coli strain C2187-69...	167	9e-39
gi|30059917|gb|AY250023.1| Escherichia coli strain 4106-54 ...	167	9e-39
gi|30059893|gb|AY250011.1| Escherichia coli strain HW32 Fli...	167	9e-39
gi|30059887|gb|AY250008.1| Escherichia coli strain HW27 Fli...	167	9e-39

pFliC4.1

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD X.nematophilus fliC and fli...	295	1e-77
gi|16903563|gb|AF425736.1|AF425736 Salmonella enterica subs...	212	2e-52
gi|882142|gb|U17176.1|SCU17176 Salmonella choleraesuis ATCC...	212	2e-52
gi|882138|gb|U17174.1|SCU17174 Salmonella choleraesuis ATCC...	212	2e-52
gi|882132|gb|U17171.1|SCU17171 Salmonella choleraesuis ATCC...	212	2e-52
gi|16420488|gb|AE008787.1| Salmonella typhimurium LT2, sect...	204	4e-50
gi|16421311|gb|AE008826.1| Salmonella typhimurium LT2, sect...	204	4e-50
gi|47470|emb|X04505.1|SRH1R S. rubislaw H-1(r) gene for pha...	204	4e-50
gi|4001806|gb|AF045151.1|AF045151 Salmonella typhimurium ph...	204	4e-50
gi|882144|gb|U17177.1|STU17177 Salmonella typhimurium phase...	204	4e-50
gi|882140|gb|U17175.1|SCU17175 Salmonella choleraesuis ATCC...	204	4e-50
gi|882136|gb|U17173.1|SCU17173 Salmonella choleraesuis ATCC...	204	4e-50
gi|217062|dbj|D13689.1|STYFLICS S.typhimurium gene for phas...	204	4e-50
gi|153978|gb|M11332.1|STYFLGH1I Salmonella typhimurium H-1-...	204	4e-50
gi|30041418|gb|AE016984.1| Shigella flexneri 2a str. 2457T ...	202	2e-49
gi|30059927|gb|AY250028.1| Escherichia coli strain C2187-69...	202	2e-49
gi|30059917|gb|AY250023.1| Escherichia coli strain 4106-54 ...	202	2e-49
gi|30059893|gb|AY250011.1| Escherichia coli strain HW32 Fli...	202	2e-49
gi|30059887|gb|AY250008.1| Escherichia coli strain HW27 Fli...	202	2e-49
gi|30059869|gb|AY249999.1| Escherichia coli strain E 39a Fl...	202	2e-49

pFliC4.2

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD X.nematophilus fliC and fli...	305	2e-80
gi|16903563|gb|AF425736.1|AF425736 Salmonella enterica subs...	216	1e-53
gi|882142|gb|U17176.1|SCU17176 Salmonella choleraesuis ATCC...	216	1e-53
gi|882138|gb|U17174.1|SCU17174 Salmonella choleraesuis ATCC...	216	1e-53
gi|882132|gb|U17171.1|SCU17171 Salmonella choleraesuis ATCC...	216	1e-53
gi|16420488|gb|AE008787.1| Salmonella typhimurium LT2, sect...	208	3e-51
gi|16421311|gb|AE008826.1| Salmonella typhimurium LT2, sect...	208	3e-51
gi|47470|emb|X04505.1|SRH1R S. rubislaw H-1(r) gene for pha...	208	3e-51
gi|4001806|gb|AF045151.1|AF045151 Salmonella typhimurium ph...	208	3e-51
gi|882144|gb|U17177.1|STU17177 Salmonella typhimurium phase...	208	3e-51
gi|882140|gb|U17175.1|SCU17175 Salmonella choleraesuis ATCC...	208	3e-51
gi|882136|gb|U17173.1|SCU17173 Salmonella choleraesuis ATCC...	208	3e-51
gi|217062|dbj|D13689.1|STYFLICS S.typhimurium gene for phas...	208	3e-51
gi|153978|gb|M11332.1|STYFLGH1I Salmonella typhimurium H-1-...	208	3e-51
gi|27448112|gb|AF336929.1| Salmonella enterica subsp. enter...	200	6e-49
gi|16226012|gb|AF420426.1|AF420426 Salmonella enterica subs...	200	6e-49
gi|8895078|gb|AF159459.1|AF159459 Salmonella choleraesuis f...	200	6e-49
gi|882134|gb|U17172.1|SCU17172 Salmonella choleraesuis ATCC...	200	6e-49
gi|47233|emb|X03395.1|SMH1D Salmonella muenchen H1-d gene f...	200	6e-49
gi|46855|emb|X03394.1|SCH1C Salmonella cholerae-suis H-1c g...	200	6e-49

pFliC4.3

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD X.nematophilus fliC and fli...	303	6e-80
gi|16903563|gb|AF425736.1|AF425736 Salmonella enterica subs...	218	3e-54
gi|882142|gb|U17176.1|SCU17176 Salmonella choleraesuis ATCC...	218	3e-54
gi|882138|gb|U17174.1|SCU17174 Salmonella choleraesuis ATCC...	218	3e-54
gi|882132|gb|U17171.1|SCU17171 Salmonella choleraesuis ATCC...	218	3e-54
gi|16420488|gb|AE008787.1| Salmonella typhimurium LT2, sect...	210	7e-52
gi|16421311|gb|AE008826.1| Salmonella typhimurium LT2, sect...	210	7e-52
gi|47470|emb|X04505.1|SRH1R S. rubislaw H-1(r) gene for pha...	210	7e-52
gi|4001806|gb|AF045151.1|AF045151 Salmonella typhimurium ph...	210	7e-52
gi|882144|gb|U17177.1|STU17177 Salmonella typhimurium phase...	210	7e-52
gi|882140|gb|U17175.1|SCU17175 Salmonella choleraesuis ATCC...	210	7e-52
gi|882136|gb|U17173.1|SCU17173 Salmonella choleraesuis ATCC...	210	7e-52
gi|217062|dbj|D13689.1|STYFLICS S.typhimurium gene for phas...	210	7e-52
gi|153978|gb|M11332.1|STYFLGH1I Salmonella typhimurium H-1-...	210	7e-52
gi|27448112|gb|AF336929.1| Salmonella enterica subsp. enter...	202	2e-49
gi|16226012|gb|AF420426.1|AF420426 Salmonella enterica subs...	202	2e-49
gi|8895078|gb|AF159459.1|AF159459 Salmonella choleraesuis f...	202	2e-49
gi|882134|gb|U17172.1|SCU17172 Salmonella choleraesuis ATCC...	202	2e-49
gi|47233|emb|X03395.1|SMH1D Salmonella muenchen H1-d gene f...	202	2e-49
gi|46855|emb|X03394.1|SCH1C Salmonella cholerae-suis H-1c g...	202	2e-49

pFliC4.4

GAATTCGCCCTTCGCTGTAGGTTGTTGTTGATTTCGTTCAGAGCGCCTTCA
GTAGTCTGAGCAATAGAGATACCGTCGTTTGCGTTACGAGAAGCCTGAGT
CAGACCTTTAACGTTCGCAGTAAAACGGTTAGCGATCGCTTGACCAGCAG
CGTCATCCTTCGCGCTGTTAATACGTAAACCAGAAGACAGACGCTCGATA
AGGGC

Sequences producing significant alignments:	Score (bits)	E Value
gi|1136223|emb|X91047.1|XNFLICD X.nematophilus fliC and fli...	276	1e-71
gi|16903563|gb|AF425736.1|AF425736 Salmonella enterica subs...	184	4e-44
gi|882142|gb|U17176.1|SCU17176 Salmonella choleraesuis ATCC...	184	4e-44
gi|882138|gb|U17174.1|SCU17174 Salmonella choleraesuis ATCC...	184	4e-44
gi|882132|gb|U17171.1|SCU17171 Salmonella choleraesuis ATCC...	184	4e-44
gi|30041418|gb|AE016984.1| Shigella flexneri 2a str. 2457T ...	176	9e-42
gi|16420488|gb|AE008787.1| Salmonella typhimurium LT2, sect...	176	9e-42
gi|16421311|gb|AE008826.1| Salmonella typhimurium LT2, sect...	176	9e-42
gi|30059927|gb|AY250028.1| Escherichia coli strain C2187-69...	176	9e-42
gi|30059917|gb|AY250023.1| Escherichia coli strain 4106-54 ...	176	9e-42
gi|30059893|gb|AY250011.1| Escherichia coli strain HW32 Fli...	176	9e-42
gi|30059887|gb|AY250008.1| Escherichia coli strain HW27 Fli...	176	9e-42
gi|30059869|gb|AY249999.1| Escherichia coli strain E 39a Fl...	176	9e-42
gi|26108544|gb|AE016762.1| Escherichia coli CFT073 section ...	176	9e-42
gi|24052307|gb|AE015215.1| Shigella flexneri 2a str. 301 se...	176	9e-42
gi|47470|emb|X04505.1|SRH1R S. rubislaw H-1(r) gene for pha...	176	9e-42
gi|8071787|gb|AF228494.1|AF228494 Escherichia coli strain A...	176	9e-42
gi|8071785|gb|AF228493.1|AF228493 Escherichia coli strain D...	176	9e-42
gi|8071781|gb|AF228491.1|AF228491 Escherichia coli strain A...	176	9e-42
gi|8071779|gb|AF228490.1|AF228490 Escherichia coli strain A...	176	9e-42

Figure 5.39 The RT-PCR amplified FliC gene fragment sequence from uninfected (ui) and 4 hour infected (4) *G.mellonella* total RNA with the first 20 BLAST scores. The restriction site at the beginning of the construct is highlighted.

* indicates an amino acid match across all sequences

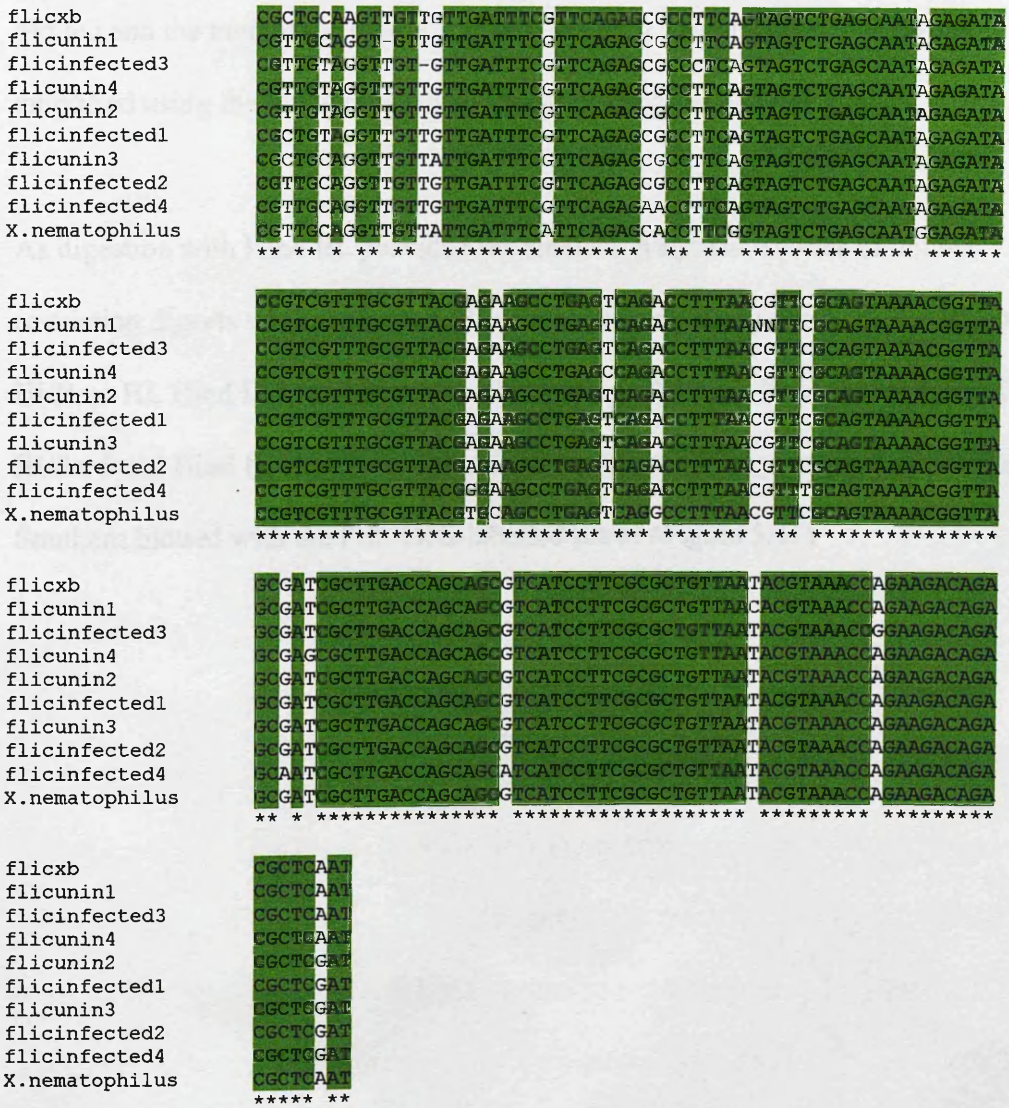
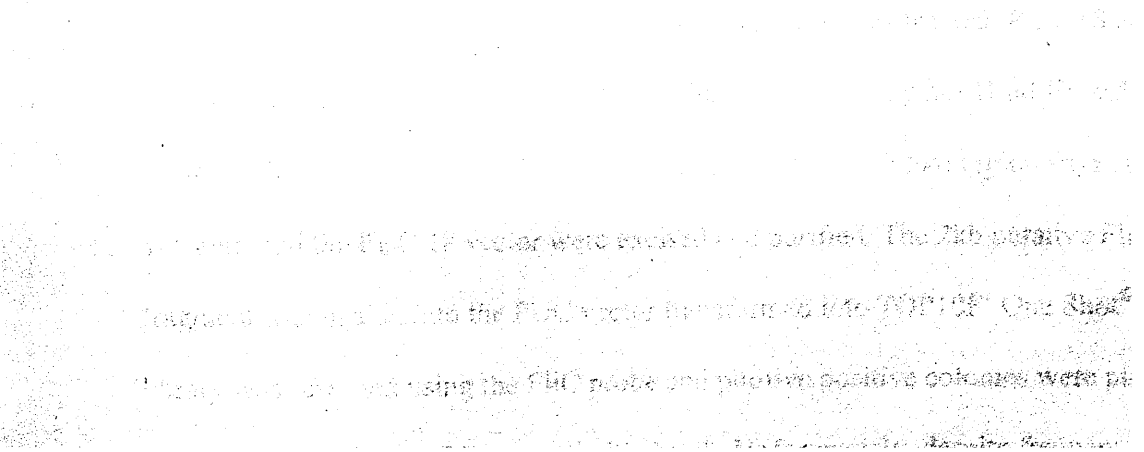


Figure 5.40 *ClustalX* alignment of 10 FliC nucleotide sequences. The green highlights are where all the residues are identical.

5.3.4 Cloning of a FliC gene fragment

To isolate a fragment of DNA from the *X. bovienii* genome containing a FliC fragment, a number of different single and double restriction digests were performed. Restriction digests using Eco RI, Hind III, Bam HI, Nco I, Xho I, Xba I, Sal I, Pst I and Kpn I, were electrophoresed on a 1% agarose gel which was then Southern blotted and the membrane hybridised with the PCR DIG-labelled FliC probe amplified using the FliC4 forward and reverse designed primers.

As digestion with Hind III provided the smallest fragment a series of double restriction digests were performed. Restriction digests using Hind III/Eco RI, Hind III/Bam HI, Hind III/Nco I, Hind III/Xho I, Hind III/Xha I, Hind III/Sal I, Hind III/Pst I and Hind III/Kpn I were electrophoresed on a 1% agarose gel which was then Southern blotted with the FliC DIG-labelled probe (Figure 5.41)



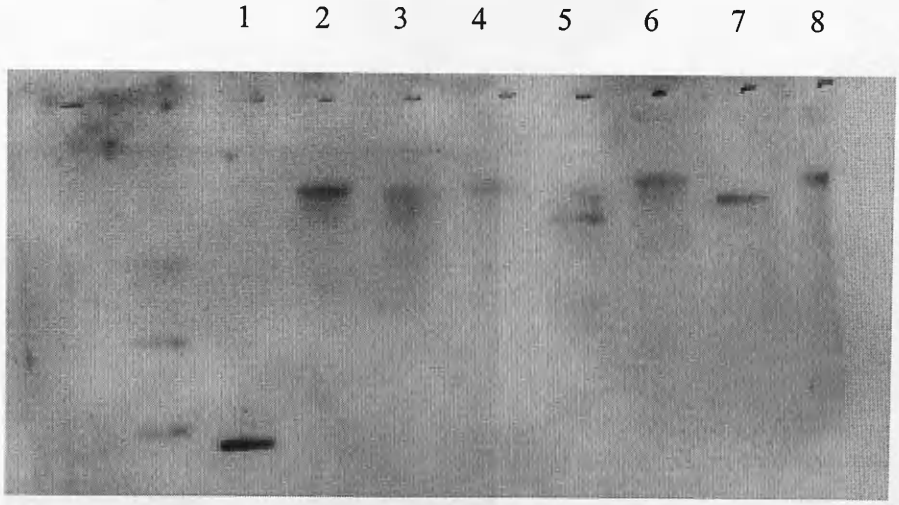


Figure 5.41 Southern blots with the FliC probe of restriction digests of *X. bovienii* genomic DNA. Lane 1: Hind III/Eco RI, lane 2: Hind III/Bam HI, lane 3: Hind III/Nco I, lane 4: Hind III/Xho I, lane 5: Hind III/Xba I, lane 6: Hind III/Sal I, lane 7: Hind III/Pst I, lane 8: Hind III/Kpn I.

A hybridising fragment of approximately 2kb produced using the restriction enzymes Hind III and Bam HI was chosen for isolation from the genomic *X. bovienii* DNA. A partial genomic library in TOP10F' One ShotPUC® was constructed. PUC 18 and *X. bovienii* genomic DNA were both cut with the restriction enzymes Hind III and Bam HI were electrophoresed on a 1% gel (Figure 5.42), the 1.5-2.5kb region from *X. bovienii* and the PUC 18 vector were excised and purified. The 2kb putative FliC fragment was ligated into the PUC vector transformed into TOP10F' One Shot®. The library was screened using the FliC probe and putitive positive colonies were picked and grown overnight in LB + amplicillin broth. Unfortunately, despite frequent repetition, none of the positive colonies were able to grow overnight in the selective growth media.

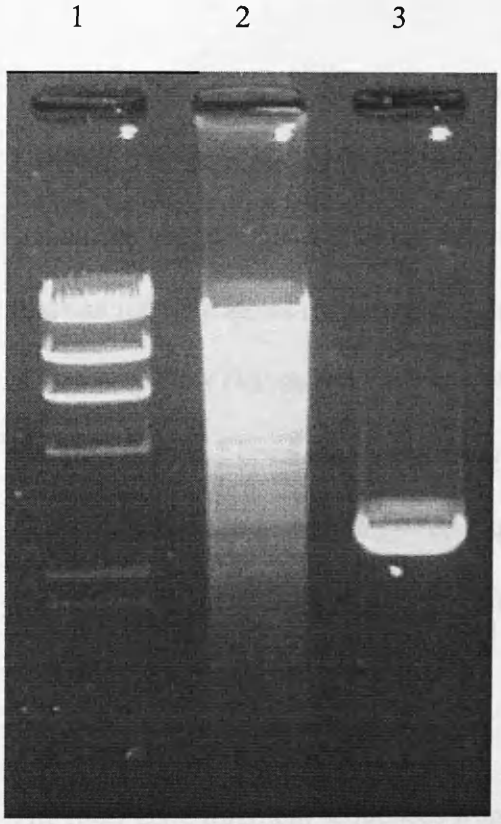


Figure 5.42 Ethidium bromide stained agarose gel with Bam HI/Hind III digested *X. bovienii* template DNA lane 1 and Bam HI/Hind III digested PUC18 template DNA, lane 3. Lane 1 shows lambda Hind III markers.

5.4 Discussion

This is the first time a fragment of the rpoS gene has been sequenced from *X. bovienii*. The extreme similarity between the *X. bovienii* and the *X. nematophilus* sequences suggest that it is likely that the same σS controls regulation in *X. bovienii* that can mediate stress resistance or host interactions (Hengge-Aronis 1993) as that in *X. nematophilus* (Vivas & Goodrich 2001). It is also the first time that a fragment of the FliC gene has been sequenced from *X. bovienii*, again, the similarity between the *X. bovienii* and the *X. nematophilus* sequences suggests that it is likely that the Phase I specific FliC gene from *X. nematophilus* (Givaudan *et al* 1996) is also active in *X. bovienii*.

The rpoS gene fragment sequences from both the infected and uninfected *G. mellonella* showed the greatest homology with the rpoS gene from *X. nematophilus*. A few differences in single nucleotide bases were observed, which is as expected as the *Galleria* were infected with *X. bovienii* not *X. nematophilus*. As the rpoS gene from *X. bovienii* has yet to be entered on the database, no match to *X. bovienii* could take place.

The results from the sequencing of the rpoS clones are suported when examined in conjunction with the sequence data from the FliC gene fragments. As with the rpoS sequence data, the gene fragments from both the infected and uninfected *Galleria* showed greatest homology with *X. nematophilus*, again as no database sequence for FliC from *X. bovienii* exists, this was expected.

These results go some way to explain the positive RT-PCR amplification of the supposed *X. bovieinii* rpoS and FliC gene fragments from the uninfected *Galleria.* These results indicate that there is a bacterium which is very similar to *X. bovienii* already present within the *Galleria* prior to infection with the entomopathogenic nematode. The bacteria must be part of the general gut flora and minute quantities of its RNA have been amplified by the RT-PCR reaction, resulting in false positives when the RNA from the time series of infection was subjected to RT-PCR. The question remains, what part, if any, does this uncultured bacterium, which is so similar to *Xenorhabdus* play in the role of infection with entomopathogenic nematodes?

5.4.1 Future work

Successful cloning of a larger region of the FliC and rpoS genes, as was attempted in this study would lead to the design of better RT-PCR primers characterisation of the regulation of the FliC and rpoS genes.

The use of real time PCR would perhaps eliminate the false positive results obtained when the RNA from the time series of infection was subjected to RT-PCR. Real time PCR would allow the amount of differing RNA to be quantified throughout the cycle, thus establishing the amount of the different types of RNA present at any given time.

The isolation, cloning and sequencing of the *Xenorhabdus*-like bacterium from *Galleria* would lead to better understanding of the infection parameters.

CHAPTER SIX

2-Dimensional characterisation of the expressed proteins from Phase I and II of *X. bovienii* (Strain UK76)

6.1 Introduction

The differences in gene expression between Phase 1 and Phase II *X. bovienii* were further characterised through investigation of the different proteins produced during culture in liquid medium. Due to the large number of proteins produced by both P I and PII *X. bovienii* the differences in expression were visualised using 2-Dimensional gel electrophoresis, which results in far superior separation and characterisation of the expressed proteins than a regular SDS-PAGE gel would provide.

As in previous chapters, a reduction in the osmolarity of the *X. bovienii* culture medium results in a transfer between Phase I and Phase II, which ultimately lead to the down regulation of a number of genes affecting the ability of *Xenorhabdus* to produce antibiotics, toxins and a flagellum. The switching off of these genes would result in a different protein expression pattern, which would be evident in the protein spots visualised on the 2-dimensional gel.

6.2 Culture of Phase I and II *X. bovienii* and protein extraction

Phase I and Phase II *X. bovienii* were cultured in DSMZ and LM broth (Section 2.1.2) respectively. Cells were grown to mid-log phase and were plated on NBTA agar to check that phase change had not occurred. Phase I *X. bovienii* will adsorb the bromothymol blue from the agar plates to produce blue/green colonies, whilst Phase II will adsorb the tetrazolium chloride from the agar plates and to produce red colonies (Kaya & Gaugler, 1993).

Two different methods of solublising bacterial proteins for 2D-SDS-PAGE analysis were tested. 2-D lysis buffer (Section 2.7.1) proved to be the most effective as the SDS lysis buffer (3% SDS, 20 mM DTT, 112 mM Tris, pH 8.8, 1.5 mM EDTA, 11% sucrose, 0.0% bromophenol blue) produced a blue stained smear on the 2-D SDS-PAGE, probably due to overloading the sample with SDS.

6.3 Optimisation of 2-Dimensional gel electrophoresis

The first dimension of the 2-Dimesional gel, the isoelectric focusing (IEF) was optimised through testing a number of different voltage combinations on the Multiphor™ II (Amersham Pharmacia) flatbed system. An optimal voltage programme for 7cm and 13cm, pH 3-10 Immobiline DryStrips and 7 and 13 cm pH 4-7 strips was obtained (Table 6.1).

Immobiline Strip	Step	V	mA	W	Time (h)	KVh
pH 3-10, 7cm	1	200	2	5	0.01	
	2	3500	2	5	1.30	2.8
	3	3500	2	5	1.05	3.7
pH 3-10, 13cm	1	200	2	5	0.01	
	2	3500	2	5	1.30	2.9
	3	3500	2	5	4.00	14.1
pH 4-7, 7cm	1	200	2	5	0.01	
	2	3500	2	5	1.30	2.8
	3	3500	2	5	1.30	5.2
pH 4-7, 13cm	1	200	2	5	0.01	
	2	3500	2	5	2.30	2.9
	3	3500	2	5	4.20	18.1

Table 6.1 Optimal voltage programming for the separation of PI and PII *X. bovienii* protein on the Multiphor II electrophoresis system.

pH 3-10, 13 cm strips produced the clearest 2-D SDS-PAGE gel, showing the greatest number of individual protein spots with the best separation between the spots and the greatest distinction between the Phase I (Figure 6.1) and Phase II (Figure 6.2) total protein extractions. For both Phase I and II the majority of proteins were detected between pH 4-7 and ranged in mass from 120-21.5 kDa. Phase II appeared to express a number of large, >55 kDa, proteins, which although some were also expressed by Phase I, expression was not as high. Phase I expressed a highly abundant protein at 31 kDa and pH 4.5, however a protein of similar size and pH was also expressed, though in much lower amounts by PII, so this very obvious protein was not chosen for further study. An abundant protein expressed only by Phase I *X. bovienii* was excised from the gel, solubilised in SDS loading buffer and different volumes were electrophoresed on a 1.5mm NOVEX Bis-Tris 10% (Invitrogen) gel (Figure 6.3).

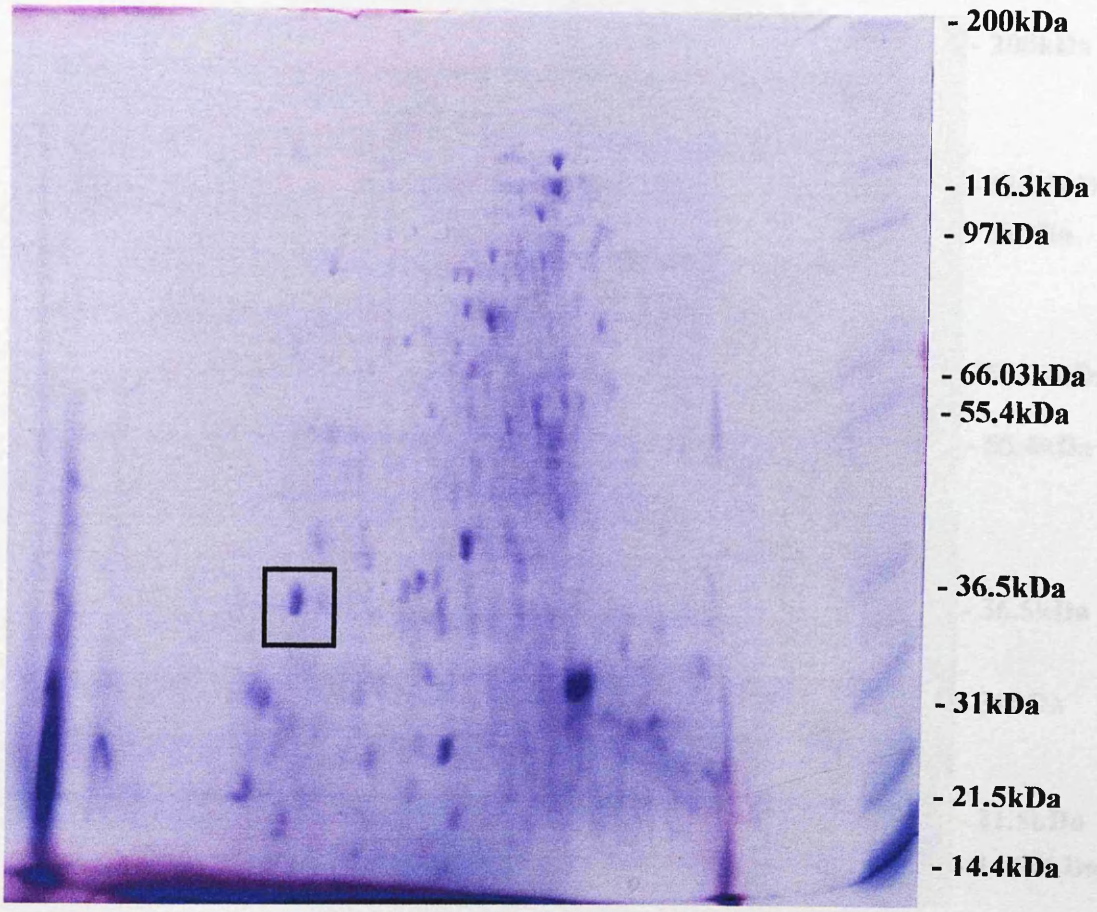


Figure 6.1 Bromothymol blue stained 2-Dimensional SDS-PAGE gel showing total protein extraction from Phase I *X. bovienii* cells cultured in DSMZ until late log phase. Proteins were focused by IEF on a 13 cm pH 3-10 Immobiline™ DryStrip followed by separation according to apparent molecular mass on a 10% SDS-PAGE. Mark 12 protein standards are indicated to the right of the gel. The protein spot of interest is highlighted by a black square.

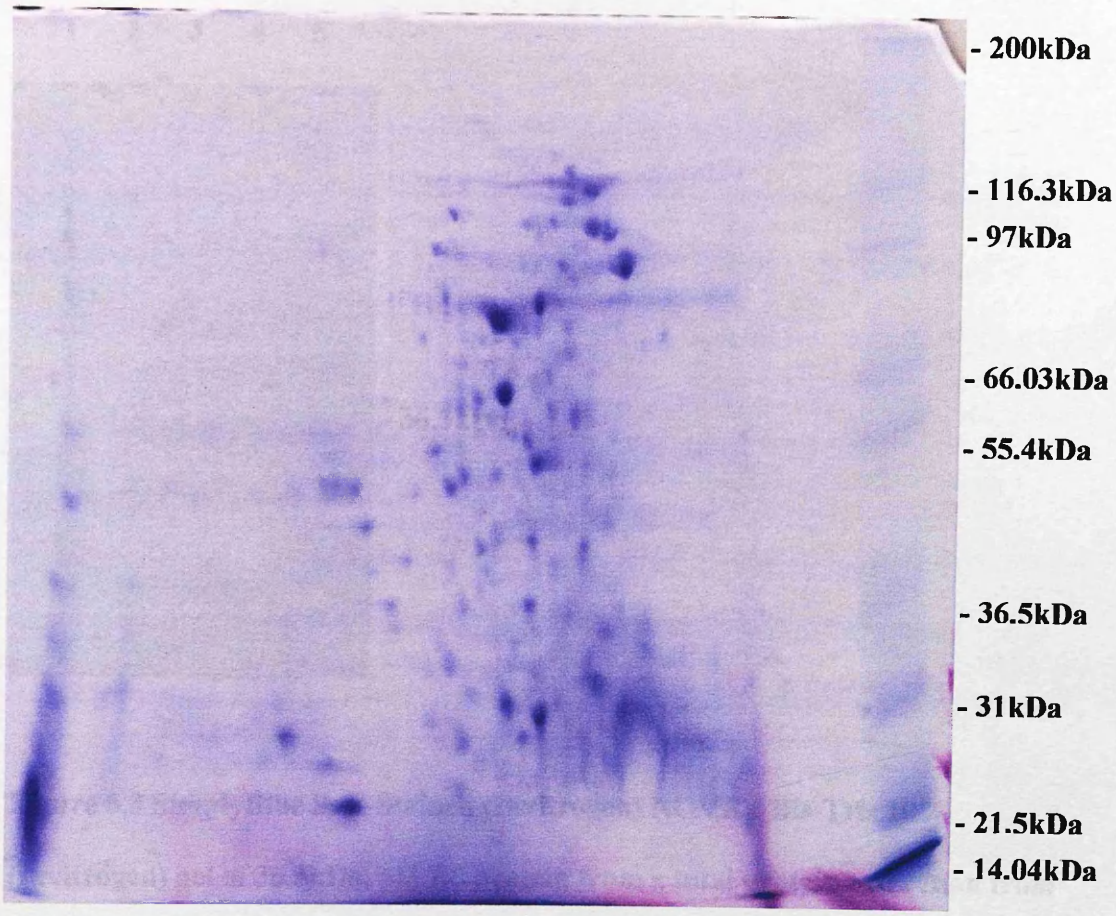


Figure 6.2 Bromothymol blue stained 2-Dimensional SDS-PAGE gel showing total protein extraction from Phase II *X. bovienii* cells cultured in LM until late log phase. Proteins were focused by IEF on a 13 cm pH 3-10 Immobiline™ DryStrip followed by separation according to apparent molecular mass on a 10% SDS-PAGE. Mark 12 protein standards are indicated to the right of the gel.

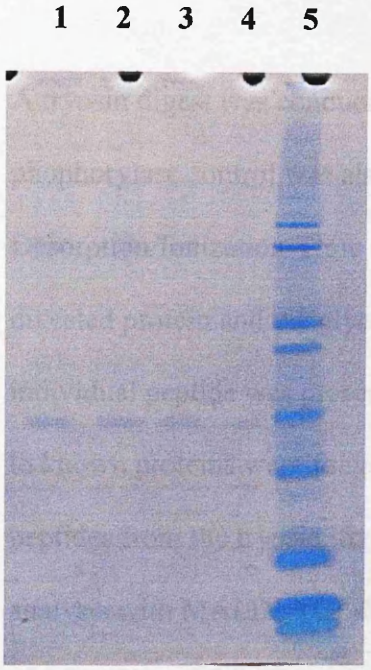


Figure 6.3 SimplyBlue Safe Stained (Invitrogen) NOVEX Bis-Tris 10% (Invitrogen) gel of 36.5kDa, pH 7.0 protein from a total protein extraction from *X. bovienii* Phase I cells cultured in LM until late log phase, focused by IEF on a 13 cm pH 3-10 Immobiline™ DryStrip followed by separation according to apparent molecular mass on a 10% SDS-PAGE. Lanes 1, 2, 3 and 4 show 80 µl, 60 µl, 40 µl and 20 µl loadings respectively. Lane 5 contains Mark 12 (Invitrogen) protein standards.

6.4 Proteomic analysis of the 36.5kDa protein from Phase I *X. bovienii*

A trypsin digest was conducted on the excised protein spot (Figure 6.4), a glycogen phophorylase control was also performed (Figure 6.5). A Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) analysis was performed on the digested protein and the glycogen phosphorlylase control, the mass in Daltons of each individual peptide was presented as a percentage of the total protein mass. No matches to known proteins were found from the MALDI-TOF analysis, therefore, three peptides from the trypsin digest of Phase I *X. bovienii* were chosen to undergo further analysis with MALDI-TOF-MSMS, 1046.56 (Figure 6.6), 688.38 (Figure 6.7) and 555.32 (Figure 6.8), the mass in Daltons of each individual amino acid was presented as a percentage of the total peptide mass. Automated database searching with the MALDI-TOF-MSMS data proved unsuccessful, suggesting that the protein has little homology with anything in the database. This lead to de-novo sequencing of the three peptides, 1046.56, 688.38 and 555.32 (Figure 6.9) and identifying the protein by homology rather than exact sequence match using the SWISS-PROT database (Figures 6.10 – 6.12).

Three matches from the SWISS-PROT database were thought to be of interest: an Ammonium transporter, ID number: Q8H6Y4, from *Phytophora infestans*, a potato blight fungus which has a 62.8% identity with the peptide 1046.56 and a PAM 100 alignment score of 71 out of a possible 113 (Figure 6.13); an ABC transporter, ID number: Q891F0, from *Clostridium tetani*, the causative agent of tetanus, which had a 82.1% identity with peptide 555.32 and a PAM 100 alignment score of 64 out of a

possible 78 (Figure 6.14); a Phosphate transport ATP-binding protein B, Q9PQU3, from *Ureaplasma parvum*, a mucosal pathogen of humans, which had a 96.2% identity with peptide 555.32 and a PAM 100 alignment score of 75 out of a possible 78 (Figure 6.15).

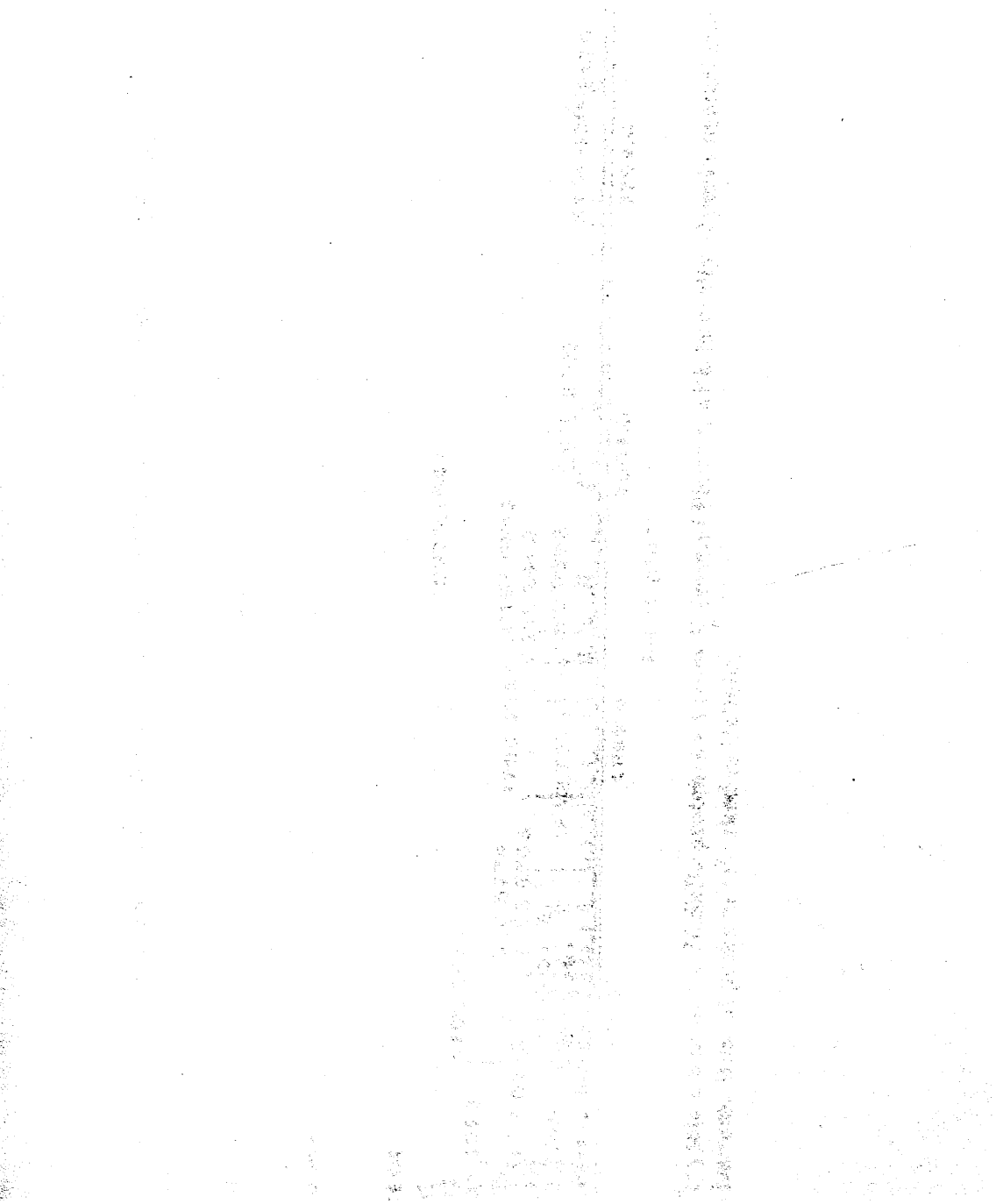
The ammonium transporter (ID no Q8H6Y4), protein comprised 518 amino acids and was 54.345 kDa in mass. The graph of the domain regions (Figure 6.16) showed that peptide 1046.56 fell within the Ammonium transporter region, PF00909, according to the numbering system attributed by the SWISS-PROT database.

The ABC transporter ATP-bi protein (ID no Q891F0) comprised 641 amino acids and was 74.078 kDa in mass. The graph of the domain regions (Figure 6.17) showed that peptide 555.32 fell within the ABC Transporter 2 region of the protein.

The phosphate transport ATP-binding protein B (ID no Q9PQU3), comprised 329 amino acids and was 37.837 kDa in mass. The graph of the domain regions (Figure 6.18) showed that the peptide fell within the same ABC transporter 2 region of the protein as Q891F0.

Although protein Q891F0 is too large to be homologous with the 36.5 kDa protein isolated from *X. bovienii*, the graph of the domain region shows that the ABC Transporter 2 region is spilt into two working domains. Peptide 555.32 falls within the first of the two domains, which is 259 amino acids long. The ABC transporter 2 region is exactly the same as the ABC transporter 2 region from Q9PQU3, note accession number PS50893 for both ABC transporter 2 regions. This suggests that with a identity match of 96.2% and 82.1% to the 555.32 peptide from Phase I *X.*

bovienii the 36.5 kDa protein is most likely to be an ABC transporter ATP binding protein.



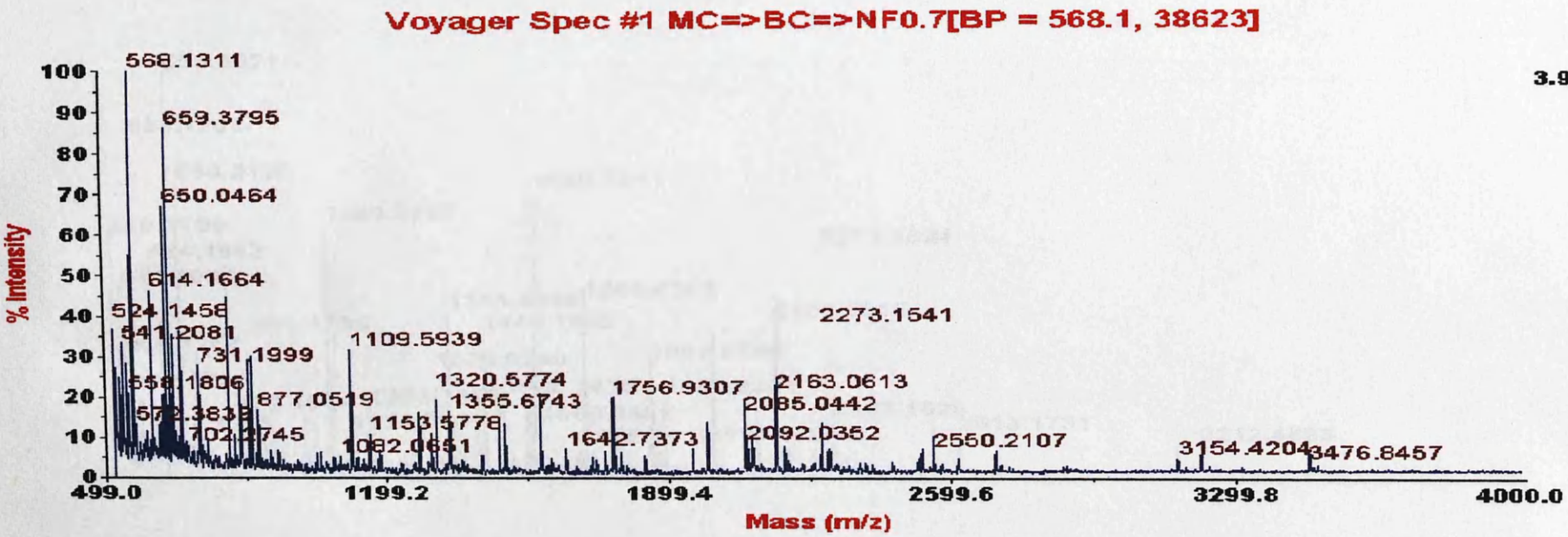


Figure 6.4 Trypsin digest of the 36.5kDa protein spot from *X. boveinii* Phase I, with intensity of peaks against mass of peptides in Daltons. Mass of peaks are marked in Daltons.

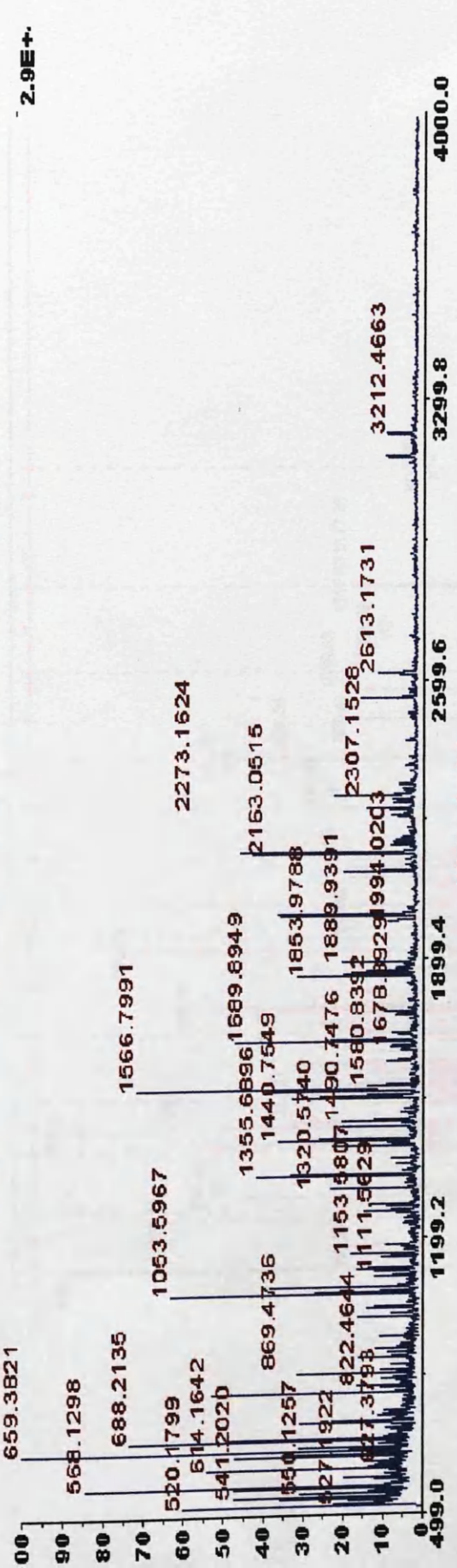


Figure 6.5 MALDI-TOF analysis of trypsin digest of Glycogen phosphorylase, with intensity of peaks against mass of peptides in Daltons. Mass of peaks are marked in Daltons.

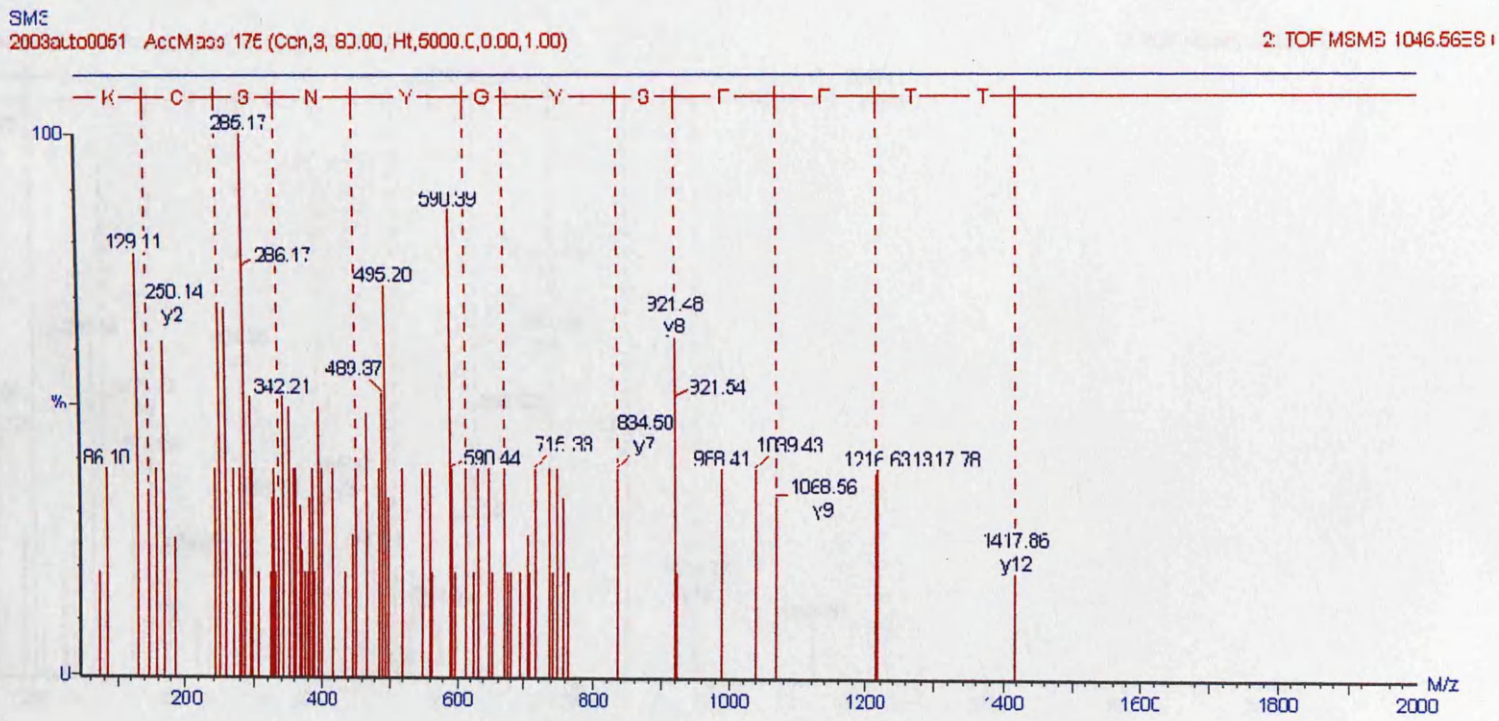


Figure 6.6 MSMS analysis of peptide 1046.56 from the trypsin digest of the 36.5kDa protein from *X. boveinii*, with intensity of peaks against mass of amino acids in Daltons. Mass of peaks are marked in Daltons.

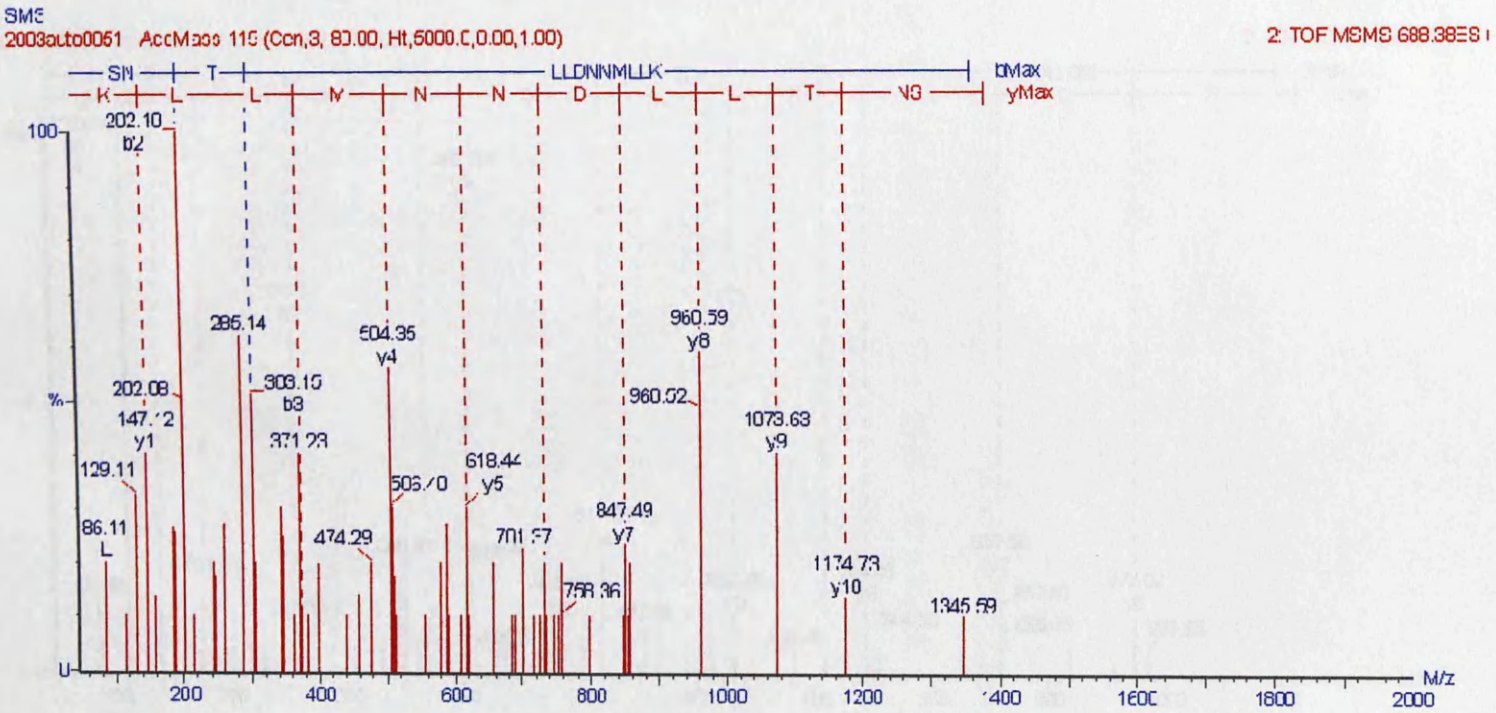


Figure 6.7 MSMS analysis of peptide 688.38 from the trypsin digest of the 36.5kDa protein from *X. boveinii*, with intensity of peaks against mass of amino acids in Daltons. Mass of peaks are marked in Daltons.

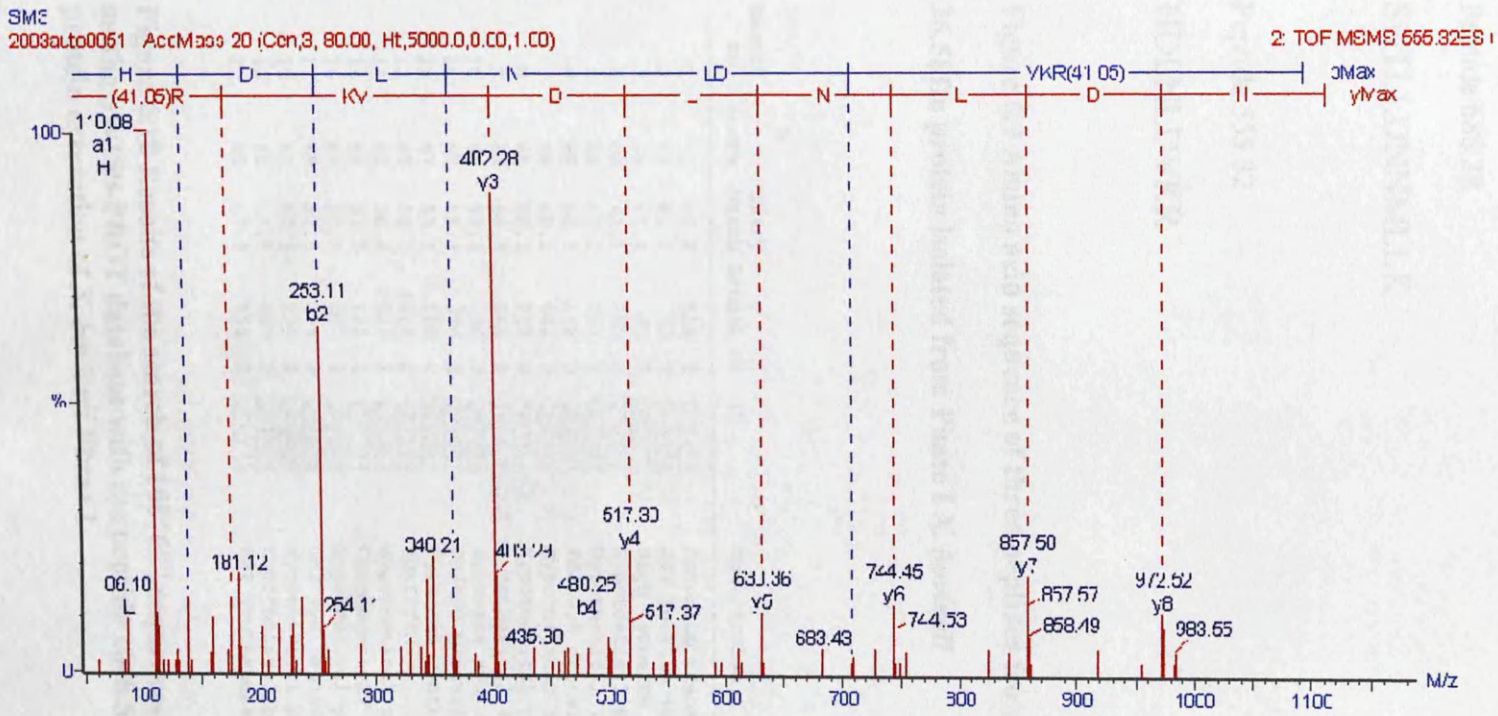


Figure 6.8 MSMS analysis of peptide 555.32 from the trypsin digest of the 36.5kDa protein from *X. boveinii*, with intensity of peaks against mass of amino acids in Daltons. Mass of peaks are marked in Daltons.

Peptide 1046.56

TTFFSYGYNSCK

Peptide 688.38

SNTLLDNNMLLK

Peptide 555.32

HDLNLDVKR

Figure 6.9 Amino acid sequence of three peptides from the trypsin digest of the 36.5kDa protein isolated from Phase I *X. bovienii*

Result No.	Score	% Query Match	Length	DB	ID	Description	Pred. No.
1	71	62.8	518	2	Q8H6Y4	Ammonium transporter.	7.25e-01
2	69	61.1	53	2	O08633	HGT keratin (Fragment)	1.66e+00
3	69	61.1	63	2	Q8IUC2	High tyrosine glycine	1.66e+00
4	69	61.1	369	2	Q8G6D1	Hypothetical protein.	1.66e+00
5	68	60.2	257	2	Q8T808	Hypothetical 28.9 kDa	2.50e+00
6	68	60.2	161	2	Q9LPM7	F2J10.6 protein.	2.50e+00
7	68	60.2	442	2	Q9LVK6	Hypothetical 50.6 kDa	2.50e+00
8	68	60.2	257	3	AAO51543	Hypothetical protein.	2.50e+00
9	67	59.3	388	1	Y464_MYCCA	Hypothetical 44.5 kDa	3.76e+00
10	67	59.3	86	2	Q8TUN3	Pyruvate synthase, sub	3.76e+00
11	67	59.3	384	2	Q9VWJ7	CG7890 protein (RE0173	3.76e+00
12	67	59.3	110	2	Q8PX81	Pyruvate synthase delt	3.76e+00
13	67	59.3	4816	2	Q8T103	BMKETTIN.	3.76e+00
14	66	58.4	1530	2	Q8IBS2	Hypothetical protein.	5.62e+00
15	65	57.5	132	2	Q08734	Chromosome XV reading	8.39e+00
16	65	57.5	202	2	Q8BRJ9	Hypothetical protein.	8.39e+00
17	65	57.5	555	2	Q8IJR9	GMP synthetase.	8.39e+00
18	65	57.5	279	2	Q8XKQ5	Hypothetical protein C	8.39e+00
19	65	57.5	600	2	Q8I816	Hypothetical protein (	8.39e+00
20	65	57.5	555	2	Q9U775	GMP synthetase (EC 6.3	8.39e+00

Figure 6.10 Results of the search of 1087687 sequences and 346235233 residues on the SWISS-PROT database with the peptide 1046.56 isolated from the total protein extraction of *X. bovienii* Phase I .

Result No.	Score	% Query Match	Length	DB	ID	Description	Pred. No.
1	70	70.7	6473	2	Q8IKH9	Dynein beta chain, put	9.11e-01
2	69	69.7	491	1	NMT_CRYNE	Glycylpeptide N-tetrad	1.41e+00
3	69	69.7	370	3	BAC68899	Putative regulatory pr	1.41e+00
4	68	68.7	734	2	Q96TA0	Protocadherin-psi2.	2.18e+00
5	68	68.7	2569	2	Q8IBG8	Hypothetical protein.	2.18e+00
6	67	67.7	188	2	Q9AZD5	Orf25.	3.34e+00
7	67	67.7	309	2	Q9APS3	UvrB-like protein (Fra	3.34e+00
8	67	67.7	188	2	Q9CI39	Unknown protein.	3.34e+00
9	66	66.7	314	2	Q8VGV2	Olfactory receptor MOR	5.12e+00
10	66	66.7	1784	2	Q9C6R1	Hypothetical 201.8 kDa	5.12e+00
11	66	66.7	117	2	Q8G351	Conserved domain prote	5.12e+00
12	66	66.7	932	2	Q8GZ49	Hypothetical protein.	5.12e+00
13	66	66.7	1791	2	Q9U6D4	Merozoite surface anti	5.12e+00
14	66	66.7	143	2	Q8YEN1	Hypothetical protein B	5.12e+00
15	66	66.7	1787	2	Q25645	Merozoite surface prot	5.12e+00
16	66	66.7	1216	3	AAO51431	Similar to Dictyosteli	5.12e+00
17	66	66.7	932	3	AAO64911	At1g58190.	5.12e+00
18	65	65.7	4725	1	DYHC_DICDI	Dynein heavy chain, cy	7.81e+00
19	65	65.7	246	2	P97611	Granzyme J.	7.81e+00
20	65	65.7	121	2	Q8IK97	Ribosomal protein L20,	7.81e+00

Figure 6.11 Results of the search of 1104069 sequences and 350750477 residues on the SWISS-PROT database with the peptide 668.38 isolated from the total protein extraction of *X. bovienii* Phase I .

Result No.	Score	% Query Match	Length	DB	ID	Description	Pred. No.
1	75	96.2	329	2	Q9PQU3	Phosphate transport AT	1.17e-03
2	64	82.1	641	2	Q891F0	ABC transporter ATP-bi	3.92e-01
3	63	80.8	465	2	Q9K7T7	Xaa-His dipeptidase.	6.49e-01
4	62	79.5	328	2	Q8S341	Purple acid phosphatas	1.07e+00
5	62	79.5	328	2	Q8GWH8	Putative purple acid p	1.07e+00
6	61	78.2	610	2	Q87XT5	ABC transporter, ATP-b	1.75e+00
7	61	78.2	310	2	Q89II5	ABC transporter ATP-bi	1.75e+00
8	60	76.9	2199	1	DPOE_SCHPO	DNA polymerase epsilon	2.86e+00
9	60	76.9	70	1	RL31_SALTY	50S ribosomal protein	2.86e+00
10	60	76.9	70	1	RL31_ECOLI	50S ribosomal protein	2.86e+00
11	60	76.9	71	1	RL31_YERPE	50S ribosomal protein	2.86e+00
12	60	76.9	424	2	Q9CXN5	3110050K21Rik protein.	2.86e+00
13	60	76.9	323	2	Q9F1R2	CbbR homolog.	2.86e+00
14	60	76.9	734	2	Q8BHW0	Similar to hypothetica	2.86e+00
15	60	76.9	313	2	P78873	Unknown protein (Fragm	2.86e+00
16	60	76.9	70	3	AAO71029	50S ribosomal protein	2.86e+00
17	60	76.9	70	3	AAN45447	50S ribosomal subunit	2.86e+00
18	60	76.9	70	3	AAP18753	50S ribosomal subunit	2.86e+00
19	59	75.6	280	2	Q8PGP5	ABC transporter ATP-bi	4.64e+00
20	59	75.6	2841	2	Q9FB33	Peptide synthetase NRP	4.64e+00

Figure 6.12 Results of the search of 1087687 sequences and 346235233 residues on the SWISS-PROT database with the peptide 555.32 isolated from the total protein extraction of *X. bovienii* Phase I .

```
                **..**..*
Database     120 FFAFGYSAC 128
Query          3 FFSYGYNSC 11
```

Figure 6.13 Smith-Waterman alignment of peptide 1046.56 from the 36.5kDa protein isolated from Phase I *X. bovienii* (Query) and the Ammonium transporter, ID number: Q8H6Y4 (Database)

```
                *..***.**
Database     353 HNINLDIKR 361
Query          1 HDLNLDVKR 9
```

Figure 6.14 Smith-Waterman alignment of peptide 555.32 from the 36.5kDa protein isolated from Phase I *X. bovienii* (Query) and the ABC transporter ATP-bi, ID number: Q891F0 (Database)

```
          ******.**
Db       99 HDLNLDIKR 107
Qy        1 HDLNLDVKR 9
```

Figure 6.15 Smith-Waterman alignment of peptide 555.32 from the 36.5kDa protein isolated from Phase I *X. bovienii* (Query) and the Phosphate transport ATP-binding protein B, ID number: Q9PQU3 (Database)

6.5 Discussion

ATP-powered pumps are ATPases that use the energy produced by the hydrolysis of ATP to move small molecules or ions across the cell membrane against the chemical concentration gradient or electrical potential of the cell (Lodish *et al* 2000). Of the four classes of ATP-powered pumps: P, F, V and ABC, the ABC transporter is the only one to transport not only ions but small molecules as well (Higgins 1995). The ABC class of transporter is present within the plasma membrane of bacteria where the transport of amino acids, sugars and peptides is known to occur (Linton & Higgins 1998).

The putative ABC transporter ATP binding protein isolated from Phase I *X. bovienii* in this study could have many uses, without the isolation of the gene encoding the protein it is difficult to assign which molecules the membrane protein is used to transport. However, the expression of the putative ABC transporter by Phase I and not Phase II *Xenorhabdus* narrows down the field of molecules which may be transported. It is unlikely to be amino acids or sugars, as these are both elements which would be necessary for the survival of both Phases of the bacterium, however the exportation of peptides into the growth medium by Phase I *Xenorhabdus* has been reported in previous work, among others: Bowen *et al* 1998, Bowen *et al* 2000, ffrench-Constant & Bowen 1999, Waterfield *et al* 2001, Bowen & Ensign 1998, Hotchkin & Kaya 1984, Blackburn *et al* 1998, Guo *et al* 1999, have all reported protein complexes secreted directly into the growth medium.

The secreted proteins are known as insecticidal Tc toxin complexes (Bowen *et al* 1998) and in combination with a range of antimicrobials, establish the insect cadaver as a monoculture breeding ground for both bacteria and nematodes (ffrench-Constant & Bowen 2000). The secretion of these protein complexes is restricted to Phase I of the bacteria (Waterfield *et al* 2001) therefore, the absence of expression of the putative ABC transporter for these complexes would be expected.

6.5.1 Future work

More information about the regulation of Phase change could be gathered through the further characterisation of the 36.5kDa protein from Phase I *X. bovienii* by cloning and sequencing of the gene and performing a BLASTn sequence similarity search. This could be carried out by two methods:

1. By designing sense and anti-sense primers to Q9PQU3 and Q8H6Y4 and performing PCR reactions with the four combinations of primers, sequencing of multiple clones containing all four possible PCR products.
2. Screening a *X. bovienii* genomic library with a P^{32} 5' labelled degenerate probe generated from Q9PQU3. Cloning of the positive hybridisation gene fragment into PUC18 followed by cloning and sequencing of the positive colonies.

Hopefully one of the sequences would match with an ABC transporter in a similar organism. Following this, knockout mutants could be constructed using antibiotic resistance loci, which would allow the phenotypic properties of the gene to be elucidated.

Chapter Seven

General Discussion

The aim of this work was to characterise an infection of *Galleria mellonella* with the entomopathogenic nematode *Steinernema feltiae* (Strain UK76) carrying the pathogenic symbiont *Xenorhabdus bovienii*. An investigation into the mechanisms of Phase change in *X. bovienii* was conducted through following the expression of *X. bovienii* FliC and rpoS genes during the course on an infection in *Galleria*. The isolation and characterisation of a Phase I specific 36.5kDa protein was also carried out.

It has been demonstrated that there is a distinct difference in the infection of *Galleria* depending on the number of nematodes and method of infection employed (Chapters Three and Four). The isolation of the rpoS and FliC gene fragments from *X. bovienii* show that it is likely that *X. bovienii* displays similar mechanisms of phase variation as that of *X. nematophilus* (Chapter Five). The isolation and characterisation of the putative Phase I specific ABC transporter protein from *X. bovienii* is thought to probably be used in transport of toxins or antibiotics from the bacterium to the surrounding culture medium (Chapter Six).

The isolation of the different species of bacteria from the injection infection: *Stenotrophomonas maltophilia* and *Xanthomonas axonopidis*, and the natural infection: *S. maltophilia* and *X. bovienii*, contradicts previous work by several authors (Akhurst & Boemare 1990, Forst & Nealson 1996) who state that *Xenorhabdus* will

establish a monoculture within the host cadaver to the exclusion of all other bacteria. This study is somewhat supported, however, by work conducted by Jackson *et al* in 1995, who isolated *Providencia rettgeri* a *Psuedomonas*-like bacterium, from a late stage infection in *Heterorhabdus* spp. Although isolated from an infection with a different species of entomopathogenic nematode, the bacterium must have originated from the gut of the infected host; therefore, it is highly likely that such a bacterium could also be isolated from an infection with *Steinernema*.

The results from the bacterial colony counts of *Xenorhabdus* (Chapter Three) and the *Xenorhabdus* 16S rRNA expression for the 100 nematode natural infection (Chapter Four) support each other, in that both show the emergence of *Xenorhabdus* following 24 hours of infection. This is also the case for the natural infection with 1000 nematodes, where both the results from the colony counts and the 16S rRNA expression show emergence of *Xenorhabdus* after 12 hours of infection. In both cases, however, the results from the 16S rRNA expression fail to show the fluctuations in the *Xenorhabdus* population which is highlighted by the colony count results.

The results from the colony counts of *S. maltophilia* and the *Eubacteriaceae* 16S rRNA expression for the natural infection with 100 nematodes are in agreement with each other; both show the emergence of *S. maltophilia/Eubacteriaceae* at 36 to 48 hours following infection and a decrease from 48 to 72 hours after infection. A similar agreement of results exists for the natural infection with 1000 nematodes; emergence of *S. maltophilia/Eubacteriaceae* occurs after 36 hours of infection. However, after 48 hours the results differ from each other, the colony counts show

the *S. maltophilia/Eubacteriaceae* decreasing to almost zero, while the 16S rRNA expression results show an increase in *S. maltophilia/Eubacteriaceae* expression at 72 hours.

The results from the colony counts of *S. maltophilia* and *X. axonopidis* and the *Xenorhabdus* 16S rRNA expression from the injection infection, are highly contradictory, as expression of *Xenorhabdus* is detected throughout the infection, but no *Xenorhabdus* colonies were isolated on the agar plates. One explanation for the discrepancy in these results is that *Xenorhabdus* 16S rRNA could be undergoing expression throughout the entire infection, as the Northern slot blots suggest, however, the main constituents of the bacterial population could be *S. maltophilia* and *X. axonopidis*, as were isolated on the agar plates.

Comparison of the results from the colony counts of *S. maltophilia* and *X. axonopidis* and the *Eubacteriaceae* 16S rRNA expression from the injection infection, also leads to a highly contradictory conclusion. The Northern slot blots show that expression of the *Eubacteriaceae* does not occur until 18 hours after infection, whilst colonies are recorded to emerge after 2 hours of infection. The exposure time of the Northern blot may have been too short to detect hybridisation to the *Eubacteriaceae*16S rRNA early in the infection, as the amount of *Eubacteriaceae* rRNA would have been minimal until the bacteria were released from the *Galleria* gut. The results of the *Xenorhabdus* 16S rRNA Northern slot blots, show that there is detectable *Eubacteriaceae* RNA on the blot, as *Xenorhabdus* is a member of the *Eubacteriaceae* family. Longer exposure time would have led to the increased hybridisation to the later infection times, which would have resulted in overexposure of the entire blot.

It is clear that further work which leads to a better understanding of the coordination between the colony types which are isolated on agar plates and the 16S rRNA expression of *Xenorhabdus* and general *Eubacteriaceae* is needed.

This study is the first time that the isolation of rpoS and FliC gene fragments from *X. bovienii* have been cloned and sequenced (Chapter Five). The cloning of these two gene fragments from *X. bovienii* is a clear indication that the mechanisms of Phase change are probably similar to that of *X. nematophilus*. No literature is yet available on the control of Phase change in *Xenorhabdus*, however it can be concluded that Phase I *X. bovienii* can be identified by ability to swarm of media plated and the transcription of the FliC gene. Phase II *X. bovienii* can be identified by the ability to resist environmentally stressful environments, such as reduced osmolarity or nutrient availability and the transcription of the rpoS gene, which is known to activate the σS.

The isolation of the *Xenorhabdus*-like bacterium from both the *Galleria* RNA rpoS and FliC RT-PCR clones supports the evidence from the colony counts and 16S rRNA expression time series of natural and injection infections in *Galleria*, that a monoculture of *Xenorhabdus* does not exist during infection of *G. mellonella* with *S. feltiae*. The presence of a bacterium which is very similar to *Xenorhabus* can only be attributed to the normal gut flora of the host, further work is needed to ascertain what role this bacterium may play in *Galleria* once infection has taken place.

This is the first time that the isolation of a putative Phase I specific 36.5kDa ABC transporter from *X. bovienii* has occurred. The isolation of this ABC transporter as Phase I specific leads to further characterisation of the differences between the two

phases for *X. bovienii*. The possibility that such Phase specific transporters may exist across all species of *Xenorhabdus* and *Photorhabdus* should be investigated further through generation of species specific primers for the ABC transporter region.

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