Ochrobactrum anthropi: a soil bacterium for the study of *Brucella* virulence

by

Mohamed N. Seleem

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Dr. N. Sriranganathan, Chair

Dr. S. M. Boyle

Dr. G. Schurig

Dr. B. Mukhopadhyay

Dr. S. Witonsky

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Abstract

The species of *Brucella* were isolated and characterized almost 120 years ago and their genomes sequenced for almost 4 years. Compared to other bacterial pathogens relatively, little is known about the factors contributing to their persistence in hosts and multiplication within phagocytic cells. Also, many aspects of the interactions between *Brucella* and its host remain unclear. Molecular characterization of intracellular survival processes of *Brucella* will provide guidance for additional prevention and control measures. One of the features that distinguishes *Brucella* is that they do not express classic virulence factors. Thus identification of virulence factors has been elusive and some of the identified virulence genes are putative. Disruption of putative virulence genes and studying the consequent effect on attenuation in cell lines or mouse models is a widely used method. However, in most cases it is not apparent whether the mutated genes encode virulence factors or merely affect normal metabolic or biological functions. Some mutations in *Brucella* can be compensated by redundancy or backup mechanisms. One method for identifying putative virulence genes involved in pathogenesis is to express these genes in a nonpathogenic host and isolate recombinants with increased virulence or survival ability either in cell culture or animal model.

We hypothesize that over-expression of *Brucella* putative virulence genes in the non-pathogenic and close phylogenic relative *Ochrobactrum anthropi* should enhance its survival in infection models *in vivo*.

O. anthropi is one of the closest *Brucella* relatives based on DNA, rRNA, and protein analyses but it is unable to establish chronic infection and considered as opportunistic pathogen that, under certain circumstances, may produce disease in immunocompromised humans. Therefore, we established enhanced expression system in

Brucella and *Ochrobactrum* to identify *B. suis* virulence genes. We created an enhanced expression system that can be used for cloning and expression of heterologous genes in *Brucella* and *Ochrobactrum*. We studied the transcriptional activity of several promoters and created some tools to enhance the expression, detection and purification of *Brucella* recombinant protein in *Ochrobactrum*.

The presumable importance of alkyl hydroperoxide reductases encoded by *ahpC* and *ahpD* genes and their contribution to intracellular survival of *Brucella* were studied by over-expressing them. The recombinant *O. anthropi* expressing *B. suis ahpC* and *ahpD* genes were able to resist *in vitro* killing by H_2O_2 and or cumene hydroperoxide and survived longer in the macrophage J774 A.1 cell line. The control *O. anthropi* was cleared from BALB/c mice in five days while the recombinants were recovered from spleens, livers and lungs of infected mice up to eight days post-infection.

We tested the contribution of *B. suis* cyclic glucan synthetase gene (*cgs*) to virulence by over-expressing it in *O. anthropi.* We studied the ability of the recombinant *O. anthropi* to resist killing *in vitro* and *in vivo*. We generated evidence that *B. suis cgs* when over-expressed in *O. anthropi* increased the amount of cyclic glucans synthesized and accumulated in the periplasmic space. This accumulation changed the virulence of the microorganism from a soil bacterium that cleared from mice in less than five days into a pathogenic organism that could survive up to 9 days and at higher doses killed the mice.

In summary, several vectors have been constructed for gene expression and protein purification in *Brucella* and *Ochrobactrum*. Novel useful tools for enhancement of heterologous gene expression were created and demonstrated to work in *Brucella* and *Ochrobactrum*. *Brucella* putative virulence genes were studied in *Ochrobactrum* using the newly constructed vectors and tools. *Ochrobactrum* as a gain of function model for studying putative virulence genes of intracellular pathogens in general and for *Brucella* in particular proved to be a very useful model.

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
LITERATURE REVIEW

Brucella: a pathogen without classic virulence genes

MOHAMED N. SELEEM, STEPHEN M. BOYLE AND NAMMALWAR SRIRANGANATHAN. Accepted Journal of Veterinary Microbiology.

Abstract

The species of *Brucella* were isolated and characterized almost 120 years ago and have been sequenced for almost 4 years. Compared to other bacterial pathogens relatively little is known about the factors contributing to their persistence in the host and multiplication within phagocytic cells. Also, many aspects of interaction between *Brucella* and its host remain unclear. Molecular characterization of the intracellular survival process of *Brucella* is important as it will provide guidance for additional prevention and control measures. One of the features that distinguishes *Brucella* is that they do not express classic virulence factors. Thus identification of virulence factors has been elusive and some of the identified virulence genes are putative. Disruption of putative virulence genes and studying their effects on attenuation in cell lines or mouse models is a widely used method. However, in most cases it is not apparent whether the mutated genes encode virulence factors or merely affect normal metabolic or biological functions. Some mutations in *Brucella* can be compensated by redundancy or backup mechanisms. This review will address the most important virulence genes known to date (true and putative) and their mechanisms of action that contribute to the intracellular survival of *Brucella* and its ability to establish chronic infection.

Historical overview of Brucella:

Examination of the skeletal remains of the Roman residents of Herculaneum killed by the catastrophic volcanic eruption of Mt. Vesuvius in late August 79 A.D. revealed vertebral bone lesions typical of brucellosis in more than 17% of the residents. A likely explanation for the high incidence of the disease was explained by scanning electron microscopy. The buried carbonized cheese made from sheep's milk found with the bones revealed the presence of cocco-bacillary forms that were morphologically similar to *Brucella spp*. (Capasso, 2002). Eighteen centuries later, Sir David Bruce isolated *Micrococcus melitensis* from a British soldier who died from Maltese fever in Malta, an island not far away from Herculaneum (Godfroid et al., 2005). The zoonotic nature of the brucellosis was again demonstrated in 1905 by isolating *Brucella melitensis* from goat's milk in Malta (Godfroid et al., 2005).

General characteristic of Brucella:

Brucella spp. are facultative intracellular Gram negative bacteria that are pathogenic for a variety of mammalian species including humans. They belong to the alpha-2 subdivision of the Proteobacteria, along with *Ochrobactrum*, *Rhizobium*, *Rhodobacter*, *Agrobacterium*, *Bartonella*, and *Rickettsia*. They cause a chronic infectious disease known as brucellosis, a major zoonosis still existing in many countries (Corbel, 1997). Eight *Brucella* species are currently recognized. Six of them infect terrestrial animals: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Verger et al., 1987) and two infect marine mammals: *B. cetaceae and B. pinnipediae* (Verger et al., 2000). Within these species, seven biovars are recognized for *B. abortus*, three for *B. melitensis* and five for *B. suis* (Verger et al., 1987). The remaining species have not been differentiated into biovars.

Zoonosis:

All *Brucella* species with the exception of *B. ovis*, *B. neotomae* and *B. pinnipediae* can infect humans. *B. melitensis* is responsible for the most serious human symptoms (Brew et al., 1999). *B. melitensis*, *B. suis* and *B. abortus* are listed as potential bioweapons by the Centers for Disease Control and Prevention (Kaufmann et al., 1997; Kortepeter and Parker, 1999). This is due to the highly infectious nature of all three

species, as they can be readily aerosolized, moreover an outbreak of brucellosis would be difficult to detect because the initial symptoms are easily confused with those of influenza (Chain et al., 2005). In places where brucellosis is endemic, humans can get infected via contact with infected animals or consumption of their products, mostly milk and milk products. All three *Brucella* species cause a severe human disease known as undulant fever (Mediterranean sea fever, Malta fever) characterized in its acute phase by undulant fever and in its chronic phase by intracellular localization of the pathogen and damage of different organs (Corbel, 1997; Roop et al., 2004). Brucellosis in humans is primarily a disease of the reticuloendothelial system in which the bacteria multiply inside the phagocytic cell; the intermittent release of bacteria from the cells into the bloodstream causes undulant fever (Smith and Ficht, 1990). *Brucella* infection is treated with a combination of antibiotics; however, in its chronic phase, eradication is difficult since *Brucella* spp. are localized intracellularly within phagocytic cells making treatment difficult, since most antibiotics and specific antibodies, although highly active *in-vitro*, do not actively pass through cellular membranes (Hall, 1990; Kohler et al., 2003; Roop et al., 2004). Brucellosis does not spread among humans; consequently, eradication of the disease from the natural animal reservoirs leads to a decrease in the incidence of human infection (Briones et al., 2001). Although, *B. abortus* strain RB51 and strain 19 are effective in preventing disease in cattle, no vaccine is available to protect against human brucellosis.

Animal brucellosis:

Beside the public health concern *Brucella* infections in animals have an important economic impact especially in developed countries as they cause abortion in the pregnant animals and infertility in males due to orchitis and epididymitis (Roop et al., 2004). In regions with high prevalence of the disease, the only way of controlling and eradicating this zoonosis is by vaccination of all susceptible hosts and elimination of infected animals (Briones et al., 2001). The most commonly used vaccines against bovine brucellosis are *B. abortus* strain 19 and the recently USDA approved strain RB51; the latter unlike strain 19 doesn't interfere with serological diagnoses (Moriyon et al., 2004). *B. melitensis* strain Rev1 although highly infectious to human it is still the only vaccine currently available for sheep and goats (Blasco, 1997).

Pathogenesis of Brucella

The pathogenicity of the brucellae is due to their ability to adapt to the environmental conditions encountered in its replicative niche including low levels of nutrients and oxygen, acidic pH and reactive oxygen intermediates (Kohler et al., 2002). Macrophages are the primary target cells in which *Brucella* multiply and cause persistent infection. In addition, *Brucella* can infect a variety of nonprofessional phagocytes, including NIH 3T3, HeLa, Vero, DMCK, ruminant trophoblast and BHK cells (Anderson and Cheville, 1986; Meador and Deyoe, 1989; Pizarro-Cerda et al., 2000).

Smooth *Brucella* inhibit host cell apoptosis, favoring bacterial intracellular survival by escaping host immune surveillance, while rough *Brucella* induce necrosis in macrophages (Pei et al., 2006). However, the mechanisms and virulence factors that mediate macrophage cell death have not been identified. In contrast to other pathogenic bacteria, no classic virulence factors, such as exotoxins, cytolysins, capsules, fimbria, plasmids, lysogenic phages, drug resistant forms, antigenic variation, endotoxic lipopolysaccharide (LPS), Pathogenicity islands or inducers of apoptosis have been described in *Brucella* (Moreno and Moriyon, 2002). *Brucella* uses number of mechanisms for avoiding or suppressing bactericidal responses inside macrophages. We will address the most important virulence factors that help *Brucella* invade and survive in macrophages.

1-Lipopolysaccharides (LPS) /O-polysaccharides

Lipopolysaccharide is vital to the structural and functional integrity of the Gram negative bacterial outer membrane (Cardoso et al., 2006). The smooth phenotype of *Brucella* is due to the presence of a complete LPS in the outer cell membrane. The LPS is composed of lipid A, a core oligosaccharide, and an O-side-chain polysaccharide. LPSs of rough *Brucella* strains do not contain O-side-chains. The LPSs of *Brucella* have structures and properties distinct from other LPSs. In contrast to classical enterobacterial LPSs, they have low endotoxicity measured at approximately several-hundred-times less active and toxic than *Escherichia coli* LPSs (Lapaque et al., 2005). The O-side chain of *B. abortus* is a linear homopolymer of β-1,2-linked 4,6-dideoxy-4-formamido-β-D-

mannopyranosyl subunits averaging between 96 and 100 subunits in length in comparison to average of about 40 subunits per chain in *E. coli* (Caroff et al., 1984; Goldman and Leive, 1980; McQuiston et al., 1999). Highly purified *B. abortus* LPS is a poor inducer of respiratory burst, bactericidal nitrogen intermediates and lysozyme secretion (Rasool et al., 1992; Riley and Robertson, 1984) and is highly resistance to degradation in the macrophage. *Brucella* LPS has been shown to impair anti-microbial host responses by inhibiting complement activity, anti-bacterial-peptide attacks and by preventing the synthesis of immune mediators (Forestier et al., 2000; Forestier et al., 1999; Lapaque et al., 2005; Moreno et al., 1981). *B. melitensis* LPS doesn't stimulate production of tumor necrosis factor-α or nitric oxide (Tumurkhuu et al., 2006). *Brucella* rough mutants are more sensitive than smooth strains to normal serum and complement attack (Allen et al., 1998; Corbeil et al., 1988; Eisenschenk et al., 1995). *Brucella* LPS plays a role in protecting against bactericidal cationic peptides (defensin NP-2, lactoferrin, cecropines, lysozyme, bactenecin-derived peptides, the defensin-like antibiotic polymyxin B and the crude lysosomal extracts from polymorphonuclear leukocytes) (Freer et al., 1996; Lapaque et al., 2005; Martinez de Tejada et al., 1995; Riley and Robertson, 1984).

B. suis smooth LPS interacts with lipid-rafts through an unknown receptor on the surface of macrophages and enter cells via a pathway allowing *Brucella* to avoid fusion with lysosomes (Lapaque et al., 2005). In contrast, the rough strain (which does not possess O-chains) seems not to enter by lipid-rafts, and fuses rapidly with lysosomes (Lapaque et al., 2005). The *Brucella* LPS is an immuno-dominant antigen, which leads to production of non-protective IgG, basically subverting the host immune response towards Th2 biased response instead of protective Th1 or cell mediated immune responses.

The smooth *Brucella* strains inhibit host cell apoptosis by the action of the O-polysaccharides through a TNF-∝ independent mechanism that is missing from rough brucellae. By inhibiting host cell apoptosis, smooth brucellae escape host immune surveillance and avoid the activation of immune system by factors released from dead cells and also avoid triggering antigen presenting cells (Gross et al., 2000; Pei et al.,

2006). These properties of LPS constitute key virulence mechanisms for intracellular survival and replication of *Brucella*.

2- Type IV secretions systems virB

Type 4 secretions systems (T4SSs) are a family of multiprotein complexes responsible for secretion of bacterial macromolecules and proteins across the bacterial cell envelope (Cascales and Christie, 2003). The T4SSs in *Brucella* are typified by the *virB* operon encoding 12 proteins; they have significant homology to other T4SSs (Delrue et al., 2001; Hong et al., 2000; O'Callaghan et al., 1999). The mechanism of assembly and the factors secreted by T4SSs in *Brucella* are still unknown. However, the similarity with the well-studied plant pathogen *Agrobacterium tumefaciens* suggests that *Brucella* uses them for the translocation of virulence factors into mammalian cells (Cascales and Christie, 2003; Celli et al., 2003; Celli and Gorvel, 2004; Christie, 2004; Christie et al., 2005; O'Callaghan et al., 1999). Expression of the *virB* operon is believed to be regulated by quorum-sensing (QS) regulator (VjbR) (Delrue et al., 2005) .

After entering macrophages via lipid rafts, the *Brucella*-containing vacuole (BCV) avoids fusion with the lysosomes (Celli et al., 2005) and follows a novel intracellular trafficking pathway. The BCV interacts with the endoplasmic reticulum (ER), leading to the creation of a specialized vacuole in which the bacteria multiply (Kohler et al., 2002). The BCVs interact at the ER export sites to generate an ER-derived intracellular organelle permissive for nutrient acquisition and intracellular replication. The acquisition of ER membranes depends on a functional type IV secretion system *virB* and a Sar1 host factor. The mechanism governing the biogenesis of this compartment remains elusive (Celli et al., 2003). *Brucella virB* mutants were unable to acquire ER membranes, and lost their ability to multiply in HeLa cells and were not recovered from the spleens of infected BALB/c mice (Celli and Gorvel, 2004; O'Callaghan et al., 1999; Sieira et al., 2000). A *vjbR* QS regulator mutant down regulated *virB* expression and was attenuated in cellular and mouse models (Delrue et al., 2005).

3- Cyclic β-1,2-glucans (CβG)

Osmoregulated periplasmic glucans (OPGs) can be divided into 4 families (I-IV) on the basis of backbone organization and are constituents of the envelopes of Gram-negative bacteria. Mutants deficient in OPG synthesis show altered chemotaxis and motility and reduced outer membrane stability and synthesis of exopolysaccharides (Bohin, 2000).

Moreover, the mutants are unable to establish successful pathogenic or symbiotic associations with their animal or plant hosts (Arellano-Reynoso et al., 2005). *Brucella* cyclic β-1,2-glucans (CβG) belong to the family II OPGs and are neither O-substituted nor osmoregulated (Bohin, 2000; Briones et al., 1997). *B. abortus* deficient in CβG is attenuated in mice and unable to multiply in HeLa cells (Briones et al., 2001). The CβG interfere with cellular trafficking by acting on lipid rafts found on host cell membranes and preventing phagosome-lysosome fusion cycle (Arellano-Reynoso et al., 2005). The BCV does not fuse with the lysosomes, instead it follows a novel intracellular trafficking pathway, which interacts with the endoplasmic reticulum (ER), leading to the creation of a specialized vacuole in which the bacteria multiply. Fusion between the endoplasmic reticulum and the BVC depended on the *Brucella virB* encoded T4SSs but not on CβG expression (Arellano-Reynoso et al., 2005).

4-Superoxide dismutase

Brucella spp. are not normally found as a free living or commensal organisms (Gorvel and Moreno, 2002). The preferred ecological niche for the brucellae is within the phagosomal compartment of host macrophages, and the capacity of this organism to establish and maintain chronic infections is dependent upon its ability to survive and replicate within these phagocytic cells (Roop et al., 2004). Experimental evidence indicates that the production of reactive oxygen intermediates (ROIs) represents one of the primary mechanisms utilized by host macrophages for limiting the intracellular replication of the brucellae (Gee et al., 2005; Jiang et al., 1993). Reactive oxygen intermediates (ROIs) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) are harmful to *Brucella* because they damage macromolecular structures. Any mechanism that help the brucellae avoid oxidative killing would help them to establish and maintain residence in their intracellular niche (Farr and Kogoma, 1991;

Hornback and Roop, 2006). Bacteria generally have two lines of defense against damage by ROIs. The first line of defense includes enzymes such as catalase, superoxide dismutase and peroxidase that directly detoxify these ROIs. The second line of defense includes enzymes that repair oxidative damage to cellular components and those that degrade the oxidatively damaged components. Such enzymes include DNA repair enzymes and some of the stress response proteases (Davies and Lin, 1988; Demple et al., 1986; Hornback and Roop, 2006; Storz et al., 1989).

The SODs are a family of metalloenzymes containing either iron, manganese, or copper and zinc at their active sites (Benov and Fridovich, 1994; Keele et al., 1970; Yost and Fridovich, 1973). These enzymes catalyze the dismutation of the superoxide (O_2^-) to oxygen and hydrogen peroxide (H_2O_2) (McCord et al., 1971). Even though superoxide itself is not very toxic and in the ionized form cannot easily pass through membranes, it can react with H_2O_2 in the presence of transition metals to produce hydroxyl radical, which is very toxic to cells (Badwey and Karnovsky, 1980; Beaman and Beaman, 1984; Huang et al., 1992).

The genome sequences of *B. melitensis*, *B. suis*, and *B. abortus* (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002) revealed two separate genes encoding SOD, the cytoplasmic Mn-Fe cofactored SOD serves to detoxify endogenous superoxide arising as a by-product of aerobic metabolism (Fridovich, 1995) and the periplasmic Cu-Zn cofactored SOD protects the brucellae from the respiratory burst of host macrophages (Beck et al., 1990; Bricker et al., 1990). Although the expression of Cu-Zn SOD in *Brucella* is increased up to five fold in response to oxidative stress (Kim et al., 2000), conflicting results exist regarding the importance of SOD in *Brucella* virulence in both cell line and animal model (Gee et al., 2005; Latimer et al., 1992; Tatum et al., 1992). *Brucella SodC* (Cu-Zn SOD) mutants are attenuated in C57BL/6J mice and corresponding peritoneal macrophages in contrast to BALB/c mice and J774 A.1 cell lines. This difference can be explained by the fact that, the macrophages isolated from the peritoneal cavities of the C57BL6J mice produce a more robust oxidative burst in primary culture than the J774 A.1 and HeLa cell lines (Gee et al., 2005). In addition, BALB/c mice experience an interruption in IFN-γ production during infection with *Brucella* strains that is not observed in C57BL/6J mice (Murphy et al., 2001).

Considering the link between IFN-γ activation, ROI production, and the brucellacidal activity of murine macrophages (Jiang and Baldwin, 1993; Jiang et al., 1993), it is possible that the *sodC* mutant *B. abortus* experiences a less intense exposure to O_2^- of phagocyte origin in BALB/c mice than it does in C57BL/6J mice (Gee et al., 2005). Vaccination of mice with live *E. coli* expressing the *Brucella* Cu-Zn SOD indicated a protective role for this antigen against *Brucella* infections (Onate et al., 1999).

5-Catalase

Catalase is well documented to play an important role in protecting cells from oxidative stress. Pathogenic bacteria use this enzyme to catalyze the decomposition of hydrogen peroxide into water and oxygen as a defensive tool against attack by the host (Kim et al., 2000). The only known catalase activity in *Brucella* is restricted to the periplasmic space and due to their periplasmic location, Cu-Zn SOD and catalase are thought to be involved in protecting the bacteria from external sources of oxidative compounds (Sha et al., 1994). *Brucella* catalase is regulated in response to external H_2O_2 and addition of exogenous catalase protects brucellae from being killed by cultured murine peritoneal macrophages and J774 A.1 cells (Jiang et al., 1993). Although a *Brucella* catalase mutant exhibited increased sensitivity to hydrogen peroxide (Kim et al., 2000), catalase does not appear to be a major virulence determinant for the brucellae in either the experimental murine model or in the natural ruminant host (Gee et al., 2004). This can be explained by the fact that catalase is most effective at protecting bacterial cells from H_2O_2 when the bacterial cell density is high, but is inefficient at protecting these cells when their density is low (Gee et al., 2004). It has been also reported that DNA repair mechanisms are more important for avoiding oxidative killing in the phagosomal compartment of host macrophages than catalase (Gee et al., 2004). Another possibility is that other cellular defenses are sufficient to protect the catalase mutant from the oxidative stress encountered during its interaction with host phagocytes. The function of catalase in *E. coli* can be substituted by alkyl hydroperoxide reductase (*ahpC)* as it is kinetically a more efficient scavenger of trace H_2O_2 than catalase and therefore is likely to be the primary scavenger of endogenous H_2O_2 (Seaver and Imlay, 2001).

6-Base excision repair (BER)

Exonuclease III, encoded by the *xthA* gene, plays an important role in the base excision repair (BER) of DNA by removing oxidative lesions from the bacterial DNA (Haring et al., 1994; Hornback and Roop, 2006). The genome sequences of *Brucella* spp. revealed two non-identical *xthA* (XthA1 and XthA2) genes (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002).

A *xthA1* gene product participates in protecting *B. abortus* from oxidative damage *in-vitro* and the *xthA1* mutant exhibits increased susceptibility to ROIs and ROI/reactive nitrogen. However, a *xthA1* mutant displayed equivalent spleen colonization profiles as wild type did in BALB/c mice and equivalent intracellular survival and replication profiles in cultured murine macrophages (Hornback and Roop, 2006). The redundancy in *xthA* in *Brucella* may have compensated for the lose of function of the *xthA1* mutant. It has been speculated that *xthA1* is not required for wild-type virulence of this strain in the mouse model (Hornback and Roop, 2006). Further experiments with double *xthA1* and *xthA2* mutant and C57BL/6J mice are necessary to clarify this issue.

7-Urease

As a result of sequencing and annotation of the *Brucella* genomes (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002) putative virulence genes were identified by finding homologs reported in other pathogens. Among those homologs, the *Brucella* ureases are interesting candidates to consider as they are the only bacterial pathogen to possess two distinct non-identical urease operons. Microbial ureases are multisubunit metalloenzymes that hydrolyze urea to form carbonic acid and two molecules of ammonia; the ammonia molecules protonate to form ammonium causing the pH to increase. Thus the degradation of urea provides ammonium for incorporation into intracellular metabolites and facilitates survival in acidic environments. In prokaryotes, ureolysis can provide a source of assimilable nitrogen (Cruz-Ramos et al., 1997), and can protect against lethal acidification (Sissons and Hancock, 1993); in some bacteria ureolysis can support ATP synthesis driven by the gradients established by ammonia generation (Smith et al., 1993).

Urease activity has been implicated in the pathogenesis of a number of human clinical infections. The virulence due to urease of *Proteus mirabilis* is related to

promoting direct toxicity to renal epithelium and stone formation; for *Helicobacter pylori* (Reyrat et al., 1996) urease allows colonization of the acidic environment of the stomach. Two nonadjacent urease gene clusters were identified in *B. suis* genome (Paulsen et al., 2002) in chromosome I separated by 1 Mb of DNA. Both loci of urease1 (5.2kb) and urease2 (5.5kb) encode three structural gene homologues, *ureA*, *ureB*, and *ureC*, as well as four accessory gene homologues, *ureD*, *ureE*, *ureF*, and *ureG*, similar to a majority of microbial ureases (Mobley et al., 1995). Based on translated nucleotide sequences, the *ure1* products are most similar to the homologues of *Mesorhizobium spp*., *Rhizobium leguminosarum*, *Klebsiella aerogenes* and *Pseudomonas aeruginosa* which tends to be regulated by nitrogen limitation (Atkinson and Fisher, 1991; Collins et al., 1993). In parallel the *ure2* encoded subunits are most similar to *Yersinia* species which tends to have maximal urease activity during the stationary phase of growth which coincides with the period of maximum acid tolerance of the bacteria (de Koning-Ward and Robins-Browne, 1997). In spite of the presumed importance of urease, and its abundance in *Brucella*, its role and contribution to virulence in *Brucella*, if any, has not yet been demonstrated.

8- Two components regulatory system (BvrR/BvrS)

The two component regulatory system in *Brucella* is essential for sensing the phagosomal environment and changing from extracellular to intracellular life style. This system is responsible for turning off the unnecessary genes for extracellular survival and turning on the essential genes for invasion and intracellular survival (Mekalanos, 1992; Sola-Landa et al., 1998). *Brucella* genome sequencing has revealed 21 putative two-component regulatory systems (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002), So far BvrR/BvrS system is the best-characterized two-component system associated with virulence of *Brucella*. The *Brucella* BvrR/BvrS two-component regulatory system is homologous to the ChvI/ChvG systems of *Sinorhizobium meliloti* and *A. tumefaciens* necessary for endosymbiosis and pathogenicity in plants (Guzman-Verri et al., 2002). Mutation of the *bvrR/bvrS* system hinders the production of Omp25 and Omp22, diminishes the characteristic resistance of *Brucella* to bactericidal polycations and increases its permeability to surfactants. Hence, BvrR/BvrS mutants are

attenuated in mice, show reduced invasiveness in cells, and are unable to inhibit lysosome fusion and to replicate intracellularly (Guzman-Verri et al., 2002; Sola-Landa et al., 1998). Also the Omp25 protein has been shown to be involved in the virulence of *Brucella* through a putative mechanism to inhibit TNF-∝ release from macrophages (Edmonds et al., 2001; Edmonds et al., 2002a; Edmonds et al., 2002b; Jubier-Maurin et al., 2001).

9- Alkyl hydroperoxide reductase (*ahpC &D)*

Alkyl hydroperoxide reductase (*ahpC* & *ahpD*) belongs to a family of peroxidases that are beginning to receive intense research attention due to their roles in degrading damaging hydrogen peroxides and related hydroperoxides (Sherman et al., 1996). The reductase plays an important role as a scavenging enzyme for protection against oxygen radical damage (Jacobson et al., 1989) and elimination of reactive nitrogen (Chen et al., 1998). The *ahpC* gene has been highly conserved in evolution and is found in organisms ranging from bacteria to humans (Chae et al., 1994). Inactivation of *ahpC* gene in various bacteria results in increased sensitivity to organic peroxide killing and to spontaneous mutagenesis (Mongkolsuk et al., 2000). *Brucella* sequencing (DelVecchio et al., 2002; Halling et al., 2005; Paulsen et al., 2002) revealed the presence of *ahpC* and *ahpD* that share no sequence homology to each other and are organized in an operon and appears to be controlled by the same promoter. Very little is known about the *Brucella* alkylhydroperoxidases (*ahpC* & *ahpD*) in terms of their structure, catalytic mechanism and contribution to virulence if any, as the proteins have not yet been purified and investigated. Like *Brucella* SOD there are conflicting results about *ahpC* & *ahpD* mutants and mice clearance model (BALB/c and C57BL/6J) (Roop and Bandara, personal communication).

10- Nitric oxide reductase (*nirD*)

Bacterial denitrification is the process of reduction of nitrate to dinitrogen gas that could allow bacteria to grow under low-oxygen conditions by respiration of nitrate. This is catalyzed by four distinct enzymes: nitrate reductase, nitrite reductase, NO reductase, and nitrous oxide reductase (Stevanin et al., 2005). Nitric oxide (NO) production by

infected macrophages is a major defense system in control of *Brucella* infection (Gross et al., 1998; Loisel-Meyer et al., 2006). The *Brucella* nitric oxide reductase (*norD*) is the key player in the "denitrification island" consisting of four reductases: Nar (nitrate reductase), Nir (nitrite reductase), Nor (nitric oxide reductase), and Nos (nitrous oxide reductase) (Loisel-Meyer et al., 2006). The denitrification island could also allow *Brucella* to survive under very low oxygen (which is a more favorable electron acceptor) tension encounter inside macrophages, using NO as terminal electron acceptors (Loisel-Meyer et al., 2006). The nitric oxide reductase of *Neisseria meningitidis* confers intracellular resistance to NO and allowed its utilization, resulting in the optimal survival of this bacterium in nasopharyngeal mucosa. The conclusion is supported by the fact that either deletion of nitric oxide reductase gene or inhibition of NO syntheses by macrophages reduced survival ability of *N. meningitides* in nasopharyngeal mucosa (Stevanin et al., 2005). A *Brucella norD* mutant was attenuated in BALB/c mice and in activated J774 A.1 cells (Loisel-Meyer et al., 2006).

11- *Brucella* virulence factor A (*BvfA*)

Recently *Brucella* virulence factor A (*BvfA*) a small 11 KDa periplasmic protein unique to the genus *Brucella* with no homologues in GenBank and no conserved domains or structural features was reported (Lavigne et al., 2005). This factor is tightly regulated (only in response to acid shock or within macrophages) and suggests it may play a role in the establishment of the intracellular niche (Lavigne et al., 2005). Although *BvfA* was essential for *Brucella* virulence in both *in vitro* and *in vivo*, its assumed role in virulence is still unknown (Lavigne et al., 2005).

12- Other genes

- Genes responsible for iron acquisition, binding and storage (siderophore, *Omp31* haemin-binding proteins (HBPs) and bacterioferritin BFR respectively) have been reported in *Brucella* (Delpino et al., 2006; Denoel et al., 1995; Lopez-Goni et al., 1992); their contribution to virulence is still not clear.

- Presence of phosphatidylcholine (PC) serves as an important structural component of the *Brucella* membranes and might also contribute to pathogenicity by modulating the host immune response (Comerci et al., 2006).
- Although *Brucella* have all the structural genes for building a flagellum (Letesson et al., 2002) and assembly of flagellar apparatus was demonstrated in early log phase culture (Fretin et al., 2005), the function and role of the *fla* genes in virulence still remain to be investigated.
- Mutational analysis demonstrated that HdeA (periplasmic chaperone that functions at low pH) contributes to acid resistance in *Brucella*, although it wasn't required for wild-type virulence in the BALB/c mouse model (Valderas et al., 2005).
- Other *Brucella* genes like carboxyl-terminal processing protease *(ctpA*) and d-alanyl-d-alanine carboxypeptidase (DAP or *dacF*) involved in the formation of the peptidoglycan layer plays a role in the maintaining cell integrity and they are required for the intracellular growth of the bacterium (Bandara et al., 2005; Kikuchi et al., 2006) although mutants are attenuated in animal model, they are considered as genes encoding essential biological function rather than virulence genes.
- The same can be described for acetohydroxyacid synthase (AHAS) that participates in the biosynthesis of isoleucine, leucine, and valine (Boigegrain et al., 2005) and for erythrulose phosphate dehydrogenase gene responsible for erythritol catabolism (Sangari et al., 2000). The preferential use of erythritol is characteristic of the genus *Brucella* except for the attenuated mutant *B. abortus* strain 19 used to vaccinate cattle (Sangari et al., 2000).

When all is said and done, the brucellae do not have classic virulence factors but they do have genes whose products are necessary for pathogenicity and virulence in the well-developed symbiotic pathogenesis in the infected host. *Brucella* might have evolved from other organisms belonging to Archea that have well developed symbiotic lifestyle in plants. This symbiosis may be the basic mechanism by which *Brucella* establishes itself

in the infected animal after the initial abortion. That is how this well adapted primary pathogen as well as a zoonotic agent survives in the environment.

Ochrobactrum anthropi:

Characteristic:

The genus *Ochrobactrum* belongs to the family *Brucellaceae* within the alphaproteobacterial order Rhizobiales, and currently comprises five described species, *Ochrobactrum anthropi*, *O. intermedium*, *O. tritici*, *O. grignonense* and *O. gallinifaecis* (Lebuhn, Bathe et al. 2006). They are non-fermentative, strictly aerobic, motile, oxidase-positive and indole-negative, Gram-negative rods (Teyssier, Marchandin et al. 2005). *Ochrobactrum* strains were recovered from diverse habitats including soil, plants and their rhizosphere, wastewater, animals and humans (Lebuhn, Bathe et al. 2006).
O. anthropi has been isolated from various clinical specimens and is recognized as an opportunistic pathogen (Teyssier, Marchandin et al. 2005) and under certain circumstances, may produce disease in immunocompromised humans (Gill, Ly et al. 1997).

Ochrobactrum as a bioremediation agent and a biopesticide

From a biotechnological point of view, *Ochrobactrum* strains are of particular interest for bioremediation (Lebuhn, Bathe et al. 2006). They are capable of degrading organophosphorus pesticides such as parathion and methylparathion (Zhang, Cui et al. 2005), phenol (El-Sayed, Ibrahim et al. 2003), the toxic organic solvent dimethylformamide (DMF) (Veeranagouda, Emmanuel Paul et al. 2005) petroleum waste (Katsivela, Moore et al. 2005) the soil contaminant chlorothalonil (Kim, Park et al. 2004). Various isolates have been reported to be potent degraders of pollutants (Lechner, Baumbach et al. 1995; Muller, Jorks et al. 1998; Song, Palleroni et al. 2000; Baek, Kim et

al. 2003; Smejkal, Seymour et al. 2003). They are capable of removal of chromium, cadmium, copper and toxic metals from the environment (Ozdemir, Ozturk et al. 2003).

In recent decades, *O. anthropi* has become a source of interesting biocatalysts. Among these are a number of amide bond-hydrolyzing enzymes; D-aminopeptidase (Asano, Nakazawa et al. 1989) L-aminopeptidase (DmpA) (Fanuel, Thamm et al. 1999), the D-amino acid amidase (Asano, Mori et al. 1989) and the L-amidase (Sonke, Ernste et al. 2005). These properties have generated considerable interest in the use of *O. anthropi* as a bioremediation agent and a biopesticide.

Ochrobactrum and Brucella

O. anthropi is one of the closest *Brucella* relatives by DNA, rRNA, and protein analyses (Yanagi and Yamasato 1993; Velasco, Diaz et al. 1997; Velasco, Romero et al. 1998; Cloeckaert, Tibor et al. 1999) but they unlike *Brucella* are unable to establish chronic infection and considered as opportunistic pathogens that, may produce disease in immunocompromised humans (Gill, Ly et al. 1997; Saavedra, Garrido et al. 1999). Due to the close relationship to *Brucella*, *Ochrobactrum* has also been used as a potential vaccine vector for the delivery of *Brucella* antigens to mice (He, Vemulapalli et al. 2002) and as diagnostic tool for brucellosis (Delpino, Fossati et al. 2004).

Gain of Function:

One method for identifying putative virulence genes involved in pathogenesis is to express these genes in a nonpathogenic host and isolate recombinants strains with increased virulence either in cell culture or animal models (Miller and Shinnick 2001). This technique was first established by using non pathogenic *Escherichia coli* K-12 strain to identify *inv* gene that enables *Yersinia pseudotuberculosis* to invade HEp-2 cells (Isberg and Falkow 1985). Using the same technique the ability of *M. tuberculosis* to gain entry into mammalian cells and survive inside the macrophage was revealed by cloning two distinct loci of *M. tuberculosis* into nonpathogenic *E. coli* (Arruda, Bomfim et al. 1993) and once again the *M. leprae* and *M. tuberculosis* virulence genes were identified using *E. coli* and *M. smegmatis* respectively as an expression host and looking for

enhanced survival of the recombinants strains (Mundayoor and Shinnick 1994; Wei, Dahl et al. 2000).

General rational of the present research

Despite intensive work, the mechanisms allowing *Brucella* to survive as an intracellular pathogen have not been clearly elucidated. For example, to date, very little is known about the bacterial factors contributing to the persistence and multiplication of *Brucella* within phagocytic cells. Also, many aspects of interaction between *Brucella* and its host remain unclear. Molecular characterization of intracellular survival processes of *Brucella* is important, because it will provide additional guidance for their prevention, control and development of more effective vaccines as well as provide insight into other intracellular pathogens. Deletion of putative virulence genes and studying the effect of deletion on attenuation of the virulence in cell lines or mouse models is a widely used method. However, in most cases it is not apparent whether the mutated genes encode virulence factors or merely affected general biological functions. In contrast some deletion in *Brucella* can be compensated by redundancy or backup mechanisms. One of the features that distinguishes *Brucella* is that they do not display obvious virulence factors. Thus, identification of classical virulence factors has been elusive and some of the identified virulence genes are putative. To overcome this deficiency we propose to use *O. anthropi* as a gain of function model for studying putative virulence genes of intracellular pathogens with particular focus on *B. suis*.

We hypothesize that by introducing *Brucella* putative virulence genes into *O. anthropi*, the survival ability in mice should be enhanced only if those genes were true virulence genes.

New vectors and methodologies for the transformation of *Ochrobactrum* have been developed that allow for the study of *Brucella* putative virulence genes in their

genetically close homologous host. *O. anthropi* was selected for use in these studies because it grows rapidly in the laboratory, readily expresses genes from *Brucella* (He, Vemulapalli et al. 2002) and can be genetically manipulated by various techniques. (Chapter 2 and 3)

O. anthropi vs O. intermedium

Although *O. intermedium* is more closely related to *Brucella* than *O. anthropi*, the presence of the native plasmid and its resistance to many antibiotics makes it hard to transform and express heterologous genes. On the other hand, *O. anthropi* strain 49237 the second closest strain to *Brucella* is susceptible to many antibiotics and doesn't contain the native plasmid which makes it a more suitable host for heterologous gene expression (Velasco, Romero et al. 1998; He, Vemulapalli et al. 2002).

Chapter 2 in the dissertation is focused on establishing an enhanced expression system in *Brucella* and *Ochrobactrum* and studying the transcriptional activity of different promoters in *Ochrobactrum.* The final goal of this chapter will be constructing a stable expression vector that carries functional strong promoter to express and detect heterologous genes in *Ochrobactrum* .

Chapter 3 in the dissertation is focused on developing tools for enhancement of heterologous gene expression and protein purification in *Brucella* and *Ochrobactrum*. Also to establish a rapid and simple method for preparation of highly electrocompetent *O. anthropi* cells that could be utilized for a variety of genetic procedures.

Chapter 4 in the dissertation is focused on cloning and expression of two *Brucella* putative virulence genes (*ahpC* & *ahpD*) in *O. anthropi.* Secondly we tested the effect of over-expression of the recombinant *O. anthropi* to resist killing by oxygen radicals *in vivo* and *in vitro* and the survivability of the recombinants in a mouse model.

Chapter 5 in the dissertation is focused on cloning and expression of *Brucella* cyclic glucan in *O. anthropi* and testing the survival ability of the recombinant in a mouse model.

In this dissertation we propose to establish a gain of function model to evaluate the function of putative virulence genes of intracellular pathogens and gain better understanding of their significance, regulation and contribution to virulence. The knowledge gained in this study should provide us with critical information that enhances our understanding of the pathogenesis of the intracellular disease causing bacteria.

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CHAPTER 2

Establishment of gene expression system in *Brucella* and *Ochrobactrum*

CHAPTER 2-1
Improved expression vector for *Brucella* species.

SELEEM, M. N., VEMULAPALLI, R., BOYLE, S. M., SCHURIG, G. G. & SRIRANGANATHAN, N. 2004 *Biotechniques,* 37**,** 740-44.

Abstract

An improved vector pNSGroE has been constructed for gene expression studies in *Brucella spp*. It is derived from the broad host range cloning vector pBBR1MCS. This new plasmid has several advantages over pBBR1MCS or its derivatives, it is (*i*) smaller in size, 2.9 kb, *(ii*) possesses eight unique multiple cloning sites to facilitate directional cloning, (*iii*) expresses proteins as His-tagged fusion for easy detection and purification, (*iv*) carries the *groE* promoter for constitutive expression that is enhanced under conditions of stress *in vitro* and *in vivo*. Our expression studies using this improved vector in *B. abortus* strain RB51 indicated that the level of heterologous protein expression is higher with pNSGroE compared to pBBGroE vector. We have also demonstrated the ability of the new vector to express heterologous fusion proteins stably in *Brucella* species.

Introduction:

B. abortus is one of the six well-recognized species of the genus *Brucella* that infects cattle as well as a variety of other mammals including humans (1). *B. abortus* strain RB51 was approved by the United States Department of Agriculture (USDA) in the United States in 1996 for use as a brucellosis vaccine for cattle, replacing strain S19. Since the lipopolysaccharide of *B. abortus* strain RB51 is devoid of O-side chain, antibodies induced by strain RB51 do not interfere with the conventional serology (2,3). The stability and vaccine efficacy of *B. abortus* strain RB51 have been well studied and documented (4-8). The ability of strain RB51 to induce a Th1 type of immune response (9,10) encouraged the development of this strain as a vaccine vector for delivery of protective antigens of other intracellular pathogens in which Th1 type immune responses are essential for the protection (11).

However, the currently available pBBR1-derived plasmids for gene expression in *Brucella* species are often large (4.7 to 5.9 kb), lack promoters for differential expression, do not have a fusion tag for easy detection and have a very small number of unique multiple cloning sites. To alleviate these deficiencies, we report here the construction of a small (2.9 kb) more efficient and versatile expression vector for *Brucella* spp., designated pNSGroE, using the *rep* gene from pBBR1MCS and the *groE* promoter of *Brucella.* In pNSGroE, the cloned heterologous genes are expressed as a fusion with a His-tag at the N-terminus. Furthermore, our studies with green fluorescence protein (GFP) and β-galactosidase indicate that this plasmid, in comparison to pBBGroE, facilitated more efficient transformation and expression of heterologous proteins in *B. abortus* strain RB51.

Materials and Methods

Bacterial strains, plasmids and oligonucleotides:

All bacterial strains and plasmids used in this study are described in Table 2-1-1 and the oligonucleotides are listed in Table 2-1-2.

Vector construction

A 185 bp fragment containing the multiple cloning sites of pRSETA vector (Invitrogen) was excised from the plasmid using *Xba*I and *Hin*dIII. This fragment was cloned into *Xba*I and *Hin*dIII sites of pGEM11 vector (Promega) to form construct A. The *gro*E promoter of *Brucella spp*. was amplified from the genomic DNA of *B. abortus* strain 2308 using Platinum PCR SuperMix High Fidelity kit (Invitrogen) and the primers (Table 2-1-2). In the reverse primer, six histidine and one glycine residues were engineered after the translational start codon to facilitate the epitope tagging at the amino terminal of the expressed proteins.

The amplified *gro*E promoter was cloned into *Bam*HI and *Sal*I sites of construct A to form construct B. The *rep* gene necessary for the replication of pBBR1 plasmids was amplified along with its own promoter from the pBBR1MCS vector (12) using a primer-pair (Table 2-1-2) with *Sal*I and *Xba*I sites engineered into the forward and reverse primers, respectively, for directional cloning of the amplified fragment.

The chloramphenicol resistance gene (*cat*) along with its own promoter was amplified from the pBBR1MCS using a primer-pair with *Spe*I and *Hin*dIII sites engineered into the forward and reverse primers, respectively, for directional cloning of the amplified fragment. A *Xba*I site was engineered into the reverse primer to add an extra unique restriction site within the multiple cloning site (MCS) of the new plasmid followed by a transcriptional stop codon. The *groE* promoter along with the down stream MCS was excised from construct B using *Sal*I and *Hin*dIII sites. The amplified fragment containing the *rep* gene was digested with *Sal*I and *Xba*I, and the *cat* gene amplicon was digested with *Spe*I and *Hind*III. After the restriction enzyme digestion, the 3 fragments were purified and ligated to form plasmid pNSGroE.

Green Fluorescence Protein expression:

A promoterless Green Fluorescence Protein gene (GFP) was excised from pGFPuv vector (BD Biosciences Clontech) and cloned in frame down stream of the promoter in the multiple cloning site area of pNSGroE and pBBGroE (pNSGroE/GFP, pBBGroE/GFP) and used as visual marker of gene expression and promoter activity inside macrophage cell line J774. A1.

β-galactosidase:

In order to study the expression of *E. coli* β-galactosidase in *B. abortus* strain RB51, a 2.9 kb insert containing the *lacZ* gene was excised from pRSETB/β-gal (Invitrogen) by *Pst*I and *Spe*I restriction enzyme digestion, and cloned into the *Pst*I and *Xba*I sites of pNSGroE; the resulting plasmid was designated pNSGroE/lacZ. Strain RB51 was transformed by electroporation according to previously described method (13) using 50 μl of competent RB51 cells, 0.5 μg and 1 μg of vector DNA for each construct. The efficiency of transformation was calculated for both constructs. *In vitro* plasmid stability was determined, following 8 subcultures over 24 days on TSA without any antibiotics.

Expression of β-galactosidase in RB51*:*

Blue-colored recombinant RB51 colonies expressing β-galactosidase were selected on agar plates containing X-gal; the strain that harbors pNSGroE/LacZ was

designated as strain RB51GroE/LacZ, while strain RB51G/LacZ that harbors plasmid pBBGroE/lacZ and expresses β-galactosidase under *groE* promoter was described earlier (11). Previously described procedures were followed to determine and compare the levels of β-galactosidase expression in strains RB51GroE/LacZ and RB51G/LacZ (11)

Expression of Green Fluorescence Protein in strain RB51:

Recombinant RB51 colonies expressing GFP were identified using the UV light. The strain that expresses pNSGroE/GFP was designated RB51GroE/GFP, while the strain that expresses plasmid pBBGroE/GFP was designated RB51G/GFP.

Western blotting:

Western blotting was performed using 12% polyacrylamide gel and the same amount of protein extracted from strains RB51GroE/GFP and RB51G/GFP was loaded. Proteins were separated by gel electrophoreses, transferred to nitrocellulose membrane and blocked with 1.5% bovine serum albumin and 1.5% dry non fat milk. One of the membranes was developed with Anti-HisG-HRP Antibody (1:5000, Invitrogen) for detection of recombinant GFP containing a Polyhistadine sequence, the other membrane was developed with monoclonal Anti-GFP antibodies (1:1000, BD Biosciences Clontech). The analyses of the band intensity was accomplished with a Kodak Digital-Science Image Station (model 440CF).

Cell culture and infection of macrophages

To study the expression of the GFP *in-vivo*, macrophage cell line J774. A1 was seeded in six-well plates (Corning Incorporated) with and without glass cover slips. The J774. A1 cells were infected with recombinant strain RB51 expressing GFP for 1 hour at a ratio of 1:100 multiplicity of infection. The cells were then washed three times with DMEM medium (Sigma Chemical Co) containing 50 μg/ml gentamicin (Sigma Chemical Co) to wash off non phagocytized bacteria prior to incubation with DMEM medium supplemented with 10 % fetal calf serum and 50 μg/ml gentamicin. The infected cells were incubated at 37 ^{o} C under 5% CO_2 pressure.

Fluorescence microscopy of infected macrophages

At 4h and 24 hours, infected cells on cover slips were washed with Phosphate Buffered Saline (PBS) and fixed in 4% paraformaldhyde for 1 hour, washed twice with PBS before mounting with Flouromount-G fluid (Southern Biotechnology Associates

Inc.). Intracellular multiplication of RB51 and expression of the GFP were visualized using a Zeiss LSM 510 laser scanning microscope.

Flow cytometry

Macrophages were seeded in six-well plates for 1 day and infected as previously described above. At 4 and 24 hours after infection the medium was removed and the cells washed with PBS and fixed with 4% paraformaldhyde for 1 hour, washed twice with PBS (14) and resuspended in 1 ml of PBS. An aliquot of each sample was transferred to a TSA plate and incubated at 37^{o}C, 5% CO_2 for 3 days to check for sterility (*Brucella* non-viability). Following confirmation of non-viability, the infected cells were used in the flow cytometry.

Results

Figure 2-1-1 depicts the pNSGroE expression vector (GenBank accession no. AY576605) that was successfully introduced into *B. abortus* strain RB51 with an efficiency of 7.4×10^5 transformants per μg DNA compared to 1.6×10^3 transformants per μg for the pBBGroE plasmid. Both strains RB51GroE/GFP and RB51G/GFP were found to be stable after 8 serial passages over 24 days on TSA plates in the absence of chloramphenicol selection. Several aliquots of the plasmid were digested each with a different restriction enzyme and analyzed by agarose gel electrophoresis, the sizes of the observed bands were as predicted from the sequence (data not shown). As shown in (Figure 2-1-2 A) the expression of GFP from strain RB51GroE/GFP was 1.7 fold more than that of strain RB51G/GFP as shown by western and estimated by scanning densitomety of western blot using monoclonal Anti-GFP antibody. The Anti-His antibody was used successfully to detect expression of GFP fused with the his-tag in *Brucella* (Figure 2-1-2 B).

The expression of β-galactosidase and enzyme activity, as calculated in modified Miller units, was compared between strain RB51GroE/LacZ and RB51G/LacZ. Without any heat-shock stimulus, the enzyme activity in strain RB51GroE/LacZ was approximately twice compared to that expressed by strain RB51G/LacZ (Table 2-1-3). The enzyme activity in both strains increased by 25% after heat-shock treatment for 20 minutes, reflecting a characteristic feature of the *gro*E promoter. Fluorescence

microscopic examination and expression of the GFP in the macrophages cell line infected with RB51GroE/GFP were visualized after 4 hours and 24 hours. The fluorescence observed clearly demonstrates the ability of pNSGroE vector to express GFP intracellularly inside macrophages (Figure 2-1-3). The expression was monitored for up to 7 days (data not shown) and was not altered over this extended period of incubation. Fluorescence intensity measured by flow cytometry of the infected macrophages was 2 fold higher after 24 hours of macrophage infection with strain RB51GroE/GFP relative to those infected with strain RB51G/GFP. (data not shown)

Discussion

Almost all published literature on expression or cloning vectors that are used on a regular basis in *Brucella* species are based on the large 4.7 kb cloning vector pBBR1MCS (12). Most of the derivatives of this vector have included modifications involving the addition of genes or promoters into the original plasmid, that lead to larger plasmids exemplified by pBBGroE that is a 5.9 kb plasmid (11). The stability and the expression of the cloned genes using pBBR1MCS or pBBGroE vector were fairly well established. However, the amount of heterologous protein that was expressed and the ease of detection wasn't sufficient to facilitate development of *Brucella* as a platform vaccine (11). Therefore, to increase the level of heterologous protein expression as well as to improve the ease of detection, we constructed the pNSGroE expression vector. The pNSGroE expression vector is based on the broad host range cloning vector pBBR1MCS and its derivative pBBGroE expression vector, which enable cloning and efficient expression of genes in RB51 and other *Brucella spp*. The vector is less than 2.9 kb in size with a His-tagged fusion in the N-terminus and 8 unique multiple cloning sites for directional cloning. In this work we clearly demonstrated that a recombinant His-tagged fusion protein could be stably expressed in *Brucella* with higher level of expression than previous *Brucella* vectors. Previous studies have shown that pBBR1MCS replicates and is stably maintained in all *Brucella spp* (12). Therefore pNSGroE expression vector should also be useful in all *Brucella* spp. So far we have expressed GFP and Red Fluorescence Protein (RFP) successfully in *B. abortus* strain 2308 and *Ochrobactrum anthropi* (Figure 2-1-2C). The economy in size of the new vector was achieved by

combining only the very essential elements for replication and expression (minimum origin of replication, promoter, antibiotic selection marker and multiple cloning sites) and removing all nonessential elements in the original vector pBBR1MCS or pBBGroE (*lacZ* gene, mobilization gene and 1 kb up stream of the *groE* promoter). With the minimization of the size, the transformation efficiency of pNSGroE increased 452 times compared to pBBGroE vector. Moreover, due to the lack of *mob* gene, pNSGroE would have no chance of transferring to other bacteria, especially when the recombinant RB51 strains containing this plasmid are used for vaccination of animals. We have shown the usefulness of His-tagged fusion protein expression in *Brucella* by demonstrating the specificity, sensitivity and ease of detection of the expressed fusion protein with Anti-His antibodies (Figures 2-1-2 B and C), which also can be exploited for affinity purification as well as for post-translational modification studies of the proteins expressed inside *Brucella spp.*

The β-galactosidase enzyme activity in strain RB51GroE/LacZ was enhanced compared to strain RB51G/LacZ (Table 2-1-3). This result also correlated with the intensity of the fluorescence light expressed by pNSGroE/GFP and was confirmed by western blotting (Figure 2-1-2 A) These data suggest that heterologous genes cloned in pNSGroE plasmid are expressed at higher levels than the same genes cloned in pBBGroE, even though in both these plasmids, expression of the cloned genes was driven by the same *Brucella gro*E promoter. The difference may be attributable to the increased copy number due to decreased size of the pNSGroE plasmid.

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Table 2-1-1. Bacterial strains and plasmids

Plasmid	**Description** [a]	**Source**
construct A	pGEM11 (Promega) containing 185-bp MCS fragment	This Study
construct B	Construct A with *gro*E promoter	This study
pRSETA	Expression Vector, Amp^r	Invitrogen, Inc.
pRSETB/β-gal	Expression vector with *E. coli* β-galactosidase	Invitrogen, Inc.
pGEM11	Cloning vector, Amp^r	Promega
pBBR1MCS	Broad host range vector; Cm^r	Elzer PH *et al* 1995
pNSGroE	Broad host range vector; Cm^r	This Study
pGFPuv	Green florescence protein expression vector	BD Biosciences Clontech
pNSGroE/GFP	Broad host range vector pNSGroE expressing GFP	This Study
pBBGroE/GFP	Broad host range vector pBBGroE expressing GFP	This Study
pNSGroE/LacZ	Broad host range vector pNSGroE expressing LacZ	This Study
pBBGroE/LacZ	Broad host range vector pBBGroE expressing LacZ	Vemulapalli *et al.*
Bacterial Strain	**Description**	**Source**
B. abortus RB51	Rough, derived from 2308 (15)	Schurig *et al.*
RB51GroE/LacZ	*B. abortus* strain RB51 containing pNSGroE vector expressing *lacZ*	This study
RB51G/LacZ	*B. abortus* strain RB51 containing pBBGroE vector expressing *lacZ*	Vemulapalli *et al.*

RB51GroE/GFP	*B. abortus* strain RB51 containing pNSGroE vector expressing GFP	This study
RB51G/GFP	*B. abortus* strain RB51 containing pBBGroE vector expressing GFP	This study
2308/RFP	*B. abortus* strain 2308 containing pNSGroE vector expressing Red Fluorescence Protein	This study
Ochro/GFP	*Ochrobactrum anthropi* strain 49237 containing pNSGroE vector expressing GFP	This study

[a] Cm^r, chloramphenicol resistance and Amp^r, ampicillin resistance.

Table 2-1-2 PCR Primers Used in The Amplification Procedure

Name	Primer sequence*
groE promoter Forward	5' CCC**GTCGAC**GCTGTTCGCGCAAAAACGCCA 3'
groE promoter Reverse	5' CC**GGATCC**ACC**ATGATGATGATGATGATG**CATGGTATA ACCCTTGGTGTTATAGACGTT 3'
rep gene Forward	5`- CCC **GTC GAC** GCC TGC CCC TCC CTT TTG GTG- 3`
rep gene reverse	5`- CCC **TCT AGA** ATA GTC TGG AAC AGC GCA CTT- 3`
cat Forward	5`- CCC **ACT AGT** GCT GCA TTA ATG AAT CGG CCA- 3`
cat Reverse	5`-CCC**AAGCTT**CCC**TCTAGA**GAA**TAA**ATACCTGTGACG GAAGATCAC TTC- 3`

*Restriction sites are shown in bold font.

The His-tag fusion sequence is shown in bold font and underlined.

Table 2-1-3 β-galactosidase enzyme activity, as calculated in modified Miller units

Strain	**Without Heat-shock**	**With Heat-shock**
RB51GroE/LacZ	1941.97 ± 41.88	2401.82 ± 53.04
RB51G/LacZ	1050.43 ± 33.75	1312.61 ± 61.04

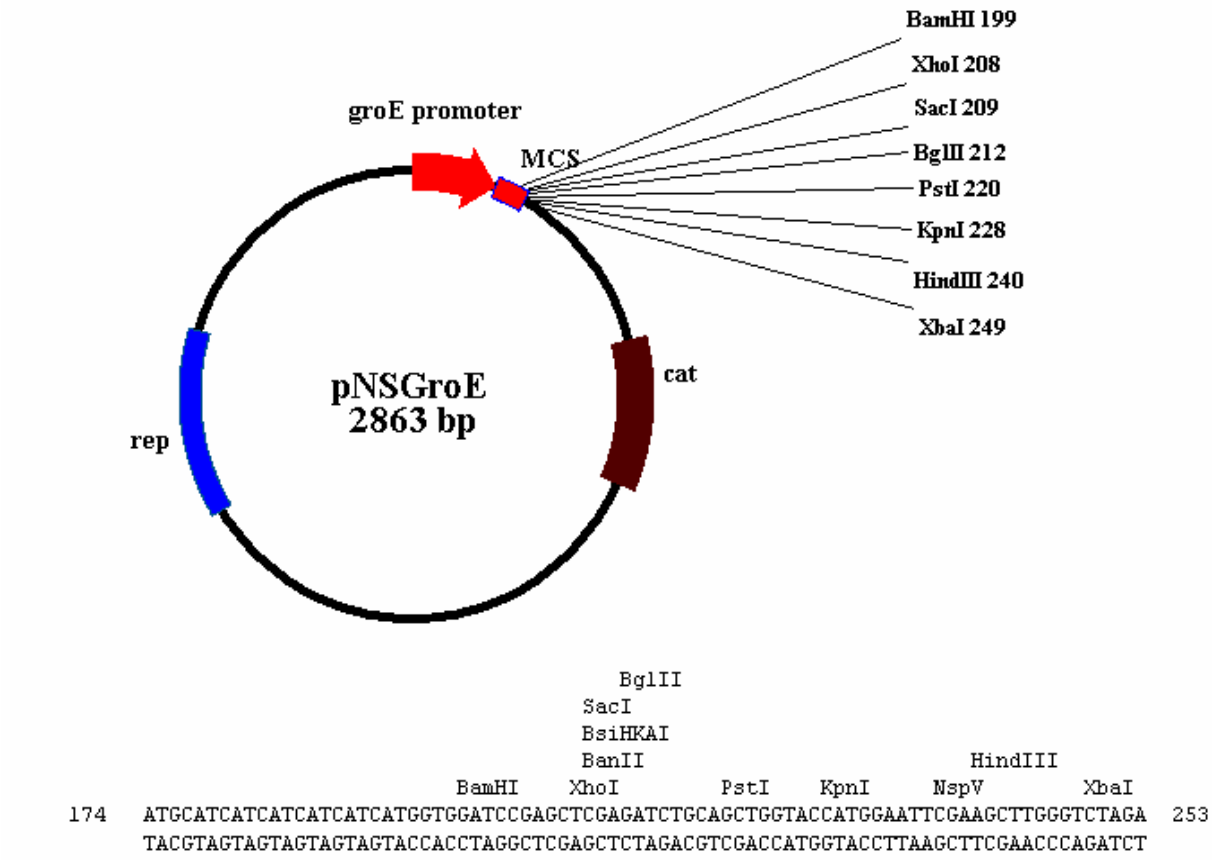

Figure: 2-1-1

The engineered pNSGroE expression vector with *Brucella gro*E promoter for constitutive expression of His-tagged N-terminal Fusion peptide in *Brucella* spp. ***MCS:*** multiple cloning site; ***cat:*** chloramphenicol acetyltransferase gene conferring chloramphenicol resistance, ***rep:*** gene required for plasmid replication.

Figure 2-1-2A Western blot using monoclonal anti-GFP- antibodies
B. abortus strain RB51 expressing GFP, Lane 1; pNSGroE/GFP, Lane 2; pBBGroE/GFP

Figure 2-1-2B Western blot using anti-His antibodies
Western blot showing the expression of GFP in *B. abortus* RB51, specificity and sensitivity of detection of the expressed fusion protein using anti-His antibodies, lane1 pNSGroE/GFP that expresses genes as a His tag fusion, *lane2* pBBGroE/GFP does not express genes as a his fusion protein and cannot be recognized by the anti-His serum.

Figure 2-1-2C Western blot showing the expression of pNSGroE vector using anti-His serum, lane1-expression of GFP in strain RB51; lane2, expression of RFP in strain *B. abortus* strain 2308; *lane3,* expression of GFP in *Ochrobactrum anthropi* strain 49237

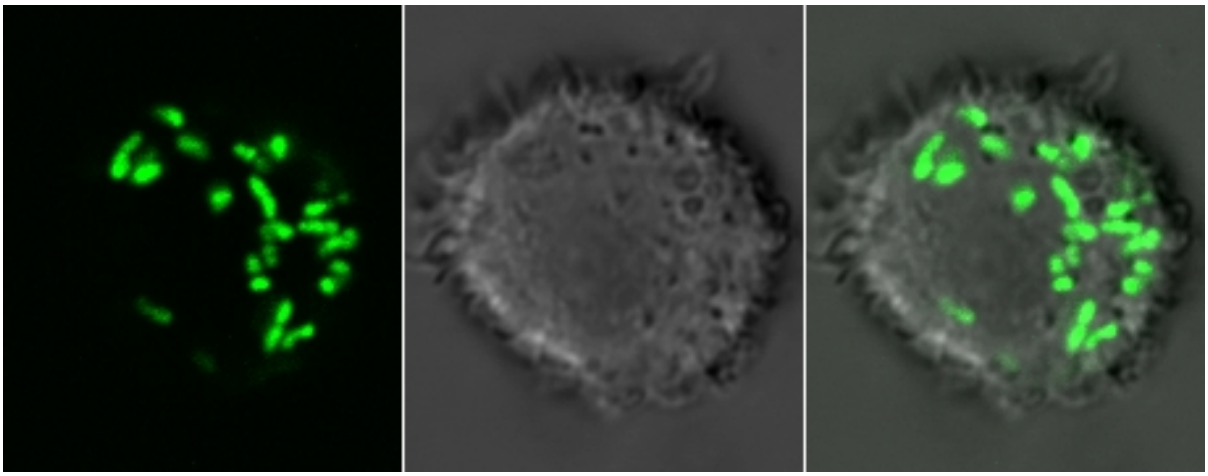

Figure 3A

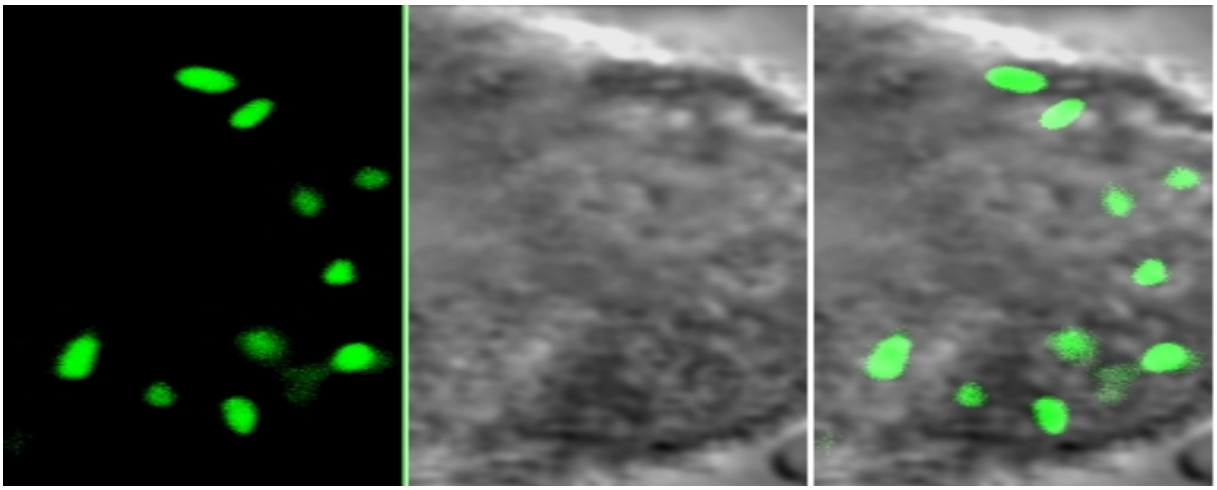

Figure 3B

Figure 2-1-3

Confocal image of J774.A1 cells infected with recombinant *B. abortus* strain RB51 that has been transformed with pNSGroE vector expressing GFP (RB51GroE/GFP). Fluorescent image on the left, transmitted light image in the middle and overlay image on the right (A) after 4 hours of infection (B) after 24 hours of infection.

Chapter 2-2: Establishment of gene expression system in *Ochrobactrum anthropi*

MOHAMED N. SELEEM, MOHAMMED ALI, STEPHEN M. BOYLE, BISWARUP MUKHOPADHYAY, SHARON G. WITONSKY, GERHARDT G. SCHURIG AND NAMMALWAR SRIRANGANATHAN. In Press Oct. 72; No.10, 2006 Journal of Applied and Environmental Microbiology.

Abstract

Genetic studies of *Ochrobactrum anthropi*, a bacterial species important in bioremediation and biopesticide, are hindered by the lack of suitable gene expression system. To date, the potential for *O. anthropi* to express and serve as a source of recombinant protein had not been investigated. In this study, we utilized the backbone of the expression vector pNSGroE to construct a set of vectors useful for gene expression and protein purification in *O. anthropi*. These vectors contain several promoters for different levels of expression and His-tag fusion in the N-terminus to facilitate protein detection and purification. We characterized the transcriptional activity of several promoters in *O. anthropi* by measuring activity of the *lacZ* gene. We demonstrated a one-step detection and purification of recombinant green fluorescence protein (GFP*)* directly from *O. anthropi*. Our study with *gfp* indicated that recombinant *O. anthropi* can be detected intracellularly in a macrophage cell line J774A.1. The new vectors should significantly enhance genetic manipulation and characterization of *O. anthropi* and other *Ochrobactrum* species.

Introduction

The genus *Ochrobactrum* belongs to the family *Brucellaceae* within the alphaproteobacterial order Rhizobiales, and currently comprised of five described species, *O. anthropi*, *O. intermedium*, *O. tritici*, *O. grignonense* and *O. gallinifaecis* (27). The bacterial species are non-fermentative, strictly aerobic, motile, oxidase-positive and indole-negative, gram-negative rods (48). *Ochrobactrum* strains are found in diverse habitats including soil, plants and their rhizosphere, wastewater, animals and humans (27). *O. anthropi* has been isolated from various clinical specimens and is recognized as an opportunistic pathogen (48) that, under certain circumstances, may produce disease in immunocompromised humans (15). From a biotechnological point of view, *Ochrobactrum* strains are of particular interest for bioremediation (27). They are capable of degrading organophosphorus pesticides such as parathion and methylparathion (55), phenol (10), the toxic organic solvent dimethylformamide (DMF) (50), petroleum waste (24), the soil contaminant chlorothalonil (25). They are also capable of removing chromium, cadmium, copper and toxic metals from the environment (35).

In recent decades, *O. anthropi* has become a valuable source of interesting biocatalysts. Among these are a number of amide bond-hydrolyzing enzymes; D-aminopeptidase (4) L-aminopeptidase (DmpA) (12), the D-amino acid amidase (3) and the L-amidase (44). These properties have generated considerable interest in the use of *O. anthropi* as a bioremediation agent and a biopesticide. Unfortunately, genetic studies with *Ochrobactrum* isolates have been hindered by the lack of an efficient system for gene expression and purification of recombinant proteins.

Our goal in this research was to establish enhanced gene expression system in *O. anthropi* to allow the study of the physiology and the cloning and over-expression of genes involved in important biochemical pathways associated in biodegradation. To allow rapid and easy one-step purification of recombinant proteins directly from *O. anthropi*, we have utilized the backbone of the pBBR1 plasmid originally isolated from *Bordetella bronchiseptica* (2), and can be maintained in a wide variety of gram-negative bacteria. We measured the transcriptional activity of various promoters in *O. anthropi*. We expressed and detected the *gfp* as His-tag fusion protein and were able to purify

recombinant *gfp* directly from *O. anthropi*. We also demonstrated that following the infection of macrophages J744.A1, *O. anthropi* carrying these plasmids could readily be detected intracellularly by fluorescence microscopy. The expression vectors used proved stably maintained in *O. anthropi*. This is the first report describing a vector for expression of heterologous genes for a member of the *Ochrobactrum spp*.

Materials and Methods

Bacterial strains, plasmids and oligonucleotides.

The bacterial strains and plasmids used in this study are listed in Table 2-2-1 and the oligonucleotides are listed in Table 2-2-2. *O. anthropi* was routinely grown at 30°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco). Chloramphenicol was used at a final concentration of 30 μg/ml.

Recombinant DNA methods

DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis were performed according to standard techniques (39). The polymerase chain reactions (PCR) were performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from QIAGEN was used for all plasmid extractions and QIAGEN PCR cleanup kit was used for all restriction enzymes removal and DNA extraction from gel.

Construction of the vector:

The sequence carrying the origin of replication (*rep*), the *cat* resistant regions and the MCS of the broad host range pNSGroE *Brucella* expression vector (GenBank accession number AY576605) (42) was used as the backbone for the construction of the new vectors. The *Brucella abortus gro*E promoter was excised from pNSGroE with *Sal*I and *Bam*HI restriction enzymes and the promoter-less pNS construct was used for promoter replacement.

Promoter replacement:

The chloramphenicol resistant gene promoter (Ch) was amplified from the broad-host-range pBBGroE expression vector (52) with primers Ch-F and Ch-R containing *Sal*I and *Bam*HI restriction sites designed in the forward and reverse primers for directional

cloning. In the reverse primer, six histidine and one glycine residues were engineered after the translational start codon to facilitate the epitope tagging at the amino terminus by adding a minimum number of amino acids fused to any expressed protein (42). To avoid the translational error or inhibition arising from rare codon bias (30), the preferred codon usage of *Ochrobactrum* spp. (http://www.kazusa.or.jp) for histidine and glycine were used. After restriction digestion and purification, the Ch promoter was cloned into the pNS construct to form pNSCh expression vector (Figure 2-2-1).

The kanamycin resistant gene promoter (Kan) and ampicillin resistant gene promoter (Amp) each containing a His-tag fusion in the N-terminus were amplified and cloned in the same way mentioned for (Ch) using the pUC4K cloning vector (Pharmacia) for Kan and the pRSETA expression vector (Invitrogen) for Amp and the primers Kan-F and Kan-R, Amp-F and Amp-R respectively.

The coliphage T5 promoter (53) with the downstream *lac* repressor, synthetic ribosomal binding site (RBSII) and the 6xHis-tag codon sequence were excised with *Xho*I and *Bam*HI from pQE-40 expression vector (Qiagen) and ligated to the pNS construct to form pNST5 expression vector (Figure2-2-1).

The hybrid TrcD promoter (1) with the downstream Bacteriophage gene10 translational enhancer (33) minicistron, reinitiation ribosome binding site (40) and lac repressor was amplified from pTrc2HisA vector (Invitrogen) and ligated to the pNS construct to form pNSTrcD expression vector.

Expression of β-galactosidase (*lacZ*)

In order to study the expression and activity of the cloned promoters inside *O. anthropi*, the promoter-less *Escherichia coli* β-galactosidase (*lacZ*) gene was amplified from pRSETB/β-gal as described before (42) and cloned into all expression vectors downstream of the various promoters. The plasmid constructs expressing *LacZ* were transformed into Mach1™ T1 Phage-Resistant (T1 R) competent *E. coli* (Invitrogen). The *lacZ* expression constructs were extracted from *E. coli* and transformed into *O. anthropi*. Black-colored recombinant *E. coli* and *O. anthropi* colonies expressing β-galactosidase were selected on S-Gal plates (Sigma) containing 30 µg cm. Colorimetric assay (52) were employed to compare the levels of β-galactosidase expression under different promoters.

GFP constructs

A promoterless Green Fluorescence Protein gene (*gfp*) was excised from pGFPuv vector (BD Biosciences Clontech) and cloned in frame down stream of the promoter in the MCS of pNSTrcD, and used as a visual marker for gene expression inside macrophages, Western blotting and protein purification.

Preparation of electrocompetent cells

The *O. anthropi* electrocompetent cells were prepared and transformed as mentioned before (31). The transformation mixture was plated on TSA plates containing 30 µg/ml Cm. Transformation efficiency and stability of the plasmids were calculated as described before (42).

Protein Purification

The pNSTrcD-*gfp* expression vector with *gfp* expressed under the TrcD promoter was transformed into *O. anthropi* as mentioned before (31). The *gfp* was purified from recombinant *O. anthropi* using Ni-NTA Agarose (Qiagen) and 6M Guanidine HCL. 20mM imidazol and 20mM ß-mercaptoethanol were added to the lysis buffer and 1% TritonX-100 and 250mM NaCl were added to the washing buffer to reduce contaminating proteins. The cells were pretreated with TE/Citrate/Zwittergent 3-14 (10mM Tris, 1mM EDTA, pH adjusted to 4 with Citric acid, 1% Zwittergent 3-14) for 2 h at 55 °C prior to purification. The concentration of the purified recombinant protein was determined by BCA Protein Assay kit (Pierce) using the manufacturer's enhanced test tube procedure after removal of urea by Microcon centrifugal filter devices YM-10 (Millipore). The purity of the extracted protein was determined by SDS-PAGE.

Western blot

To test the fusion of the His-epitope tagging and efficiency of detection, Western blot analysis was performed by separating the total cell lysates of recombinant *O. anthropi* expressing *gfp* under TrcD promoter by 10% SDS-PAGE then transfer to one of two nitrocellulose membranes (Osmonics). One membrane was incubated overnight with a mixture of Anti-HisG-HRP antibody (1:3000) (Invitrogen) and Anti-His6-Peroxidase (Roche) (1:3000). The combination of antibodies that recognize 6xHis and one glycine (Anti-HisG-HRP) with antibodies that recognize only 6xHis (Anti-His6-Peroxidase) led to more intense band and higher sensitivity of detection (unpublished data). The second

membrane was incubated overnight with Anti-His6 antibody (Roche) (1:1000) and Anti-mouse-HRP secondary antibodies (1:1000) (KPL) for 1 hour.

Cell culture and infection of macrophages

To study the expression of the gfp in-vivo, the murine macrophage-like cell line J774 A.1 was seeded at a density of ~5 x 10^5 cells per well in six- well plates (Corning Incorporated) with glass cover slips 48h prior to infection. The cells were routinely grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma Chemical Co) in a humidified 5% CO_2 atmosphere at 37°C. The J774 A.1 cells were infected with recombinant *O. anthropi* expressing *gfp* under TrcD promoter for 30 minutes at a 1:200 multiplicity of infection. The infected cells washed three times with DMEM medium containing 50 μg/ml gentamicin (Sigma Chemical Co) to wash off non-phagocytized bacteria prior to incubation with DMEM medium supplemented with 10 % FCS and 50 μg/ml gentamicin. At 4 and 24 hours post infection, the infected cells on cover slips were washed with Phosphate Buffered Saline (PBS) and fixed in 4% paraformaldhyde for 20 minutes, washed twice with PBS before mounting with Flouromount-G fluid (Southern Biotechnology Associates Inc.). Intracellular multiplication of *O. anthropi* and expression of the *gfp* was visualized using a Zeiss LSM 510 laser-scanning microscope.

Nucleotide sequence accession number

The sequences of all the vectors reported in this paper have been deposited in the GenBank database; their descriptions, sizes and accession numbers are listed in Table 2-2-3.

Results and Discussion

Vector construction

A series of vectors (Table 2-2-3) were constructed to create a functional and enhanced expression system that could be used in both *O. anthropi* and *E. coli*. Thus, to construct a set of vectors suitable for gene expression in *O. anthropi*, we decided to use the backbone of the broad host range expression vector pNSGroE, a derivative from pBBR1 (2, 42). Although *B. abortus gro*E promoter is a very strong constitutive promoter that functions well in *Brucella* and other related species, it is a weak promoter in *E. coli* (unpublished data). Hence, promoter replacement was essential to create readily

assayable expression vector in both *E. coli* and *O. anthropi*. Figure 2-2-1 depicts the pNSCh and pNST5 expression vectors that were introduced into *O. anthropi* with an efficiency of 2.1 x 10^9 transformants per μg DNA. All plasmids were stably maintained after 8 serial passages over 16 days on TSA plates in the absence of chloramphenicol selection. Several aliquots of the plasmids were digested each with a different restriction enzyme and analyzed by agarose gel electrophoresis; the sizes of the observed bands were as predicted from the sequence analysis (data not shown).

Promoter activity

A comparison was made between the relative levels of *lacZ* activity expressed under different promoters. All amplified promoters were able to express inside *O. anthropi* with various strengths (Figure 2-2-2). The *B. abortus* constitutive *gro*E promoter was the strongest promoter, as *Ochrobactrum* is the closest known *Brucella* relative (51). We have also tested the transcriptional activity of the strong coliphage T5 promoter (53) and TrcD hybrid promoter (1) in *O. anthropi* and demonstrated that both promoters were functional (Figure 4A). All promoters were functional in *E. coli*, with the TrcD and Ch promoters being the strongest promoters and *B. abortus gro*E promoter the weakest promoter (data not shown). The T5 and TrcD hybrid promoters have the *lac* operator (*lacO*) that permits binding of the *lac* repressor to repress transcription for regulated expression in bacterial strains that harbor the *lac*Iq gene encoding the *lac* repressor protein found in *E. coli* strains. Regulated expression using the T5 and TrcD hybrid promoters in *O. anthropi* is possible by incorporating the *lac*Iq gene into the plasmid (7). The results indicate that the constructed vectors may be used in *O. anthropi* as a reporter of gene expression and promoter activity. The data also suggests that all the promoters described here may be used to express genes in *O. anthropi* with different level of expression.

His-tag fusion and Protein purification

Expression and purification of recombinant proteins are fundamental techniques in molecular biology, which can be simplified by short epitope tagging of the gene (6). Histidine tags are the most commonly used as they are less likely to interfere with the function of the expressed protein (49). A tagged protein can be easily purified using

metal chelate affinity chromatography (37). The 6xHis epitope tag was expressed and detected successfully in *O. anthropi*. The Anti-His antibodies were used successfully to detect expression of *gfp* fused with the His-tag. Figure 2-2-3 shows the usefulness of the His-tagged fusion protein expression in *O. anthropi* by demonstrating the specificity, sensitivity and ease of detection with Anti-His antibodies.

Our second goal of adding the N-terminus His-tag fusion was purification of recombinant protein directly from *O. anthropi* without using *E. coli* as an expression host. Although, various expression systems are available and easy purification procedures of large amount of recombinant protein from *E. coli* are well established, it doesn't allow post-translational modification studies and protein-protein interaction in *O. anthropi*. Also differences in codon usage between *E. coli* and *O. anthropi* may interfere with the expression. The N-terminus His-tag fusion allowed one-step purification of a large amount of pure recombinant *gfp* from *O. anthropi* by nickel chelate affinity chromatography. We were able to purify 7.3 mg of recombinant *gfp* from 100 ml of bacterial culture (OD_{600} = 2) using Ni-NTA agarose and 6M Guanidine HCL. Pretreatment of the cells with TE/Citrate/Zwittergent 3-14 for 2 h at 55 °C prior to purification enhanced purity and increased the amount of purified recombinant protein by more than 50%. The 6M Guanidine HCl alone wasn't a sufficient solubilizing agent. Figure 2-2-4 shows the purity of the purified recombinant protein. This is the first report of purification of His-tagged recombinant protein directly from *O. anthropi*.

Although the presence of the His-tag fusion may affect the characteristic or function of the purified recombinant protein and downstream applications (54), it can be resolved by adding a site specific protease to cleave the N-terminal fusion. The His-tag can be exploited for affinity purification as well as for post-translational modification studies of proteins expressed in *O. anthropi* and other related species (42), without the need for *E. coli* as an expression host (18, 32). This method will facilitate the purification of important pharmaceutical enzymes and recombinant proteins directly from *O. anthropi*.

In vivo expression of GFP

Previous studies in our laboratory showed that the *O. anthropi* could survive for several hours inside the J774A.1 macrophage cell line (17). To evaluate the ability of the newly constructed vectors to express in-vivo and the possibility to monitor *O. anthropi* intracellularly, we infected J774A.1 macrophage cell line with recombinant *O. anthropi* expressing *gfp* under TrcD promoter. Macrophages were observed by confocal microscopy at 4 and 24 hours post infection. The fluorescence observed (Figure 2-2-5) clearly demonstrates the ability of our vector to express intracellularly inside macrophages and the possibility of monitoring *O. anthropi* during the course of infection. The high level of *gfp* expression did not alter the stability of the plasmid or the growth characteristic of *O. anthropi* inside macrophages compared to the wild type strain.

In this study we reported the construction of a series of expression vectors for *O. anthropi* that can be used for heterologous gene expression, protein detection and purification. The *lacZ* and *gfp* genes were introduced into *O. anthropi* and expressed under various promoters. Recombinant *gfp* protein was detected by Western blot using Anti-His antibodies and purified using Ni-NTA. The newly described vectors can be used to enhance the Ochrobactrum's degrading capabilities by over-expression of genes involved in important pathways associated in biodegradation. This will facilitate the use of *O. anthropi* as a potential bioremediation tool and a biopesticide agent. All the vectors in this study have the same reading frame and same MCS for easy transfer of genes between them. The genetic techniques described herein should be applicable to the study of other environmentally significant organisms because our expression vectors are carrying a replication protein (*Rep*) from pBBR1 plasmid (2) that shares sequence homology with replication proteins in gram-negative bacteria (28). It can replicate and be stably maintained in a wide range of Gram-negative organisms, including *Bordetella bronchiseptica, Aeromonas hydrophila, Zymomonas mobilis, Pseudomonas oleovorans, Pseudomonas putida, Pseudomonas aeruginosa, Bartonella bacilliformis, Brucella spp., Rhodobacter sphaeroides, Halomonas spp., Rhizobium leguminosarum, Agrobacterium tumefaciens, Sinorhizobium meliloti, Magnetospirillum gryphiswaldense, Burkholderia cepacia, Ralstonia eutropha, Azospirillum brasilense, Geobacter sulfurreducens, Pedobacter heparinus, Hyphomicrobium facilis, Hyphomicrobium denitrificans,*

Methylobacillus glycogenes, Methylobacterium extorquens, Methylophilus methylotrophus (2, 5, 8, 9, 11, 13, 14, 16, 19-23, 26, 28, 29, 34, 36, 38, 41, 43, 45-47, 56).

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Table 2-2-1. Bacterial strains and plasmids used in this study

Plasmid	**Description** [a]	**Source**
pNSGroE	Cm^r, Broad host range expression vector, bearing *Brucella gro*E promoter	(Seleem, Vemulapalli et al. 2004)
pBBgroE	Cm^r, Broad host range expression vector, bearing *Brucella gro*E promoter	(Vemulapalli, He et al. 2000)
pUC4K	Km^r, cloning vector	Pharmacia
pRSETA	Amp^r, Expression vector with T-7 promoter	Invitrogen
pQE-40	Amp^r, Expression vector with T-5 promoter	Qiagen
pRSETB/β-gal	Amp^r, Expression vector with *E. coli* β-galactosidase	Invitrogen
pGFPuv	Amp^r, Green florescence protein expression vector	BD Biosciences Clontech
pTrc2HisA	Amp^r, Expression vector bearing *trc* hybrid promoter	Invitrogen
Bacterial Strain	**Description**	**Source**
O. anthropi strain 49237	Originally isolated from soil	He et al., 2002
E. coli Mach1	Chemical competent cells, *endA1, recA1, ton*A, *pan*D	Invitrogen

[a] Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance.

Table 2-2-2 Primers used to amplify the promoters

Promoter	Size in bp	Source of DNA	Primers Name	Primer sequence
Ch	237	pBBgroE expression vector (Vemulapalli, He et al. 2000)	Ch-F	5`-CCC**GTCGAC**GAATAAATACCTGTGACGGAA-3`
			Ch-R	5`-CCC**GGATCC**ACC**ATGATGATGATGATGATG** CATTTTAGCTTCCTTAGCTCCTGAAAATCT-3`
Kan	149	pUC4K cloning vector (Pharmacia)	Kan-F	5`-GGG**GTCGAC**GGGGAAAGCCACGTTGTGTCT-3`
			Kan-R	5`-GGG**GGATCC**ACC**ATGATGATGATGATGATG** CATAACACCCCTTGTTAAACTGTTTATGTA-3`
Amp	233	pRSETA expression vector (Invitrogen)	Amp-F	5`-CCC**GTCGAC**AAGGGCCTCGTGATACGCCTA-3`
			Amp-R	5`-CCC**GGATCC**ACC**ATGATGATGATGATGATG** CATACTCTTCCTTTTTCAATATTATTGAAG-3`
TrcD	277	pTrc2HisA expression vector (Invitrogen)	TrcD-F	5`- CCC**GTCGAC**AGAAAAAAGATCAAAAAAATAC TTGACAATTAATCATCCGGCTCGTATAATG-3`
			TrcD-R	5`- CCC**GGATCC**ACC**ATGATGATGATGATGATG**CAT GGTTTATTCCTCCTTATTTAAT-3`

Table 2-2-3. Descriptions of the plasmids that were constructed and used in this study

Plasmid	Size bp	Promoter	Type of expression	Accession number
pNSCh	2903	*Ch*	Constitutive	DQ412050
pNSKan	2809	*Kan*	Constitutive	DQ412052
pNSAmp	2891	*Amp*	Constitutive	DQ412053
pNST5	2810	*Phage T5*	Regulated	DQ412054
pNSTrcD	2937	*trc hybrid +UP*	Regulated	DQ412056
pNSGroE	2863	*B. abortus groE*	Constitutive	AY576605

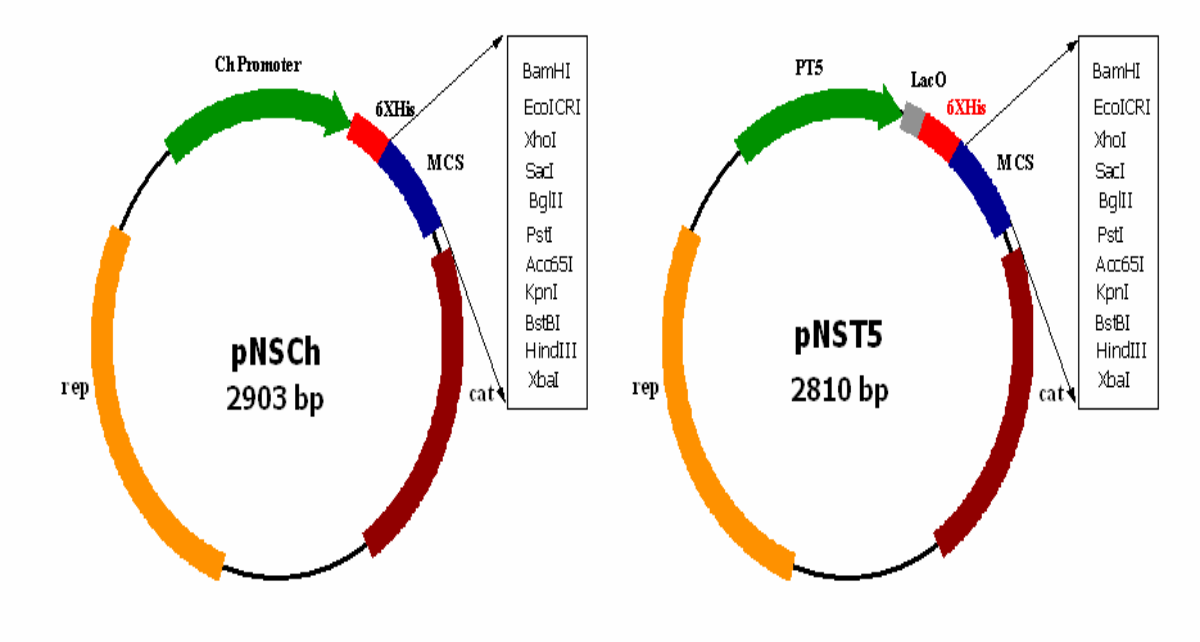

Figure 2-2-1. Plasmid Map
The engineered pNSCh and pNST5 expression vectors for constitutive and regulated expression respectively with the His-tag fusion in the N-terminus. MCS, multiple cloning site; *cat*, chloramphenicol acetyltransferase gene conferring chloramphenicol resistance; *rep*, gene required for plasmid replication; 6Xhis, His tag fusion; LacO, lac operator for regulated expression.

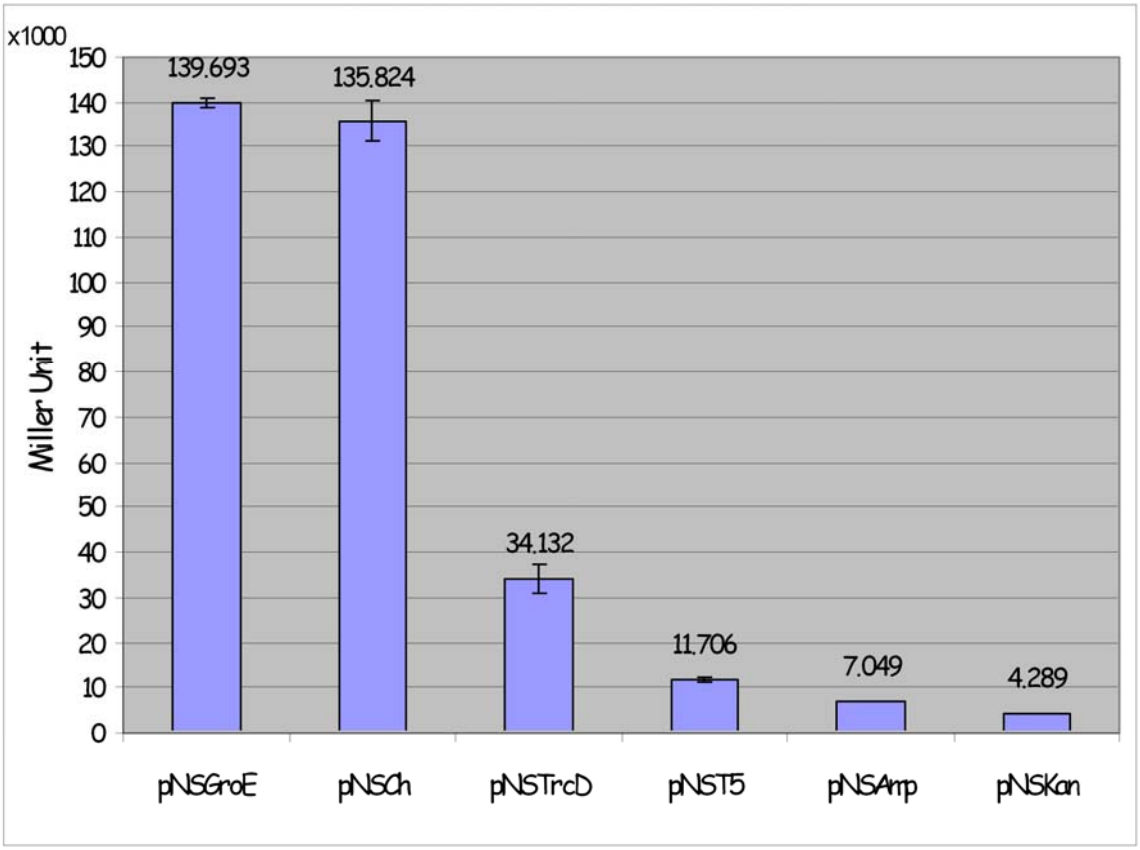

Figure 2-2-2 LacZ activity from different promoter in *O. anthropi*

LacZ activity of each construct was determined as described under expression of β-galactosidase *(lacZ*) and represented as Miller unit. The activity represents the average of three separate cultures. Triplicate cultures were used in each experiment

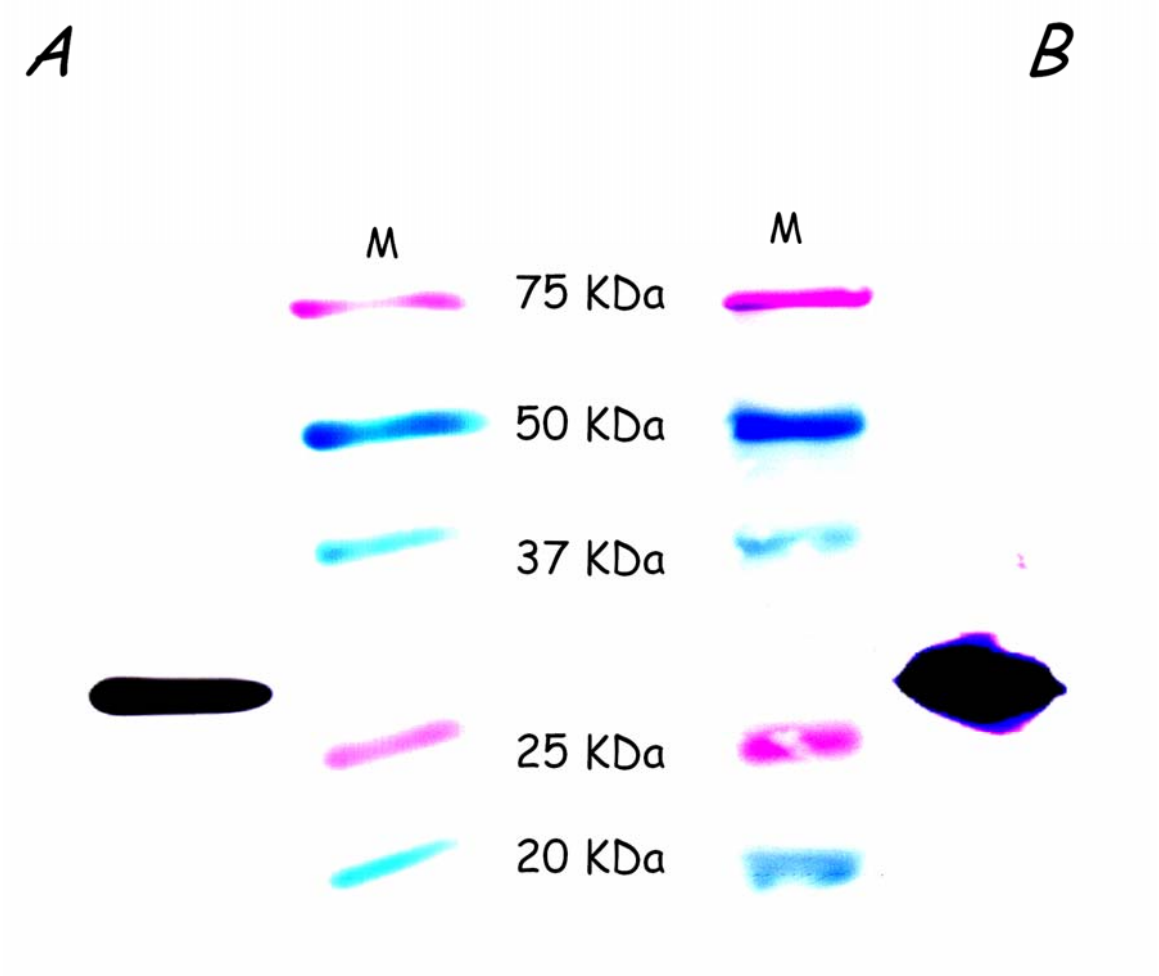

Figure 2-2-3. Western Blot of recombinant *gfp*.
Detection of His-tagged fusion *gfp* protein expressed from the TrcD promoter in *O. anthropi*. A) Membrane was incubated with Anti-His6 antibody (1:1000) overnight and Anti-mouse-HRP secondary antibodies (1:1000) for 1 hour. B) Membrane was incubated with a mixture of Anti-HisG-HRP antibody (1:3000) and Anti-His6-Peroxidase (1:3000) overnight. MW, Precision Plus Protein™ Dual Color Standards (Bio-Rad)

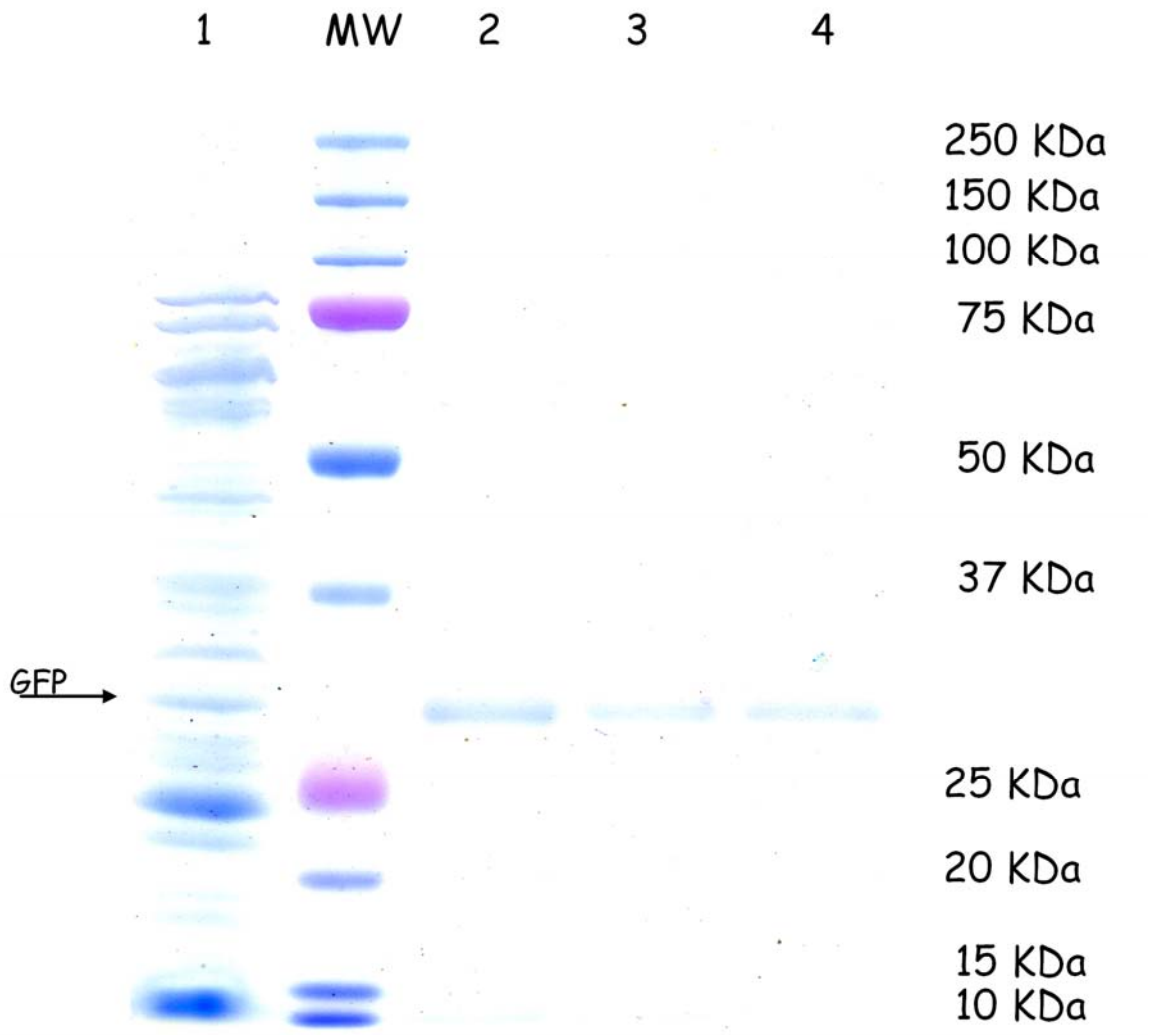

Figure 2-2-4. SDS-PAGE of purified recombinant *gfp*.

The cells were pretreated with TE/Citrate/Zwittergent 3-14 for 2 h at 55°C prior to purification of *gfp* from *O. anthropi* using Ni-NTA Agarose (Qiagen). The SDS-PAGE was stained with Coomassie blue. Lane 1; total cell lysate of recombinant *O. anthropi*; lanes 2-4, purified recombinant *gfp* elution 1-3*;* MW, Precision Plus Protein™ Dual Color Standards (Bio-Rad)

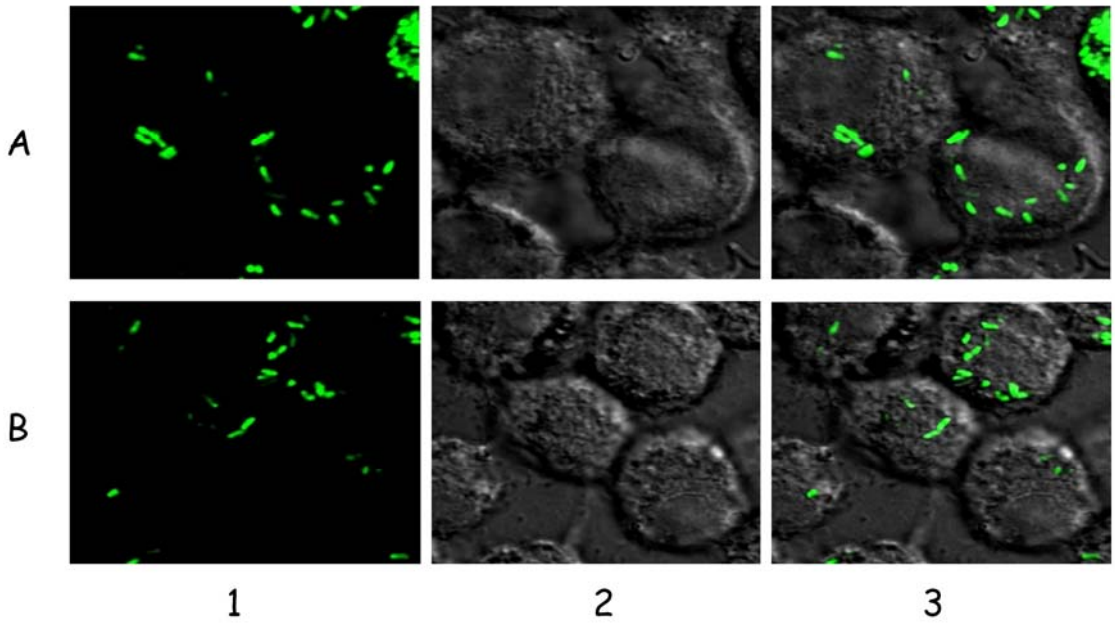

Figure 2-2-5 Confocal image of J774.A1 cell infected with *O. anthropi*.
J774.A1 macrophage cell line was infected 1:200 with *O. anthropi* expressing *gfp* from the TrcD promoter. Images were taken after 4 h (A) and 24 h (B), 1; Fluorescent image, 2; transmitted light image and 3; overlay image.

Chapter 3
Enhancement of gene expression in *Brucella* and *Ochrobactrum*

Chapter 3-1

Enhanced expression, detection and purification of recombinant proteins using RNA stem loop and tandem fusion tags

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MOHAMED SELEEM [1], M.W. ABD AL-AZEEM[2], MOHAMMED ALI[3], STEPHEN M. BOYLE [1] AND NAMMALWAR SRIRANGANATHAN [1*]. [1]Department of Biomedical Sciences and Pathobiology, Center of Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic and State University, Blacksburg, VA, [2]Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt. [3]Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Assiut University, Egypt.

Abstract

The creation of a double-His tag fusion that forms a RNA-stem loop in the mRNA encoding the N-terminus of the target protein is a novel single approach for enhancement of expression, purification and detection of recombinant protein. A tandem His-tag fusion RNA stem loop located downstream of the constitutive *gro*E and Ch promoters enhanced heterologous gene expression in *Brucella*, *Salmonella* and *E. coli* compared to a single His-tag fusion. We demonstrated a one-step detection and purification of recombinant green fluorescence protein *gfp* directly from *Brucella spp* without using *E. coli* as an expression host. The amount of purified *gfp* using tandem His-tag RNA stem loop increased more than 3 fold and detection sensitivity more than 4 fold in comparison to the single His-tag fusion form. This method has potential applications in heterologous gene expression and high-throughput protein synthesis and purification.

Introduction

Expression and purification of recombinant proteins are fundamental techniques in molecular biology. There are several approaches employed to enhance the expression, detection or purification that must be applied empirically and tested separately for each target protein. Although various expression systems are available and easy purification procedures of large amount of recombinant protein from *E. coli* are well established, it doesn't allow post-translational modification studies and protein-protein interaction in other microorganisms. Also differences in codon usage between *E. coli* and other unrelated bacteria can interfere with the expression.

Having enhanced and simple system for expression and purification of recombinant protein directly from its native microorganism without using *E. coli* as an expression host will facilitate more accurate study of the characteristics of the expressed protein as well as post-translational modification studies and protein-protein interaction. Also, the system will over-come the problem that may arise from differences in codon usage, toxicity and signal sequence associated with the target heterologous protein in *E. coli*.

Short epitope tagging of the cloned gene simplified recombinant protein purification (Braun and Suske 1999). Histidine tags are the most commonly used as they are less likely to interfere with the function of the expressed protein (Van Reeth, Dreze et al. 1998). A tagged protein can be easily purified using metal chelate affinity chromatography (Porath, Carlsson et al. 1975). On the other hand, the creation of a synthetic RNA stem loop in the mRNA encoding the N-terminus of the target protein allowed the expression of previously non-expressible genes and enhanced expression of all other studied genes (Paulus et al., 2004). The mechanisms of enhancing expression by RNA stem loop are through suppression of the long-range interactions between the translation initiation domain and gene-specific mRNA sequences and formation of accessible translational initiation domain (Paulus et al., 2004).

We developed a single approach for enhancing expression, detection and purification of recombinant protein simultaneously by combining epitope tagging and RNA stem loop approach and creating a tandem His-tag RNA Stem Loop (HRSL).

Application of HRSL downstream of various promoters enhanced heterologous gene expression in all studied strains; vaccine strains (*Brucella* and *Salmonella*), wild type strains (*Salmonella*) and genetically modified laboratory strains (*E. coli*) used for standard cloning and gene expression. The HRSL allowed one-step purification of pure recombinant *gfp* from *Brucella spp* by nickel chelate affinity chromatography. The sensitivity of detection after applying the HRSL was dramatically improved compared to a single His-tag fusion that doesn't form RNA stem loop. The HRSL should significantly enhance genetic manipulation and protein purification in many bacterial species.

Materials and Methods:

The polymerase chain reactions (PCR) were performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from QIAGEN was used for all plasmid extraction and QIAGEN PCR cleanup kit was used for all restriction enzyme removal and DNA gel extraction. RNA folding and secondary structure prediction was done by *mfold* software version 3.2 at 37 $^{\circ}$C (Mathews, Sabina et al. 1999; Zuker 2003).

Vector construction:

1- pNSGroE2His

The tandem His-fusion (2 x 6His) RNA stem loop (stem length, 6-bp; $\triangle G_0$ =-2.1 kcal/mol) (Figure 3-1-1) was introduced by PCR downstream of the first translational start codon of the *Brucella gro*E promoter in two steps using primers GroE-F and His-R1 (Table 3-1-1). The amplified DNA was gel purified and used as a template for the second PCR cycle using primers GroE-F and His-R2 (Table 3-1-1). The *Sal*I and *Bam*HI restriction sites were designed in the forward and reverse primers (GroE-F and His-R2) for directional cloning. In the reverse primers His-R1 and His-R2 (Table 3-1-1), the histidine residues were engineered after the translational start codon to facilitate the epitope tagging at the amino terminus (Seleem, Vemulapalli et al. 2004). The sequence carrying the origin of replication (*rep*), the *cat* resistant gene and the MCS of the broad host range pNSGroE *Brucella* expression vector (GenBank accession number

AY576605) (Seleem et al., 2004) was used as the backbone for the construction of the new vectors. The *Brucella gro*E promoter with a downstream single His-tag fusion was excised with *Sal*I and *Bam*HI restriction enzymes (Figure 3-1-2). The amplified GroE2His promoter from step two above with the downstream double His-tag fusion RNA-stem loop after restriction digestion and purification was cloned into the pNS construct to form the pNSGroE2His expression vector (Genbank accession number DQ412049) (Figure 3-1-2).

2- pNSCh2His:

The chloramphenicol resistant gene promoter (Ch) was amplified from the broad-host-range pBBGroE expression vector (Vemulapalli, He et al. 2000) with primers Ch-F and Ch-R (Table 3-1-1). In the reverse primer, six histidine and one glycine residues were engineered after the translational start codon to facilitate the epitope tagging of the 6His. The amplified Ch promoter was cloned into the pNS construct to form the pNSCh expression vector (Genbank accession number DQ412050). The HRSL (Figure 3-1-1) was introduced by PCR downstream of the first translational start codon of the Ch promoter and cloned in the same way mentioned for GroE2His using Ch-F and His-R2 primers (Table 3-1-1). After restriction digestion and purification the amplified Ch2His promoter with the downstream HRSL was cloned into the pNS construct to form the pNSCh2His expression vector (Genbank accession number DQ412051) (Figure 3-1-2).
The plasmids were sequenced and the presence of the predicted RNA-Stem loop was confirmed.

Expression of β-galactosidase (lacZ):

In order to study and compare the *in-vivo* expression and activity of the cloned promoters with the HRSL, the promoter-less *E. coli* β-galactosidase gene (*lacZ*) was cloned as described before (Seleem et al., 2004) into the expression vectors pNSGroE2His and pNSCh2His bearing the HRSL and the expression vectors pNSGroE and pNSCh bearing single His-tag fusion and transformed into Mach1™ T1 Phage-Resistant (T1R) competent *E. coli* (Invitrogen). Black-colored recombinant *E. coli* colonies expressing β-galactosidase were selected on S-Gal 30µg/ml Cm plates (Sigma).

Bacterial transformation:

To test the effect of the HRSL on the level of *lacZ* expression, the pNSGroE2His/*LacZ* and pNSGroE/*LacZ* were transformed into several bacterial strains: *Brucella abortus* vaccine strain RB51 (Seleem, Vemulapalli et al. 2004), *Salmonella choleraesuis* wild type and *Salmonella choleraesuis* vaccine strain 54 (SC54) (Kennedy, Yancey et al. 1999) and *E. coli* BL21 Star (Invitrogen). The pNSCh2His/*LacZ* and pNSCh/*LacZ* level of expression were compared in two strains, *S. choleraesuis* wild type and Mach1™ T1 Phage-Resistant (T1 R) competent *E. coli* (Invitrogen). *Brucella* and *Salmonella* were transformed by electrotransformation with a Gene Pulser (BTX) set at 2 KV, 25 µF and 200 Ω, using a previously described procedure for *Brucella* spp. (McQuiston, Schurig et al. 1995). *E coli* were transformed according to the manufacturer's recommendations. Black-colored recombinant *Brucella, Salmonella* and *E. coli* colonies expressing β-galactosidase were selected on S-Gal 30µg/ml Cm plates (Sigma).

Previously described procedures (Vemulapalli, He et al. 2000) were followed to determine and compare the levels of β-galactosidase expression under different promoters. Transformation efficiency and stability of the plasmids were calculated as described before (Seleem, Vemulapalli et al. 2004).

GFP construct:

A promoterless green fluorescence protein gene (*gfp*) was excised from pGFPuv vector (BD Biosciences Clontech) and cloned in frame down stream of the promoter into each of the multiple cloning sites of pNSGroE2His and pNSGroE and used as visual marker of gene expression, Western blotting and protein purification (Seleem, Vemulapalli et al. 2004).

The pNSGroE2His/*gfp* and pNSGroE/*gfp* constructs expressing *gfp* were transformed into *B. abortus* strain RB51 by electrotransformation as mentioned earlier. Green fluorescent recombinant *Brucella* colonies were selected on TSA 30µg/ml Cm plates (Sigma).

Protein Purification:

The His-tagged *gfp* was purified from recombinant *B. abortus* RB51 using Ni-NTA Spin Columns (Qiagen) under denaturing condition using 8M urea and according to

the manufacturer's instructions with some modification; Briefly, 5 ml of the recombinant *B. abortus* RB51 culture harboring pNSGroE2His/*gfp* or pNSGroE/*gfp* were collected at OD_{600} of 2.0 and resuspended in 1 ml of 8M urea pH 8. Benzonase nuclease (Novagen) 100 U/ml and lysozyme 4mg/ml were added and the cells were lysed at room temperature with shaking for 6 hours before loading into the Ni-NTA Spin Columns. The concentration of the purified recombinant protein was determined by BCA Protein Assay kit (Pierce) using the manufacturer's enhanced test tube procedure. The purity of the extracted protein was determined by SDS-PAGE. Equivalent amounts of total cell extracts or 20μg purified protein prepared from recombinant *B. abortus* RB51 were loaded and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes for Western blotting, and incubated overnight with Anti-HisG-HRP antibody (1:2000) (Invitrogen). A colorimetric method of detection was used (Sambrook J 1989). The signal intensity was measured using a Gel Logic 200 imaging system (Kodak).

Results

LacZ activity:

The expression of heterologous protein (*lacZ*) expressed in pNSGroE2His harboring the HRSL were increased as compared to single His-tag fusion pNSGroE in all tested strains as determined by Miller units. As seen in Figure 3-1-3, *lacZ* gene expression increased approximately 40% in *B. abortus* RB51, two and three fold in *S. choleraesuis* vaccine strain and wild strain respectively, 2.7 and two fold in *E. coli* Mach1 and BL21 star respectively. The expression of heterologous protein (*lacZ*) expressed in pNSCh2His harboring the HRSL were increased 45% and two fold in *S. choleraesuis* wild type and *E. coli* Mach1 respectively over the single His-tag fusion pNSCh as determined by Miller units (Figure 3-1-4). The activity represents the average of four different experiments.

Protein purification and detection:

The amount of recombinant *gfp* that was purified from *B. abortus* RB51 using HRSL pNSGroE2His/*gfp* was 3.6 fold (286 μg/spin column) higher than the single His-tag fusion pNSGroE/*gfp* (80 μg/spin column). The capacity of the column reported by

the manufacturer is 150 μg/spin column, Figure 5 shows the purity of the purified recombinant protein.

Sensitivity of detection using either same amount of total cell lysates or purified recombinant protein from *B. abortus* RB51 harboring the pNSGroE2His/*gfp* was enhanced 4.3 fold over the pNSGroE/*gfp*. (Figure 3-1-6)

The stability and the transformation efficiency of the plasmid didn't change due to the addition of HRSL (data not shown).

Discussion

It has been shown that inserting 6-bp RNA stem-loop at the appropriate place will prevent interaction or hybridization between the leader sequence and the downstream mRNA sequence leaving the leader sequence more accessible to ribosomal initiation, which leads to mRNA stabilization and enhancement of expression (Paulus, Haslbeck et al. 2004). Having a 11-15 nt. leader sequence before the RNA-stem loop is essential for successful initiation of translation (Paulus, Haslbeck et al. 2004). The presence of the RNA-stem loop within the leader sequence will down regulate gene expression by preventing the ribosome from binding to the mRNA (Sudershana, Du et al. 1999). So careful designing of the N- terminal fusion tag and appropriate codon usage should be considered in order to avoid formation of RNA-stem loop in the leader sequence.

In this study we combined two strategies (tandem fusion tag and RNA stem-loop) to improve expression, purification and detection of recombinant proteins. Although the utilization of multiple tags and RNA stem loop separately to improve expression has been described (Paulus, Haslbeck et al. 2004), their concomitant use is novel. We created a double-His tag fusion (2x6His) that forms a RNA-stem loop in the mRNA encoding the N-terminus of the target protein, we designed a 14 nt. leader sequence before the RNA-stem loop for successful initiation of translation and binding to the mRNA.

The enhanced expression of heterologous protein due to the tandem His-tag RNA stem loop was not restricted to a certain strain as we demonstrated the enhanced expression in wild type strain (*S. choleraesuis*), vaccine strains (*B. abortus* RB51 and *S. choleraesuis* SC-54) and laboratory strains used for standard cloning and expression

procedures (*E. coli* MachI and BL21 star), or specific promoter as we demonstrated the enhanced expression using *Brucella gro*E and Ch promoters.

To test the effect of tandem tag fusion on protein purification and to avoid the interference of the increased expression due to HRSL on the amount of purified protein, we chose *B. abortus* RB51 to purify *gfp* protein, as it showed the lowest level of increase in *lacZ* expression. Although there was 40% increase of heterologous gene expression, the amount of purified protein was increased 3.6 fold using the HRSL. This indicates that the increased amount of purified recombinant *gfp* was mainly due to the presence of the tandem His-tag fusion in the N-terminus and presumably increased binding of recombinant protein to the Nickel affinity column (Nakajima and Yaoita, 1997). However, the positive effect of increased expression on the amount of purified protein cannot be ignored. The two important vaccine strains *B. abortus* RB51 and *S. choleraesuis* SC-54 will benefit from the enhanced heterologous gene expression as both vaccines can be used for the delivery of protective antigens of other pathogens (bivalent vaccines) (Cardenas and Clements 1992; Vemulapalli, He et al. 2000).

Figure 3-1-6 shows the usefulness of the His-tagged fusion protein expression in *Brucella* by demonstrating the specificity, sensitivity and ease of detection of the expressed fusion protein with Anti-His antibodies. Moreover, it also shows the increased sensitivity of detection by comparing the total cell lysates or purified *gfp* using HRSL versus a single His-tag fusion. The enhanced sensitivity of detection of recombinant protein by Western blot was due to the presence of the tandem His-tag fusion as more epitope will be available for binding to the antibodies (Nakajima and Yaoita, 1997). Although the HRSL enhanced expression in the two important laboratory *E. coli* strains (Mach1 and BL21) it can be exploited for increased affinity purification yield as well as for post translational modification studies of proteins expressed in *Brucella* and *Salmonella* (Seleem, Vemulapalli et al. 2004), without the need for *E. coli* as an expression host (Higgins, Avison et al. 2001; Ogawa, Takeda et al. 2001). Importantly, the high level of protein expression achieved using the HRSL did not alter the stability of the plasmid or the growth characteristics of the recombinant strains compared to the wild type strains.

We did not study the effect of tandem His-tag fusion on the stability of the expressed protein and whether it played a role in the enhanced level of expression, but the increase in the number of epitope tags as reported by (Nakajima and Yaoita, 1997) did not result in an increase in protein production.

Although the presence of a double His-tag fusion may affect the characteristic or function of the recombinant protein and downstream applications (Wu and Filutowicz 1999), any anomalies can be resolved by adding a site specific protease to cleave the N-terminal fusion without affecting the structure of the stem loop.

We clearly demonstrate that a double His-tagged fusion with RNA stem-loop in the N-terminus of the mRNA enhanced the expression of heterologous proteins in *Brucella*, *Salmonella* and *E. coli* compared to a single His-tag fusion. We have shown the usefulness of the HRSL by demonstrating the higher level of protein expression, elevated level of purified protein and enhanced sensitivity of detection of the expressed fusion protein.

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Table 3-1-1 –Primers used to amplify the promoters

Promoter	Size in bp	Source of DNA	Primers Name	Primer sequence
GroE2His	221	pNSGroE (Seleem et al., 2004)	GroE- F	5`- CCC**GTCGAC**GCTGTTCGCGCAAAAACGCCA-3`
			His- R1	5`-**GATGATGATG**GCC**ATGATGATGATGATGATG**-3`
			His-R2	5`- CCC**GGATCCATGATGATGATGATGATG**GCC **ATGATG**-3`
Ch	237	pBBgroE expression vector (Vemulapalli et al., 2000)	Ch-F	5`-CCC**GTCGAC**GAATAAATACCTGTGACGGAA-3`
			Ch-R	5`-CCC**GGATCC**ACC**ATGATGATGATGATGATG** CATTTTAGCTTCCTTAGCTCCTGAAAATCT-3`
Ch2His	261	pNSCh (this study)		

Restriction sites are shown in bold font.
The His-tag fusion sequence is shown in bold font and underlined.

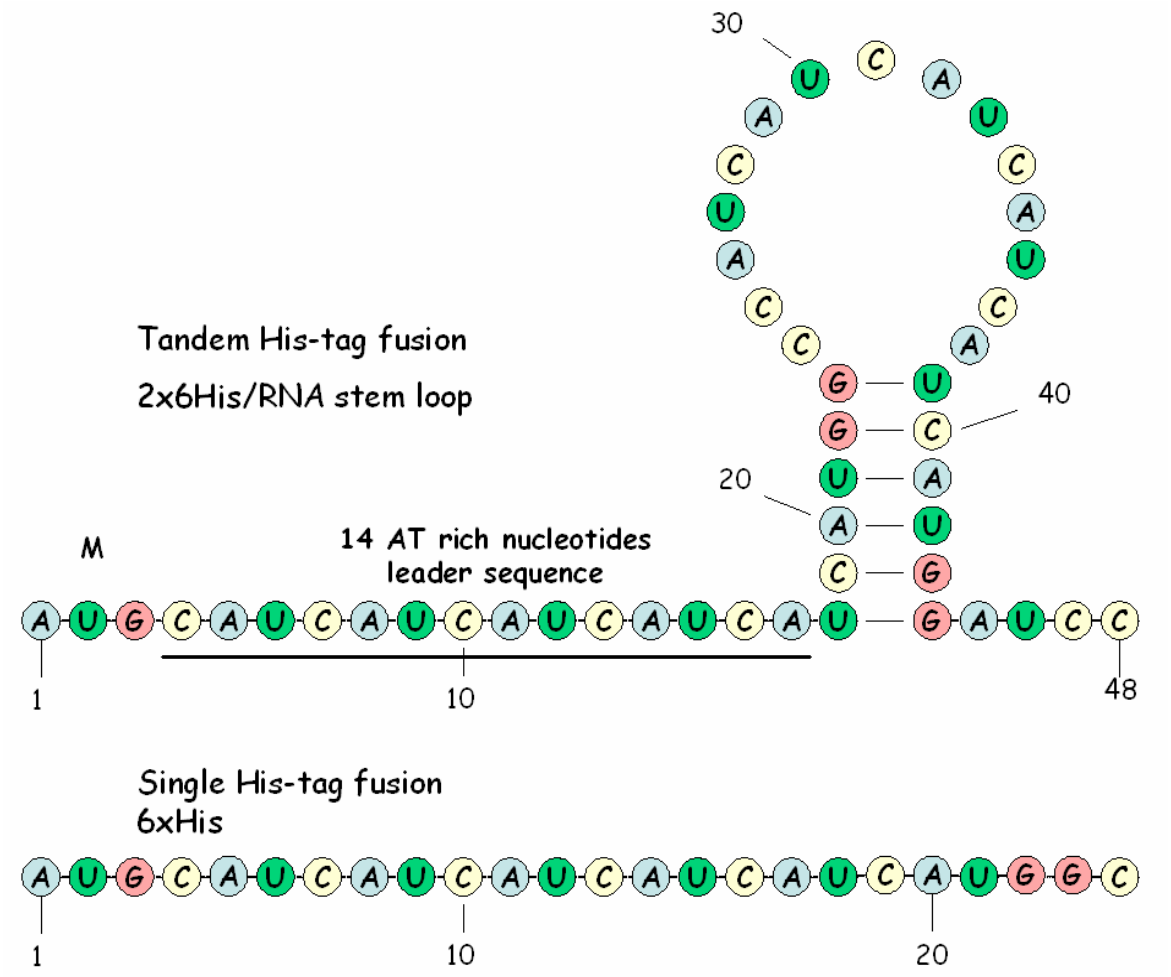

Figure 3-1-1 Double His-tag fusion that form RNA stem-loop in the N-Terminus with stem length, 6-bp; $\triangle G_0$ =-2.1 kcal/mol as predicted by *mfold* software. And the sequence of a single His-tag fusion.

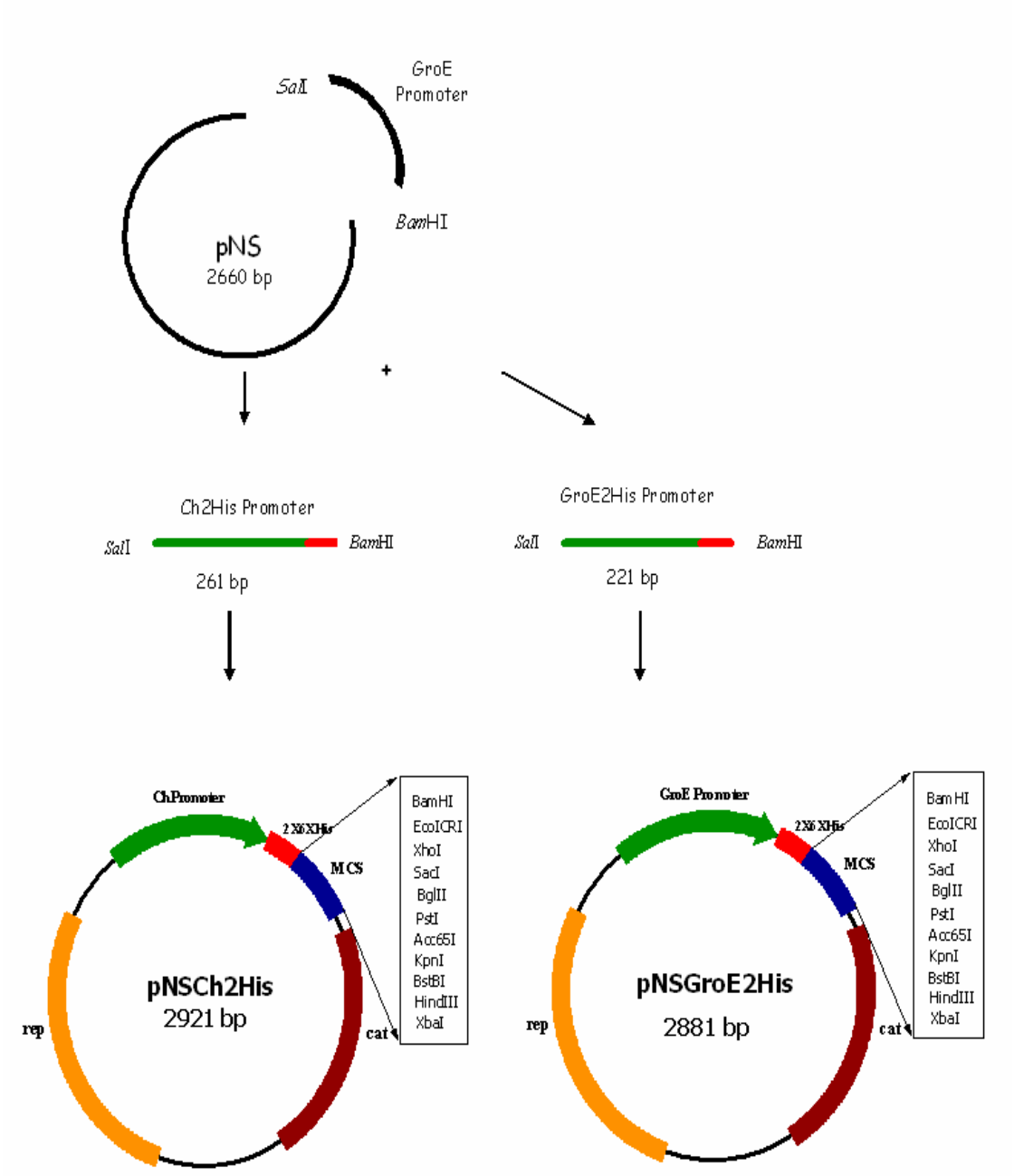

Figure 3-1-2: construction of the two expression vectors pNSGroE2His and pNSCh2His.

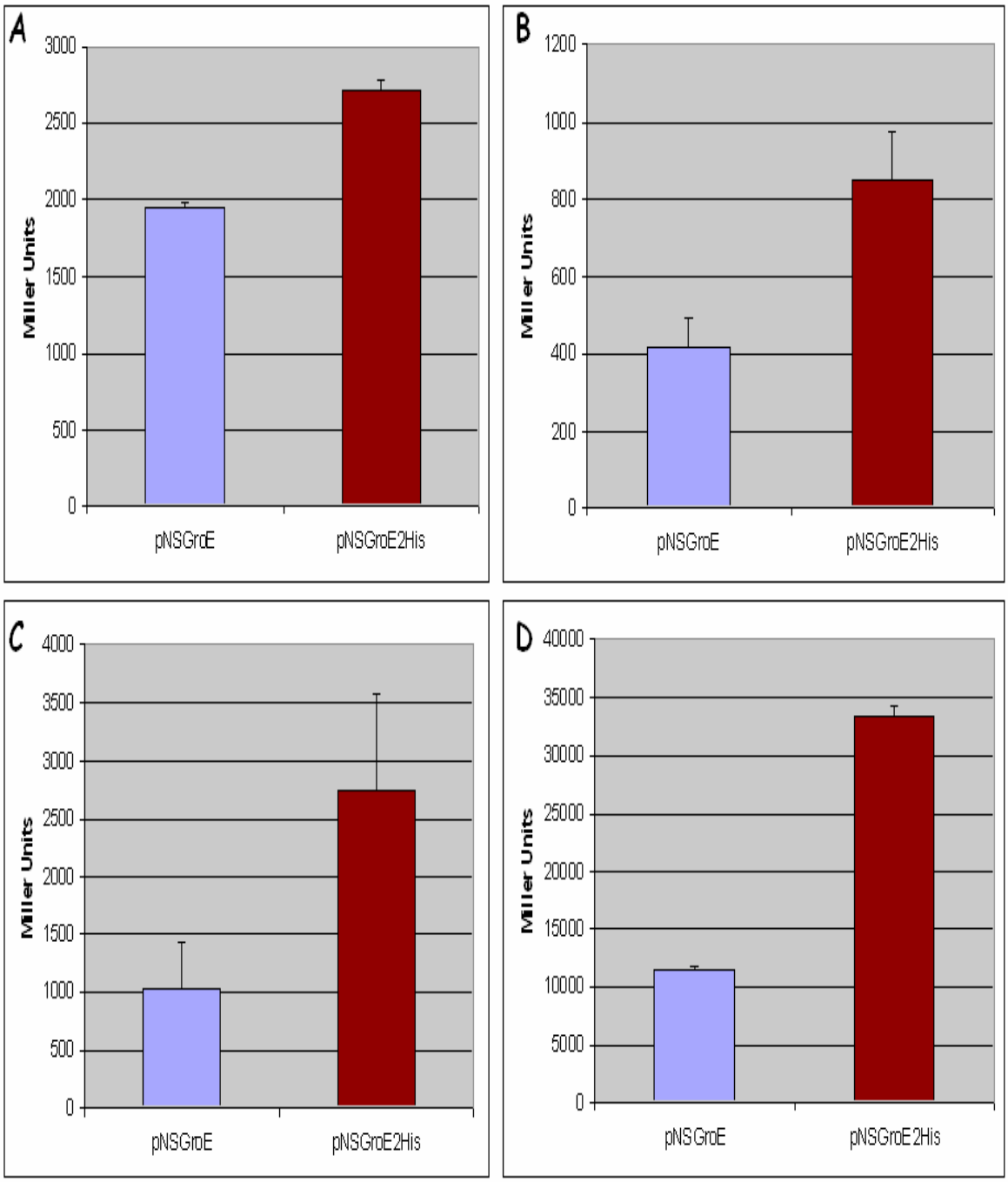

Figure 3-1-3:
LacZ activity of each construct was determined as described under expression of β-galactosidase *(lacZ*) and represented as Miller units. The activity represents the average of three cultures. A: *B. abortus* RB51, B: *S. choleraesuis* SC-54, C: *S. choleraesuis* wild type, D: *E. coli* Mach1

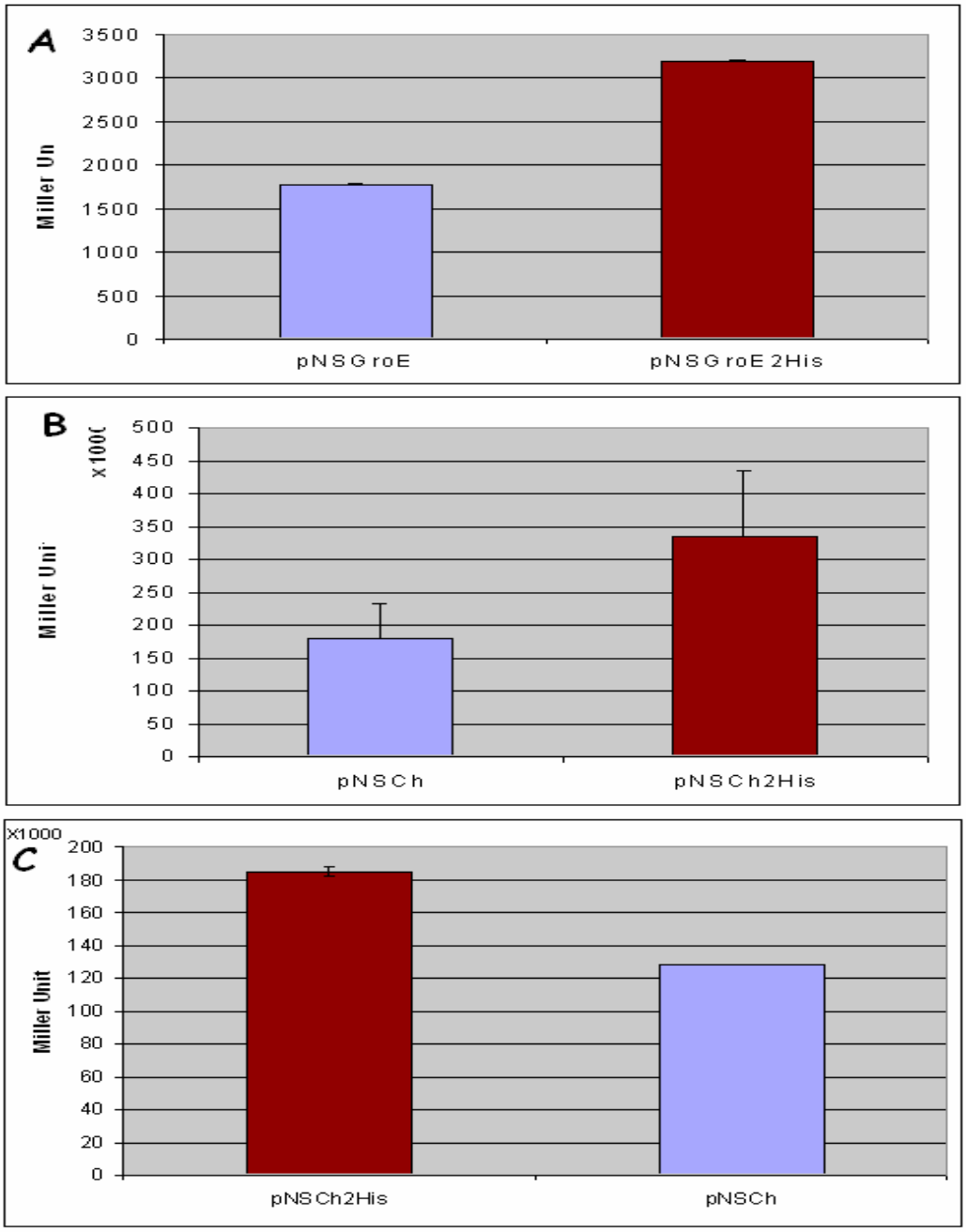

Figure 3-1-4

LacZ activity of each construct was determined as described under expression of β-galactosidase (*lacZ*) and represented as Miller unit. The activity represents the average of three cultures. A: *E. coli* BL21, B: *E. coli* Mach1, C: *S. choleraesuis* wild type.

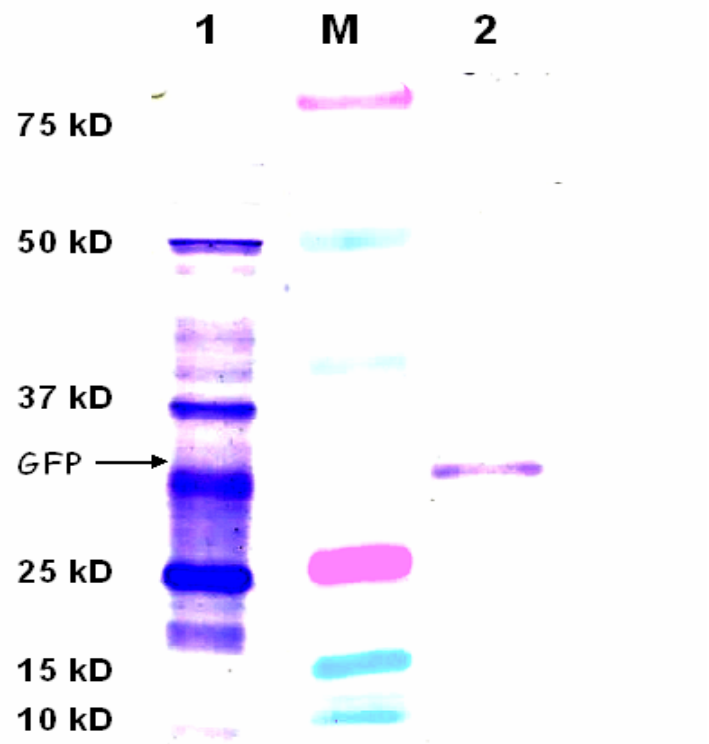

Figure 3-1-5.
SDS-PAGE of purified recombinant protein from strain RB51 stained with Coomassie blue, lane 1; total cell lysate of recombinant strain RB51. Lane 2; 20 µg of purified recombinant *gfp* with double His-tag fusion. M; Precision Plus Protein™ Dual Color Standards (Bio-Rad)

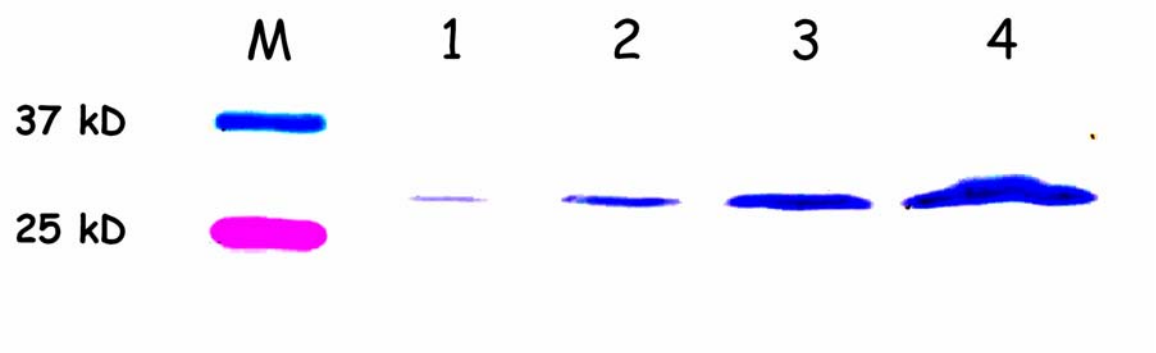

Figure 3-1-6.
Western Blot of total cell lysate and 20 µg purified recombinant *gfp* protein from *Brucella*. The membrane was incubated overnight with Anti-HisG-HRP antibody (1:2000) M; Precision Plus Protein™ Dual Color Standards (Bio-Rad) lane1; total cell lysates of strain RB51 single His-tag fusion. Lane 2; total cell lysates of RB51 double His-tag fusion. Lane 3; 20 µg purified *gfp* protein from RB51 with single His-tag fusion. 4; 20 µg purified *gfp* protein from strain RB51 with double His-tag fusion.

Chapter 3

Enhancement of gene expression in *Brucella* and *Ochrobactrum*

Chapter 3-2

High-level heterologous gene expression in *Ochrobactrum anthropi* using an A-rich UP element

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MOHAMED SELEEM [1], MOHAMMED ALI[2], M.W. ABD AL-AZEEM [3], STEPHEN M. BOYLE [1], AND NAMMALWAR SRIRANGANATHAN [1*]. [1]Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic and State University, Blacksburg, VA 24061, [2] Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Assiut University, Egypt. [3] Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt.

Abstract

DNA regions that flank a gene's promoter play an important role in determining transcription efficiency by interacting with the carboxy-terminal domain (CTD) of RNA polymerase α-subunit. We placed an adenine (A)-rich upstream element (UP) between –38 and –59 of the core *trc* promoter to enhance gene expression in *Ochrobactrum anthropi* up to 66-fold. The high level of expression achieved by the UP element and the N-terminus fusion of a 6xHis epitope tag facilitated detection and purification of heterologous proteins directly from *O. anthropi*.

Introduction

Ochrobactrum anthropi is a Gram negative bacterium that constitutes up to 2% of the cultivable bacteria from soil (Lebuhn et al., 2000). In recent decades, *O. anthropi* has become a source of interesting biocatalysts like D-aminopeptidase (Asano et al., 1989b) L-aminopeptidase (DmpA) (Fanuel et al., 1999), the D-amino acid amidase (Asano et al., 1989a) and the L-amidase (Sonke et al., 2005). The species is gaining recognition due to its capability of degrading organophosphorus pesticides (parathion and methylparathion), antibiotics, herbicides, hemicellulose, anthracene, and other complex organic molecules including those found in crude oil (Lechner et al., 1995, Asano et al., 1992, Asano et al., 1989a, Christenson et al., 1997, Gard et al., 1997, Laura et al., 1996, Schafer et al., 1996, Yu et al., 1998). Unfortunately, genetic studies of *O. anthropi* are hampered by the lack of suitable system that allows the expression and purification of recombinant protein. In this study, we have exploited the transcriptional activity of the *trc* hybrid promoter (Amann et al., 1983) in *O. anthropi* and demonstrated that the UP (upstream) element from an *Escherichia coli* ribosomal RNA operon (*rrnD*) (Estrem et al., 1998) can enhance the expression of *trc* promoter up to 66-fold. We have also shown that with the enhanced level of expression and the N-terminus His-tag fusion, recombinant proteins can be expressed, detected and purified directly from *O. anthropi* without using *E. coli* as an expression host.

Materials and methods

The polymerase chain reaction (PCR) was performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from QIAGEN was used for all plasmid extraction and QIAGEN PCR cleanup kit was used for all restriction enzymes removal and DNA extraction from gel.

Vector construction

The *trc* promoter was amplified from pTrc2HisA vector (Invitrogen) in two steps using primers Trc-F and Trc-R1 (Table 3-2-1) in the first step. The PCR amplicon DNA was gel purified and used as a template for the second PCR cycle. In the reverse primer

Trc-R2 (Table 3-2-1), six histidine and one glycine residues were engineered after the translational start codon to facilitate the epitope tagging at the amino terminus by adding minimum number of amino acids fused to any expressed protein (Seleem et al., 2004). In order to avoid the translational error or inhibition arising from rare codon bias (Makrides, 1996) the preferred codon for histidine (CAT) in *O. anthropi* (http://www.kazusa.or.jp) was used.

The *Brucella abortus gro*E promoter from the pNSGroE broad host range expression vector (Seleem et al., 2004) was excised with *Sal*I and *Bam*HI restriction enzymes and the *trc* promoter purified and cloned into the pNS construct to form the pNSTrc expression vector (Genbank accession number DQ412056).

Adding Up-stream element (UP element)

To add the A+T rich UP element sequence named 4192 (TrcB) and the A-rich sequence *rrn*D UP element (Estrem et al., 1998) (TrcD) (Figure 3-2-1), upstream of the –35 hexamer of *trc* promoter between –38 and –59 , primers were designed (Table 3-2-1) to amplify TrcB and TrcD promoters. The two promoters were cloned into pNS as mentioned above and the two new constructs were designated as pNSTrcB (GenBank accession number DQ412057) and pNSTrcD (GenBank accession number DQ412056) (Figure 3-2-2). The three vectors were sequenced and the incorporation of the UP element upstream of the *trc* promoter was confirmed.

Expression of β-galactosidase (LacZ):

In order to study the expression and activity of the cloned promoters inside *O. anthropi*, the promoter-less *E. coli* β-galactosidase (*lacZ*) gene was amplified from pRSETB/β-gal vector (Invitrogen) using forward and reverse primers (Table 3-2-1) that have *Bam*HI and *Xba*I sites for in-frame directional cloning. After restriction digestion and purification, the amplified *lacZ* gene was cloned into the 3 expression vectors and transformed into Mach1™ T1 Phage-Resistant (T1R) competent *E. coli* (Invitrogen). Black-colored recombinant *E. coli* colonies expressing β-galactosidase were selected on S-Gal 30µg/ml Cm plates (Sigma).

To investigate whether the UP element alone can act as a promoter, we incorporated the A-rich sequence *rrn*D upstream of the promoterless *lacZ* gene using

primers lacZ-UP-F and lacZ-R (Table 3-2-1). After restriction digestion and purification, the amplified product was cloned into the pNS construct as mentioned above.

GFP constructs:

A promoterless Green Fluorescence Protein gene (*gfp*) was excised from pGFPuv vector (BD Biosciences Clontech) and cloned in frame down stream of the TrcD promoter in the multiple cloning site of the pNSTrcD vector and used for Western blot and protein purification.

Transformation of Ochrobactrum:

The plasmids (pNSTrc, pNSTrcB and pNSTrcD) containing *lacZ* or pNSTrcD-*gfp* were transformed into *O. anthropi* by electrotransformation with a Gene Pulser (BTX) set at 2.7 KV, 25 µF and 200 Ω. The transformation mixture was diluted and plated on several TSA plates containing 30µg/ml Cm. Colonies growing on the plates after 36h were checked for the presence of the plasmid, by plasmid extraction and PCR (data not shown). The recombinants were evaluated using a previously described procedure for *Brucella spp*. (Vemulapalli et al., 2000) to determine the levels of β-galactosidase expression in *O. anthropi*.

Western blot:

To test the fusion of the His-epitope tagging and efficiency of detection, Western blot analysis was performed by loading the total cell lyates of recombinant *O. anthropi* expressing *gfp* under TrcD promoter into a 12% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated overnight with Anti-His6 antibody (Roche) (1:1000) and Anti-mouse-HRP secondary antibodies (1:1000) (KPL) for 1 hour.

Protein Purification:

The *gfp* was purified from recombinant *O. anthropi* using Ni-NTA Agarose (Qiagen) and 6M Guanidine-HCl according to the manufacturer's instructions. 20mM imidazole and 20mM ß-mercaptoethanol were added to the lysis buffer, and 1% TritonX-100 and 250mM NaCl were added to the washing buffer to reduce contaminating proteins. The concentration of the purified recombinant *gfp* protein was determined by BCA Protein Assay kit (Pierce) using the manufacturer's enhanced test tube procedure

after removal of Guanidine-HCl by Microcon centrifugal filter devices YM-10 (Millipore). The purity of the extracted protein was determined by SDS-PAGE.

Results

We placed an adenine (A)-rich and A+T rich upstream element (UP) (Estrem et al., 1998) between – 38 and –59 of the core *trc* promoter (Figure 1). The 'A tract' sequence (TrcD) had a positive effect on core *trc* promoter activity in *O. anthropi*, when positioned at – 38, increasing transcription 66-fold (19,658 ± 2,500 Miller unit) over the core *trc* promoter (298 ± 4 Miller unit). There was no significant increase using A+T rich TrcB promoter (319 ± 54 Miller unit). The 'A tract' sequence alone without the *trc* promoter, resulted in an increase of the *lacZ* expression (472± 97 Miller unit) over core *trc* promoter. To test the fusion with respect to epitope tagging and efficiency of detection, *gfp* was cloned downstream of the TrcD promoter. Figure 3-2-3 shows the detection of the expressed fusion protein in *O. anthropi* with Anti-His antibodies. We were able to highly purify 3.158 mg of recombinant *gfp* using Ni-NTA Agarose (Qiagen) and 6M Guanidine-HCl from 200 ml of (OD_{600} = 2) bacterial culture (Figure 3-2-4).

Discussion

The 'A tract' sequence (TrcD) had a positive effect on core *trc* promoter activity in *O. anthropi*, when positioned at – 38, increasing transcription 66-fold over the core *trc* promoter. This enhancement appears to be due to ability of the 'A-tract' to provide binding site(s) for the RNA polymerase that contributes to the wrapping of the promoter DNA around the RNA polymerase (Aiyar et al., 1998). When cloned upstream of the promoterless *LacZ* gene the 'A tract' sequence alone without the *trc* promoter, resulted in an increase of the *lacZ* expression over core *trc* promoter; however, the increase wasn't significant compared to the combined UP element and *trc* promoter. This result excludes the possibility that the 'A tract' sequence alone can act as a separate strong promoter.

Figure 3-2-3 shows the usefulness of the His-tagged fusion protein expression in *O. anthropi* by demonstrating the specificity, sensitivity and ease of detection of the expressed fusion protein with Anti-His antibodies.

The enhanced level of expression of the UP element and the *trc* promoter was strong enough to allow purification of recombinant proteins expressed under TrcD promoter from recombinant *O. anthropi* by nickel chelate affinity chromatography.

The His-tag can be exploited for affinity purification as well as for post transitional modification studies of proteins expressed in *O. anthropi* and other related species (Seleem et al., 2004), without the need for *E. coli* as an expression host (Higgins et al., 2001, Ogawa et al., 2001). Importantly, the high level of protein expression achieved in *O. anthropi* by using UP element did not alter the stability of the plasmid or the growth characteristic of *O. anthropi* compared to the wild type strain.

In summary, we used the upstream sequence that has A-tract to enhance the *trc* promoter activity up to 66-fold in *O. anthropi*. The His-tag fusion was designed post-translational start codon to facilitate minimum epitope fusion at the N-terminus of any expressed proteins using pNSTrcD vector. This method will facilitate the purification of important pharmaceutical enzymes and recombinant proteins directly from *O. anthropi.* Also, it can be used to enhance this soil bacterium's degrading capabilities by over-expression of genes involved in important biochemical pathways associated in biodegradation. This will facilitate the use of *O. anthropi* as a potential bioremediation tool and a biopesticide agent.

Acknowledgement:

We express our appreciation to Dr. Tim Larson of Virginia Tech for his critical reading of the manuscript and suggestion. We would like to thank Mrs. Kay Carlson of Virginia tech for providing us with *O. anthropi* strain and the support through out the experiment.

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Table 3-2-1 PCR Primers used in the amplification

Primer Name	Product Name	Size in bp	Primer sequence
Trc- F			5`- CCC**GTCGAC**AAATATTCTTGAAATGAGCTGT-3`
Trc- R1	Trc	272	5`- CCC**CATGGT**TTATTCCTCCTTATTTAAT-3`
Trc-R2			5`- CCC**GGATCC**ACC**ATGATGATGATGATGATG**CAT GGTTTATTCCTCCTTATTTAAT-3`
TrcB- F	TrcB	278	5`- CCC**GTCGAC**GGAAAATTTTTTTTCAAAAGTACTT GACAATTAATCATCCGGCTCGTATAATG-3`
TrcD-F	TrcD	277	5`- CCC**GTCGAC**AGAAAAAAGATCAAAAAAATACTTG ACAATTAATCATCCGGCTCGTATAATG-3`
LacZ/F	LacZ	3073	5`- CCC**GGATCC**GATCCCGTCGTTTTACAAGGTGGT-3`
LacZ/R			5`- CCC**TCTAGA**ATTTTTGACACCAGACCAACTG-3`
LacZ-UP	LacZ-UP	3095	5`- CCC**GTCGAC**CAGAAAAAAGATCAAAAAAATATGAT AGAT CCCGTCGTTTTACAA-3`

Restriction sites are shown in bold font.
The His-tag fusion sequence is shown in bold font and underlined.

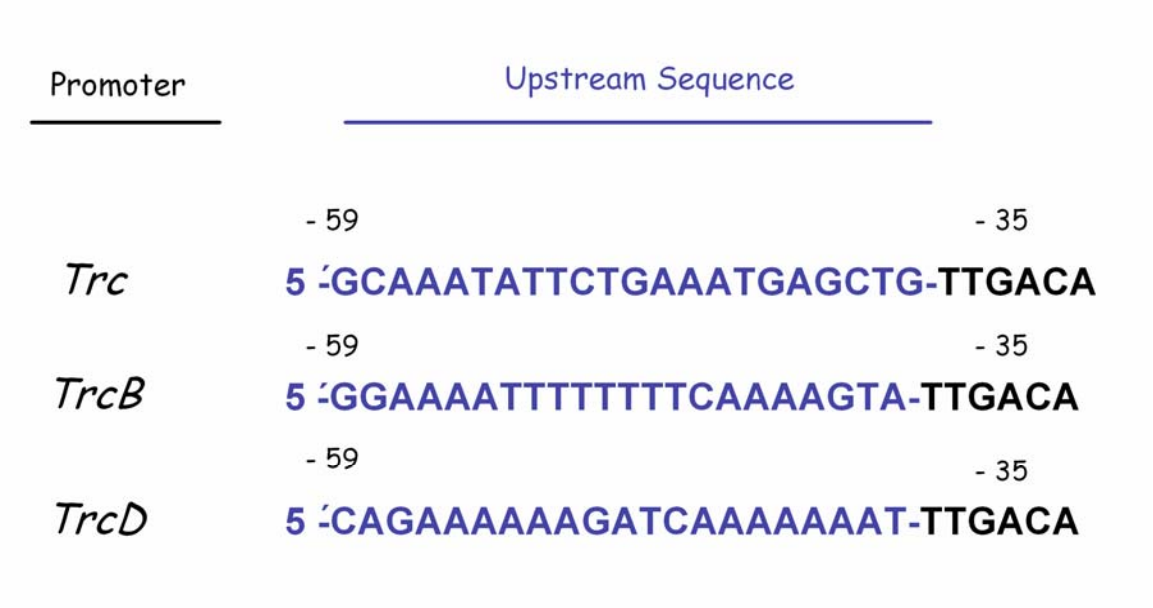

Figure 3-2-1. The organization and sequence of the UP element that was added between -38 and -59 of the core *trc* promoter. TrcD has an A-rich tract that enhanced expression of core *trc* promoter by 66-fold.

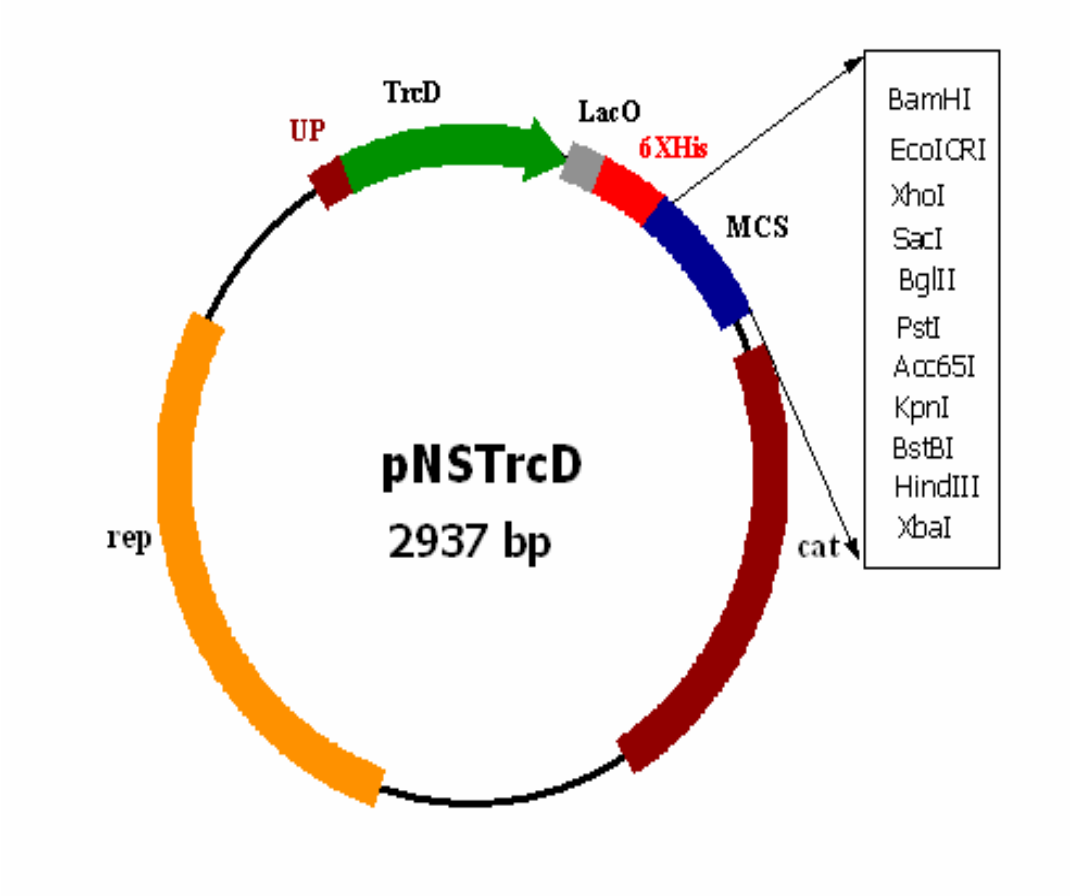

Figure 3-2-2. **Plasmid Map**

The engineered pNSTrcD expression vector with the UP element and the His-tag fusion in the N-terminus. MCS, multiple cloning site; *cat*, cholramphenicol acetyltransferase gene conferring cholramphenicol resistance; rep, gene required for plasmid replication; 6Xhis, His tag fusion; LacO, lac operator for regulated expression.

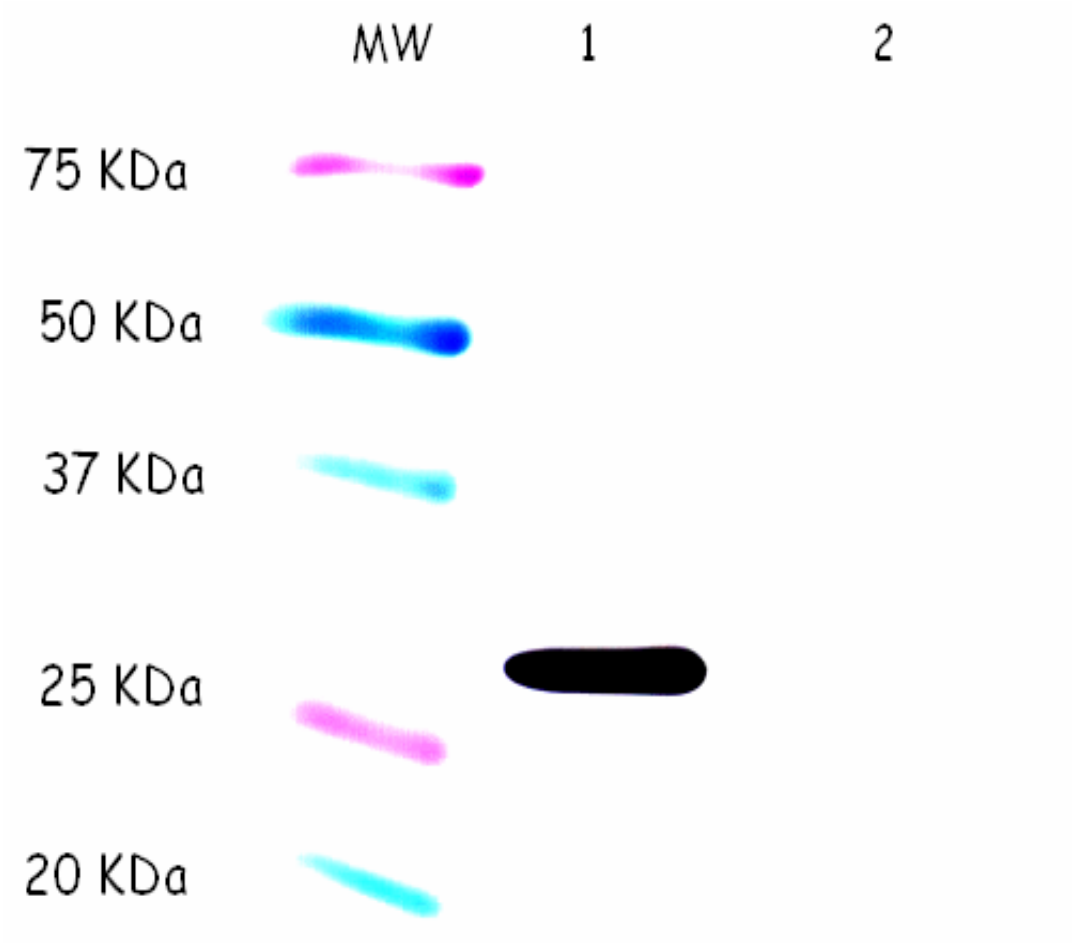

Figure 3-2-3. **Western blot of recombinant *gfp***

Detection of the His-tagged fusion *gfp* protein expressed under TrcD promoter in *O. anthropi*. Membrane was incubated with Anti-His6 antibody (1:1000) overnight and Anti-mouse-HRP secondary antibodies (1:1000) for 1 hour. Lane 1, *O. anthropi* expressing *gfp* under TrcD promoter; lane 2, negative control. MW, Precision Plus Protein™ Dual Color Standards (Bio-Rad)

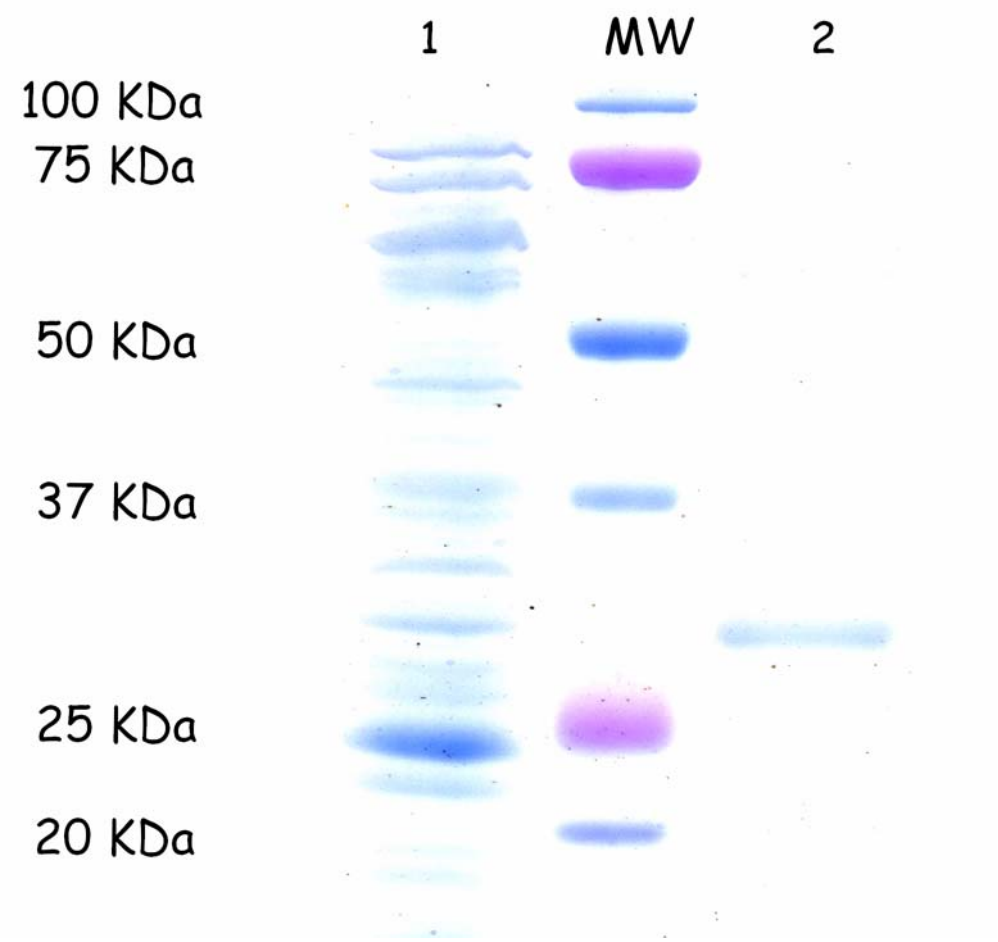

Figure 3-2-4. **SDS-PAGE of purified recombinant protein**

SDS-PAGE of purified recombinant *gfp* protein from *O. anthropi* using Ni-NTA agarose (Qiagen) and 6M Guanidine-HCl, stained with Coomassie blue, lane 1; total cell lysate of recombinant *O. anthropi*; lane 2, purified recombinant *gfp;* MW, Precision Plus Protein™ Dual Color Standards (Bio-Rad)

Chapter 3

Enhancement of gene expression in *Brucella* and *Ochrobactrum*

Chapter 3-3

Simple method for transformation of *Ochrobactrum anthropi*

MOHAMED SELEEM, STEPHEN M. BOYLE AND NAMMALWAR SRIRANGANATHAN Under review Journal of Microbiological Methods

Abstract

A rapid and simple method for preparation of highly efficient *Ochrobactrum anthropi* electrocompetent cells has been developed. The efficiency of transformation increased 100-fold when the cells were prepared from liquid culture compared to agar plates. The electrocompetent *O. anthropi* prepared by this method were 50-fold more efficient than commercial sources of competent *Escherichia coli*.

Introduction

Ochrobactrum anthropi is a Gram negative bacterium that constitute up to 2% of the cultivable bacteria from soil (Lebuhn et al., 2000). In recent decades, *O. anthropi* has become a source of useful enzymes including D-aminopeptidase (Asano et al., 1989b), L-aminopeptidase (DmpA) (Fanuel et al., 1999), and the D-amino acid amidase (Asano et al., 1989a) and the L-amidase (Sonke et al., 2005). From a biotechnological point of view, *Ochrobactrum* strains are of particular interest for bioremediation (Lebuhn et al., 2005). These strains are capable of degrading organophosphorus pesticides such as parathion and methylparathion (Zhang et al., 2005), phenol (El-Sayed et al., 2003), the toxic organic solvent DMF (Veeranagouda et al., 2005), petroleum waste (Katsivela et al., 2005) and soil contaminants like chlorothalonil (Kim et al., 2004). To date, genetic manipulation of *Ochrobactrum* spp. has been limited due to the inefficiency of DNA transformation and the lack of an efficient stably replicating vector. Transformation of *O. anthropi* using the two methods described for related species *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* (Holsters et al., 1978) weren't successful (unpublished data). Therefore, the goal of this study was to establish a rapid and simple method for preparation of highly electrocompetent cells that could be utilized for a variety of genetic procedures.

Materials and Methods

O. anthropi strain 49237 was used for preparation of the electrocompetent cells. Low speed centrifugation was done at 4°C for 15 minutes at 3500 *5 g*. Filter sterilized ice-cold 10% glycerol was used for all washing process.

To prepare *O. anthropi* competent cells, we used two different methods; A) 200 µl of *O. anthropi* culture at an OD_{600}=1 was spread on five Tryptic Soy Agar (TSA) plates (Difco), and incubated for 20 hours at 30°C. The bacterial cells were collected by scraping from the plates into 25ml ice-cold 10% glycerol and kept on ice for 30 minutes. The cells were harvested by centrifugation and washed twice with 25 ml ice-cold 10% glycerol. The cells were resuspended to a density of approximately 10^{11} cells/ml in ice-

cold 10% glycerol using approximately 3 ml 10% glycerol for the five plates (preparation 1). Preparation one was used as is for transformation or diluted further with the same volume of ice-cold 10% glycerol to obtain $5x10^{10}$ cells/ml (preparation 2) or with four volumes of ice-cold 10% glycerol to obtain $2x10^{10}$ cells/ml (preparation 3). B) For the second method, *O. anthropi* was grown in 400 ml of filter sterilized YENB salt free medium (0.75% Bacto Yeast extract and 0.8% Nutrient Broth) at 30 °C with shaking at 200 rpm. When the cells reached density of OD_{600}= 0.6 they were placed on ice for 30 minutes then collected by centrifugation. The cells were washed twice with 25 ml ice-cold 10% glycerol and resuspended to a density of approximately 10^{11} cells/ml in ice-cold 10% glycerol (approximately 1 ml 10% glycerol for the 400 ml culture) (preparation 4).

To test the efficiency of transformation of the electrocompetent *O. anthropi*, 50 µl from each preparation (1, 2, 3 and 4) were mixed gently with approximately 100 ng of pNSGroE *Brucella* expression vector (Seleem et al., 2004). The DNA was transformed into *O. anthropi* by electroporation with a Gene Pulser T-100 (BTX) set at 10 msec pulse length and field strength of 2.7 kV/cm using prechilled 1mm gap cuvettes (Eppendorf). Immediately after electroporation, 500 µl of S.O.C medium (Invitrogen) were added to the cuvettes and the cells were transferred into 50ml tubes and incubated at 30°C, with shaking at 200 rpm, for 2 h to allow the expression of antibiotic resistance gene. The transformation mixture was diluted and plated on several TSA plates containing 30µg/ml Cm. Colonies growing on the plates after 36h were checked for the presence of the plasmid, by plasmid extraction (QIAprep Spin Miniprep Kit, QIAGEN) and PCR (Platinum PCR SuperMix High Fidelity, Invitrogen).

Results and Discussion

Table 3-3-1 shows the transformation efficiency for all preparations. Although the highest transformation efficiency of (2.1×10^9 transformants per microgram of DNA) was obtained when the cells were grown in liquid media at the early growth phase (preparation 4), cells that were collected from stationary growth phase (preparation 2) exhibited an efficiency of (4.7×10^7 transformants per microgram of DNA). Although preparation 4 had the lowest transformation efficiency, it provided enough cells for at

least 300 transformation reactions with efficiency of (5 x 10^5 transformants per microgram of DNA).

We tested the ability of the electrocompetent *O. anthropi* to be transformed with ligation reaction in comparison to commercial chemical and electrocompetent *E. coli*. Standard ligation reactions were carried out as described before (Sambrook J, 1989) using pBBR1MCS broad host range cloning vector (Kovach et al., 1994) and four genes of different sizes from *Brucella suis* strain 1330 listed in Table 3-3-2. The same volume of the ligation reactions were used for the transformation of *O. anthropi* as described above and Mach1™ T1 Phage-Resistant (T1R) chemical competent *E. coli* (Invitrogen) or electrocompetent GeneHogs™ (Invitrogen) for Cyclic-β 1-2 glucan synthetase gene according to the manufacturer's instructions. The transformation reactions were spread on several TSA plates containing 30µg/ml Cm and colonies were counted and screened for presence of the plasmid and insert by plasmid extraction, restriction digestion and PCR (Figure 3-3-1). As seen in Table 2, *O. anthropi* were more efficient than commercial competent *E. coli* for transformation using the ligation reaction. The efficiency of transformation of *O. anthropi* compared to *E. coli* seemed to increase with increased size of the cloned insert. The efficiency of *O. anthropi* was 38-fold higher than commercial *E. coli* (Mach1) chemical competent cells and up to 50-fold higher than commercial *E. coli* (Genehogs) electrocompetent cells (Table 3-3-2).

We applied the same methods described above for preparation of electrocompetent cells of *Brucella, Salmonella and Pasteurella.* Table 3-3-3 shows the transformation efficiency for each species. For *Salmonella and Brucella,* tryptic soy broth (TSB) was used instead of YENB free salt medium and 2 washing steps with 10% glycerol was enough to remove the salts. Storage of the competent cells at -80°C prepared by this method did not alter transformation efficiency in cells stored over 2 years. The protocol applied here simplified the procedures for preparation and transformation of *O. anthropi* and other species that don't require any chemicals or enzymes to be added to the procedure. Coupled with efficient expression system this rapid technique will facilitate the use of *O. anthropi* as a bioremediation tool and as a biopesticide agent.

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Table 3-3-1. Efficiency of transformation of electrocompetent *O. anthropi* prepared by different methods

Preparation Method	**CFU/μg of DNA**	**No of the transformation reactions prepared**
1	9.5×10^6	60
2	4.7×10^7	120
3	5×10^5	300
4	2.1×10^9	20

Table 3-3-2. Efficiency of transformation of ligation reaction

Gene name*	**Size in bp**	**No of colonies *E. coli***	**No of colonies *O. anthropi***	**Fold increase**
sodC	522	75	693	9
ahpD	528	27	231	9
ahpD/C	1162	9	341	38
Cyclic-β 1-2 glucan synthetase	8199	4	198	50

* *sodC*; Copper/Zinc superoxide dismutase, *ahpD*; Alkylhydroperoxidase-D, *ahpD/C*; Alkylhydroperoxidase operon (C &D),

Table 3-3-3. Efficiency of transformation of different bacterial species

Bacterial strain	**Preparation method**	**Transformation efficiency CFU/μg of DNA**
Salmonella choleraesuis	4	1.2×10^8
Pasteurella multocida	1	4.5×10^4
Brucella abortus	4	2.2×10^8

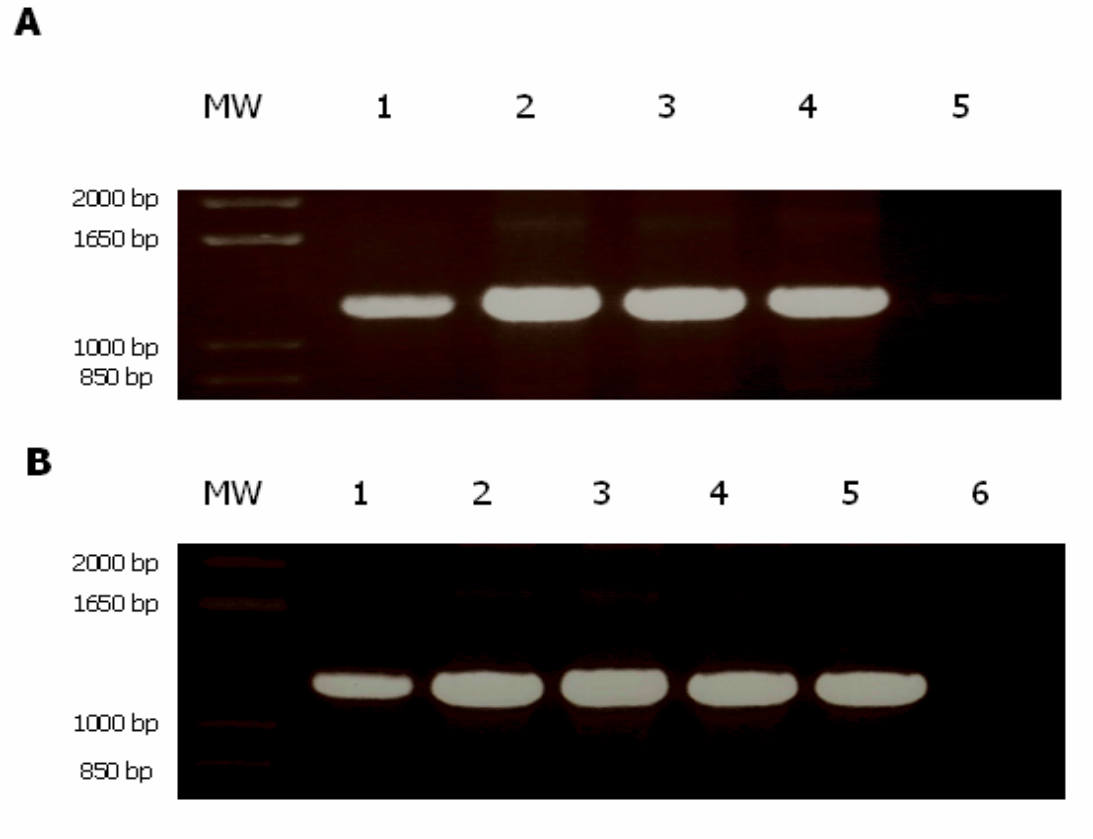

Figure 3-3-1. PCR screening of AphD/C gene that was ligated with pBBR1MCS vector and transformed into *E. coli* and *O. anthropi*. A; *E. coli* Mach 1, lane 1 positive control (*B. suis* DNA), lanes 2-4, transformants, lane 5, negative control (*E. coli* DNA no plasmid); B; *O. anthropi* , lane 1 positive control (*B. suis* DNA), lanes 2-5, transformants, lane 6, negative control (*O. anthropi* DNA no plasmid)