USING BACTERIAL LUCIFERASES AS REPORTER TO SCREEN *BRUCELLA ABORTUS* PROMOTERS

by

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USING BACTERIAL LUCIFERASES AS REPORTER TO SCREEN BRUCELLA ABORTUS PROMOTERS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

AA	amino acid
bp	base pair
cm	centimeter
CTAB	cetyltrimethylammonium bromide
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
FMN	flavin mononucleotide, oxidized form
$FMNH_2$	flavin mononucleotide, reduced form
Fp	flavin protein, oxidized form
FpH	flavin protein, reduced form
hr	hour
HtrA	high-temperature-requirement A protein
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kb	kilobase
kDa	kilodalton
kV	kilovolt
LB	Luria-Bertani broth
M	molar

mbp	million base pair
µl	microliter
µM	micromolar
min	minute
ml	milliliter
mm	millimeter
msec	millisecond
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
SOD	superoxide dismutase
TB	terrific broth
TE	Tris-EDTA
YENB	yeast extract and nutrient broth

Chapter 1

Introduction

Brucella abortus

Brucella abortus, a facultative intracellular pathogen causing bovine brucellosis as well as undulant fever in humans (1), belongs to Eubacteria; Proteobacteria; alpha subdivision; Rhizobiaceae; Brucella. It is a Gram-negative; non-motile; non-sporulating; non-encapsulated coccus, coccobacillus, or short rod; 0.6-1.5 mm in length and 0.5-0.7 mm in width. Most strains are fastidious and slow-growing and require 5-10% CO_2 at an optimal growth temperature of 36-38 °C (1).

The *B. abortus* genome has two chromosomes of 2.1 and 1.5 mbp with 58-59% GC (2). Both replicons encode essential metabolic and replicative functions (3, 4).

Like other pathogens, *B. abortus* is able to adapt to very different environments. Outside the host, it can survive for two to three months in wet soil, one to two months in dry soil, three to four months in feces, and up to eight months in liquid-manure tanks or in an aborted fetus in the shade. After entering the body through cuts and abrasions in the oral mucosa, nasopharynx, conjunctivae, genitalia or even unbroken skin, *B. abortus* specifically invades cells with ectodermal and mesodermal origin. It can be phagocytosed by neutrophils and macrophages and then carried to the regional lymph nodes. Multiplication of *B. abortus* within lymph nodes causes a lymphadenitis and then a bacteremia. The bacteremia can resolve itself or be recurrent for at least two years in some animals. During the bacteremia, *B. abortus* can be carried intracellularly in neutrophils and macrophages or free in plasma and locates in various organs, especially the gravid uterus, udder, and supramammary lymph nodes. Localization of infection in

the endometrium of the gravid uterus and in fetal membranes of cattle can extend to the placental stroma, blood vessels, and ultimately to the fetus and result in abortion, premature birth, or the birth of a viable or nonviable calf depending on the severity of placentitis. After the abortion or parturition, the uterus can be cleared of *B. abortus* within a few weeks until next outburst (1).

The capability to survive and propagate inside the phagocytic cells is essential to the virulence of *B. abortus*. The critical step of intracellular survival of *B. abortus* is to avoid fusion with lysosomes (5). Also *B. abortus* inhibits degranulation (6) and the oxidative burst in neutrophils (7) as well as prevent phagolysosomal fusion in macrophage (8), More studies are needed to understand its virulence factors and the components contribute to its intercellular survival.

Since *B. abortus* does not express pili, fimbriea or capsular material, the only two components of the outer membrane, the lipopolysaccharide and the outer membrane proteins, can be potential structural virulence factors. The association of lipopolysaccharide and intracellular survival and virulence (9-11), the toxicity of the lipid A portion of lipopolysaccharide (12-14), and the protective immunity stimulated by the O-polysaccharide portion of lipopolysaccharide (15, 16) suggest that it is one of the virulence factors. The outer membrane proteins of *B. abortus* have been identified into 3 major groups. Group 1 is a lipoprotein covalently linked to peptidoglycan (17) with molecular weight of 88 to 94 kDa; group 2 proteins, Omp2a and Omp2b, have porin activity with molecular weight of 36 to 38 kDa (18, 19); and group 3 protein is the 25 to 27 kDa Omp25 (20). But the immunological protective activity of group 2 and group 3 proteins is low (21).

Besides the structural virulence factors, other genes contribute to the intracellular survival and virulence. In *Nocardia asteroides* (22) and *Shigella flexneri* (23), superoxide dismutase can be protective by detoxifying the reactive microbicidal oxygen intermediates such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) produced by mononuclear phagocytes. Two distinct types of superoxide dismutases (24), a periplasmic Cu-Zn SOD (25) and a cytosolic Mn SOD, have been found in *B. abortus*. The Cu-Zn SOD helps infection but not persistence of *B. abortus* in mice (26). It is down-regulated within the macrophage (27). Catalase is another antioxidant enzyme found in *B. abortus* and its activity is correlated with virulence (28). Its periplasmic location draws the consideration that it functions to detoxify exogenous oxygen intermediates instead of those endogenously produced by respiration (29). Study of a catalase mutant suggests that it might play a role in the survival of *B. abortus* within activated macrophages but that it is not active enough to produce an intracellular clearance (30). RecA, the DNA recombination-repairing enzyme which contributes to the virulence of *Vibrio cholerae* (31), *Neisseria gonorrhoeae* (32), and *Salmonella typhimurium* (33), contributes to the survival of *B. abortus* in DNA damaging environment but is not crucial for the persistence (34). *B. abortus* can inhibit phagolysosome fusion, degranulation, activation of the myeloperoxidase-H_2O_2-halide system, and production of tumor necrosis factor by releasing adenosine and guanine monophosphate (6, 35) which involves *purE* gene. Also, *B. abortus purE* mutant has reduced intracellular growth (Essenberg, R. C., unpublished observation) similar to what has been found on *B. melitensis* (36). High-temperature-requirement A protein (HtrA) is a heat shock-induced serine protease functioning to degrade misfolded proteins in the periplasm (37) and plays a major role in the virulence

of *S. typhimurium* (38). Though the *B. abortus htrA* and *htrA*-like mutants are less resistant to oxidative killing, they are able to recover in 3 weeks post infection in mice and grow to an even higher spleenic level than the virulent strain 2308 in 60 days (39). It is down-regulated in macrophage (27). GroEL, another heat shock protein, correlates with survival in macrophage (40). An extracellular substance with sulfhydryl protease activity cleaves immunoglobulin G1 in whey (41), which probably helps the extracellular survival of *B. abortus* and persistence within the mammary gland (42). 2, 3-Dihydroxybenzoic acid, a siderophore produced by *B. abortus*, can prevent killing by macrophages (43).

Two-component regulatory systems consisting of a sensor and response regulator components are involved in environmental sensing and adaptive responses in bacteria. They also contribute to the virulence of many pathogens. Recently, the FeuPQ two-component system involving in the regulation of iron uptake has been cloned from *B. suis*. But the *feuP* isogenic mutant had no effect on virulence (44).

Erythritol, a four carbon polyol found elevated in the placentas and fetal fluids of pregnant cattle, is stimulatory to the growth of *B. abortus* and perhaps contributes to its tissue localization (45-48). The capacity of using erythritol as carbon source is uncommon in prokaryotes (42). The metabolic pathway of erythritol (Figure 1) is coupled with the electron transport system and requires reduced glutathione for activity (49). *B. abortus* 19 which has the *ery* locus deleted and is thus unable to metabolize erythritol has been used as vaccine for a long time. Also, *B. abortus* 19 was unable to prevent lysosome fusion and had reduced intracellular survival (5). Though pathogenic erythritol-negative *Brucella* strains have been identified (50), the relation between erythritol metabolism and virulence is still under investigation.

B. abortus, lacking fructose 6-phosphate kinase and fructose 1,6-diphosphate aldolase which function in glycolysis (51), catabolizes glucose via the hexose monophosphate pathway in conjunction with the citric acid cycle (52). It has an UDP-glucose 4' epimerase which allows it to utilize galactose (53).

Bacterial luciferases

Bioluminescent bacteria primarily live in the ocean as well as terrestrial habitats and freshwater as free-living or symbiotic species. Luciferases from these bacteria are heterodimeric flavin monooxygenases catalyzing the light-emitting reaction:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + h\nu \ (\lambda = 490nm)$$

where R is a straight-chain aliphatic moiety containing seven or more carbon atoms. There are significant advantages to the use of luciferase as a reporter gene. First, measuring gene expression with light is unparalleled both in sensitivity and linearity over many orders of magnitude. Second, the assay is nontoxic; light production can measured from the same living cells throughout an experiment. Third, it works well during stationary phase when other reporter gene products are masked by the presence of antibiotic and spore pigments. Finally, it can localize expression in space as well as in time (54).

Among the bacterial luciferases, those from *V. harveyi*, *V. fischeri*, *Photobacterium phosphoreum*, and *Xenorhabdus luminescens* have been cloned and sequenced (55-59). The high resolution crystal structure of the *V. harveyi* luciferase has been solved (60). But the mechanism by which the enzyme works is not completely solved yet.

Plasmid pBBR1

There are no native plasmids found in *B. abortus* to date. But pBBR1 from *Bordetella bronchiseptica* was reported to be able to stably replicate within *B. abortus* (61). It is a 2687 bp circular double strand DNA and contains a *mob* gene for mobilization and a *rep* gene for replication. These two genes lie in opposite orientations with the replication origin between their 5' ends. It maintains about 50 copies per cell in bacteria.

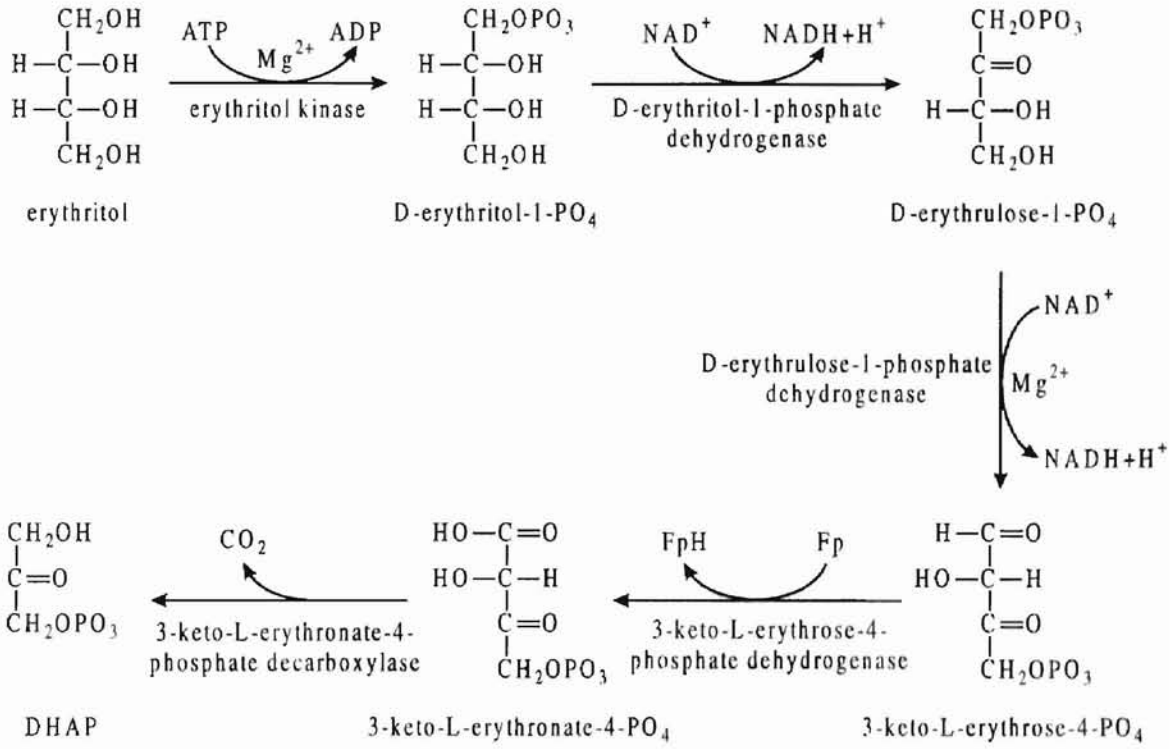

Figure 1. Erythritol catabolism by *B. abortus*. 3-keto-L-erythrose-4-phosphate dehydrogenase is membrane-bounded and catalyzes the rate-limiting step of the pathway.

Chapter 2

Materials and Methods

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1 and Table 2. The media for growing *Escherichia coli* and *B. abortus* strains are listed in Table 3. *E. coli* strains were routinely cultured at 37 °C overnight. *B. abortus* were grown at 37 °C under an atmosphere containing 5% CO_2 for 2 to 5 days. For *E. coli* strains, 50 $\mu g \bullet ml^{-1}$ ampicillin was added as required, while 100 $\mu g \bullet ml^{-1}$ ampicillin was used for *B. abortus*.

Preparation and manipulation of DNA

Plasmid DNA was purified by Wizard® *Plus* Minipreps DNA Purification Systems (Promega Co., Madison, WI), QIAprep Spin Miniprep Kits (QIAGEN Inc., Santa Clarita, CA), or the alkaline-SDS method (62) with slight modification: the mixture of diatom and cell lysate was applied to recycled Wizard® Minipreps columns followed by washing with diatom wash (50% ethanol; 200 mM NaCl; 10 mM EDTA; 50 mM Tris-HCl, pH 7.4) and elution with H_2O. The Wizard® Minipreps columns were recycled by washing with H_2O, soaking in 0.1 M HCl overnight, rinsing with H_2O, and autoclaving. Genomic DNA of *B. abortus* 2308 was prepared by the CTAB method as described in Current Protocols in Molecular Biology (63). Restriction endonucleases, exonuclease III, S1 nuclease, Klenow enzyme, Taq polymerase, calf intestinal alkaline phosphatase and T4 DNA ligase were purchased from Promega and Gibco BRL (Life Technologies, Inc., Gaithersberg, MD) and used under conditions specified by their manufacturers. *E. coli* cells were transformed by the Hanahan method (64) or the method mentioned in Dr.

Edward M. David's thesis (65). Standard techniques were used for molecular cloning and electrophoresis (66).

Electroporation of plasmid into *B. abortus*

Plasmids were introduced into *B. abortus* 2308 by electroporation (67) in an ElectroCell Manipulator 600 electroporation System (Biotechnologies & Experimental Research Inc., San Diego, CA). Plasmids were resuspended in H_2O or directly taken from a ligation reaction. *B. abortus* 2308 was harvested from YENB plates after growth at 37 °C, 5% CO_2 for 3 days and washed with chilled H_2O 3 times. One µl ligation reaction mixture or 0.2 µl supercoil plasmid and 80 µl competent cells were transferred into a 0.1 mm gap cuvette and electroporated at 12.5 $kV \bullet cm^{-1}$ with R7 resistance, giving a time constant of approximately 11.5 msec. After pulsing, cells were mixed with 1 ml SOC-B broth in a microfuge tube and incubated at 37 °C, 5% CO_2 for 10 min with the cap open then 1 hr with the cap closed. Cells were then spun down and resuspended in 100 µl SOC-B and plated directly onto tryptose plates supplemented with selective antibiotics or grown on recovery tryptose plates overnight before selection.

Detection of luminescence

Detection of luminescence by X-ray film

To detect luminous colonies, one drop or 10 µl decanal was spread on the lid of the Petri dish and then the dish was exposed to Fuji medical X-ray film RX (Fuji Photo Co. Ltd., Tokyo) for a period of time in the dark.

Detection of luminescence by Gel Doc 1000

A similar procedure as X-ray film detection was used except the images of plates were collected by the Multi-Analyst 1.0.2 (PPC) program (Bio-Rad Laboratories, Inc.,

Hercules, CA) on a PowerPC Macintosh (Apple Computer Inc., Cupertino, CA) connected with the Gel Doc 1000 (Bio-Rad Laboratories, Inc.) without the orange filter. Images were integrated for 100 sec. Three times of 3×3 noise reduction processes were applied.

Detection of luminescence by luminometer

Since decanal is not soluble in water, 10 µl 0.01% solution in 70% (v:v) ethanol : 8% (v:v) methanol (68) was injected into 0.5 ml of *E. coli* in a cuvette. The LKB 1251 luminometer and 1291 dispensers (Wallac Oy, Turku, Finland) were controlled by the Multiuse program (Bio-Orbit Oy, Turku, Finland) run on a PC. The following program was used to collect data:

Type = Luminescence Assay

Parameters

Time interval = 1.000000

Time constant = 0.500000

Procedure length = 15

Actions

Use 2nd dispenser at 0 second for 1 cycle

Mix at 0 second for 5 seconds

Measurements

$g1=signal at 0 second

Calculations

result = $g1

The 1291 dispenser is set to 2.0 which is approximately 10 µl/cycle.

Construction of promoter-probe vector using *V. fischeri luxAB*

RE945 contains plasmid pRE330 which has the *Xba*I fragment containing *V. fischeri luxAB* from pRL1063a (69) inserted into *Xba*I site of pBluescript KS+ in the same orientation as *lacZ*, while RE946 contains plasmid pRE331 in which the *luxAB* is inversely inserted into the same site. Since luminescence was detected from RE945 and RE946, there is promoter activity in the 5' uncoding region of *luxAB*. To remove this region, exonuclease III uni-direction digestion was used on *Pst*I-*Sma*I double digested pRE330. Restriction digestion was used to check the plasmids from luminous colonies and their identity was further confirmed by sequencing. In one of the plasmids, the 5' uncoding region was trimmed and left only two bp in front of the start codon of *luxA*. The *luxAB* from this plasmid had the least background luminescence in later subcloning. The plasmid was designated as pRE467. The *Sal*I-*Xba*I fragment in the multicloning site of pSP72 (Promega) was replaced by the *luxAB* fragment cut out from pRE467 with the same restriction enzymes. The resulting plasmid was digested with *Bam*HI and *Bgl*II.The long fragment was then self-ligated to eliminate some repetitive restriction sites and resulted in pRE472.

Detection of promoter activity of *glk* in *E. coli*

The glucokinase of *B. abortus* 19 was expressed when its gene, *glk*, was inserted in pUC19 in both orientations, suggesting the promoter was contained in the cloned fragment (70).

pRE65, the plasmid containing the *glk* fragment mentioned above, was digested with *Hinf*I. The sticky ends were filled in with Klenow enzyme. The 1152 bp fragment

containing the supposed promoter of *glk* was separated by agarose gel electrophoresis and inserted into the *Eco*RV site of pRE472 and confirmed by nucleotide sequencing. The constructed plasmids were transformed into *E. coli* XL-1 Blue and their luminescence was examined by 12 hr 20 min exposure to X-ray film.

Screen promoters of *B. abortus* in *E. coli*

B. abortus 2308 genomic DNA was completely digested with *Hsp*92I and inserted into the *Cla*I site of pRE472. Luminescent transformants were detected by exposure to X-ray film for 12 hr. Two plasmids from the luminescent colonies were extracted and sequenced.

Preparation of the multicloning site from pBluescript SK+

The multicloning site was prepared by PCR with T3 and T7 primers (Oklahoma State University Recombinant DNA/Protein Resource Facility) in a PTV-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). The PCR mixture consisted of 5 ng pBluescript SK+, 2.5 µl 10× Taq polymerase buffer (Promega), 2.0 mM $MgCl_2$, 0.5mM dNTPs, 1.0 µl of 20 mM each primer, and 5 unit Taq polymerase (Promega). The final volume was brought to 25 µl by H_2O. No pBluescript SK+ was added into the control. The thermocycler was programmed as follows:

1. 95 °C 4 min
2. 95 °C 30 sec
3. 46 °C 30 sec
4. 72 °C 30 sec
5. GOTO step 2 for 34 times

6. 72 °C 10 min

7. 4 °C 0 sec

8. End

The amplified DNA was separated by electrophoresis on a low melting agarose gel. The expected DNA band was extracted by freeze and thaw method followed by ethanol precipitation.

Construction of a promoter-probe vector using *V. harveyi luxAB*

Since ColE1 based replicons can not be maintained in *B. abortus*, a vector based on pBBR1 was constructed. Also due to the failure of cloning the *V. fischeri luxAB* into pBBR1 based plasmid, *V. harveyi luxAB* was used.

pBBR1MCS, a derivative from pBBR1, has *cam*, *mob*, and *lacZ* genes in opposite orientation to *rep* gene. To avoid leaking, these genes were removed. The *cam* and *mob* genes were replaced with the *bla* gene from pBluescript KS+ in the same orientation as *rep*. The resulting plasmid was designated as pJD23.

A *Bam*HI fragment containing the *V. harveyi luxAB* from pFUSLUX (71) was inserted into pBluescript KS+, then exonuclease III uni-direction digestion was performed to trim the 5' end uncoding region. The *luxAB* fragment with 17 bp left in front of the start codon was cut out from one of the resulting plasmids and replaced the *lacZ* of pJD23. The multicloning site from pBluescript SK+ was inserted in the front of *luxAB* for easier cloning and sequencing. The vector resulting was pJD27.

The background expression of luciferase in pJD27 in *E. coli* was examined by luminometer. JD27, the *E. coli* XL-1 Blue containing pJD27, and E. coli XL-1 Blue along were grown for 8 hr and their concentrations were measured by A_{600} readings in a UV-

160U UV-Visible Reording Spectrophotometer (Shimadzu Corporation, Japan) with 1 cm cuvettes.

Construction of a genomic DNA library of *B. abortus* 2308 in pJD27

The genomic DNA of *B. abortus* 2308 was partially digested with *Sau*3AI which left sticky ends compatible with *Bam*HI. Five unit *Sau*3AI (Gibco BRL) was used to digest 3.4 μg *B. abortus* 2308 genomic DNA in 50 μl or 40 μl reaction solution. Five μl was taken out every 5 min from the 50 μl reaction solution and 20 μl from the 40 μl reaction solution at 3rd and 7th min. One μl or 0.5 μl 0.5 M EDTA was used to stop the digestion in 20 μl or 5 μl respectively. The DNA between about 200 bp and 2 kb on agarose gel electrophoresis was extracted and ligated with *Bam*HI cut and dephosphorylated pJD27.

The ligation product was transformed into *E. coli* XL-1 Blue and plated on LB with ampicillin. Twenty six colonies were randomly picked to grow in 0.5 ml TB with ampicillin overnight. Cells were spun down by microfuge and resuspended in 50 μl TB with ampicillin. Forty μl phenol: chloroform saturated with TE buffer (pH 8.0) was added following by vortex and 3 min centrifuge at top speed in microfuge. The aqueous layer was taken out and incubated with 5 μl of 0.5 mg/ml RNase for 1 hr at 37 °C. Three μl 10× gel loading buffer was added and 7 μl of the mixture was run on 1% agarose gel and compared with JD27 to check the existence of inserts.

The library was constructed by electroporation of ligation product into *B. abortus* 2308.

Screen promoters of *B. abortus* 2308

The genomic DNA library was plated on tryptose plates containing ampicillin. Luminescence of colonies was examined using the X-ray film method with 15 min

exposure. The luminous colonies were transferred to minimal media containing different carbon sources. The expression of luciferase was measured by X-ray film in the same way.

Test the thermal stability of *V. harveyi* luciferase *in vivo*

JD35 was grown on LM plate with ampicillin for 9 hr at 37 °C then 4 hr at 42 °C. The luminescence was examined on X-ray film with about 30 sec exposure inside a 42 °C incubator in the dark.

Nucleotide sequencing

All nucleotide sequences were determined by Oklahoma State University Recombinant DNA/Protein Resource Facility using dideoxynucleotide chain termination method and read on an ABI PRISM™ 373 DNA Sequencer (PE Applied Biosystems, Foster City, CA).

The primers used in nucleotide sequencing were synthesized on an ABI 392 DNA/RNA Synthesizer by Oklahoma State University Recombinant DNA/Protein Resource Facility.

Inserts in pRE472 were sequenced using SP6 primer and primer 4477 (5'GGTGGTTGATACGAA3'). Inserts in pJD27 were sequenced using T3 primer and KS primer if necessary. The whole insert of pJD48 could not be covered by the sequencing from both ends. An internal primer 4660 (5'GCATAGGCATCTG3') was synthesized and used in sequencing.

Sequence editing and BLAST search

MacVector 6.0 (Oxford Molecular Group) program was used for sequence editing, restriction site and nucleic acid subsequence search, ClustalW alignment, and BLAST search through its connection to NCBI.

Strains	Characteristics	Sources or references
E. coli		
XL-1 Blue	*redA1 endA1 gyrA96 thi-1 hsdR1 supE44 relA1 lac*[F' *proAB lacI8 ZΔM15* Tn*10(Tet^r)*]	Ref. 72
RE945	XL-1 Blue containing pRE330	RCE
RE946	XL-1 Blue containing pRE331	RCE
JD6, JD9	XL-1 Blue containing pJD6, pJD9	This work
JD23	XL-1 Blue containing pJD23	This work
JD27	XL-1 Blue containing pJD27	This work
JD35, JD36	XL-1 Blue containing pJD35, pJD36	This work
B. abortus		
2308	wild type strain	OADDL
BJD27	2308 containing pJD27	This work
BJD35, BJD36	2308 containing pJD35, pJD36	This work
BJD45, BJD46, and BJD48-64	2308 containing pJD45, pJD46, and pJD48-64	This work

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OADDL, Oklahoma Animal Disease Diagnostics Laboratory, Oklahoma State University, Oklahoma.

Table 1. Bacterial strains used in this study.

Plasmids	Characteristics	Sources or references
pBluescript SK+, pBluescript KS+	cloning vector (amp^r)	Stratagene
pSP72	cloning vector (amp^r)	Promega
pRE330	pBluescript KS+ with *V. fischeri luxAB* in same orientation as *lacZ*	RCE
pRE331	pBluescript KS+ with *V. fischeri luxAB* in opposite orientation as *lacZ*	RCE
pRE467	pRE330 with the 5' uncoding region of *luxAB* cut	This work
pRE472	pSP72 carrying the *luxAB* from pRE467	This work
pBBR1MCS	broad-host-range cloning vector (cam^r)	Ref. 73
pFUSLUX	pBR322 based plasmid containing *V. harveyi luxAB* genes (tet^r)	Ref. 71
pJD6, pJD9	pRE472 with a fragment of *B. abortus* 2308 genomic DNA in front of *luxAB*	This work
pJD23	pBBR1 replication machinery + *bla*	This work
pJD27	pJD23 carrying the multicloning site from pBluescript SK+ following by a trimmed *V. harveyi luxAB*	This work
pJD35, pJD36, pJD45, pJD46, and pJD48-64	Isolations of *B. abortus* 2308 genomic DNA library in pJD27	This work

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Table 2. Plasmids used in this study.

Media for growing *E. coli*	Characteristics	References
LB		Ref. 74
LM		Ref. 64
TB		Ref. 75
Media for growing *B. abortus*		
Tryptose medium		Difco
SOC-B		Ref. 67
YENB		Ref. 76
Minimal medium with glucose		Ref. 77
Minimal medium with erythritol	Replacement of glucose in minimal medium with glucose with same molar content of erythritol	This work
Minimal medium with galactose	Replacement of glucose in minimal medium with glucose with same molar content of galactose	This work
Minimal medium with succinate	Replacement of glucose in minimal medium with glucose with same molar content of sodium succinate	This work
Minimal medium with glycerol	Replacement of glucose in minimal medium with glucose with same molar content of glycerol	This work

Table 3. Media for growing bacteria.

Chapter 3

Results and Discussion

Promoter-probe vector pRE472

pRE472 was based on pSP72 and contained the promoterless *V. fischeri* luciferase genes *luxAB* in the opposite orientation to the *bla* gene (Figure 2).

B. abortus glk promoter

The *Hinf*I fragment containing the *glk* promoter was inserted into the *Eco*RV site of pRE472 in both orientations and transformed to *E. coli* XL-1 Blue. Positive control was constructed using *lacZ* promoter from pBluescript KS+. For further comparison, *lacZ* promoter in opposite orientation was also insert into pRE472.

Luminescence was detected on pRE472lacZ+, the positive control, without IPTG, suggesting that the repression of the *lacZ* promoter is not tight. pRE472 alone had low background expression. But on pRE472lacZ-, a construct with *lacZ* promoter inverted in pRE472lacZ+, no luminescence was detected, which suggested that the low background expression of luciferase came from pSP72. As shown by pRE472glk+, the *glk* promoter was able to weakly express the downstream luciferase in *E. coli*, compared to the repressed *lacZ* promoter in pRE472lacZ+. But interestingly, pRE472glk- in which the *glk* promoter was in opposite orientation of *luxAB* could also express active luciferase at a comparable level with repressed *lacZ* promoter and stronger than pRE472glk+ (Figure 3). There are three possible explanations. First, in *E. coli*, the promoter of *B. abortus glk* is not properly recognized. The RNA polymerase binds to this region, but transcribes in both directions with even more trend to the opposite one. Second, The *Hinf*I fragment from pRE65 contains the promoter of *glk* as well as a promoter for another gene at

opposite orientation. But the upstream sequence does not give a long open reading frame. Third, the ligation of two DNA molecules creates new promoters at the place they join together. Though the second and the third explanations are less possible, to rule them out completely needs some more experiments.

B. abortus DNA fragments containing promoter activity in _E. coli_

Some *Hsp*92I fragments of *B. abortus* genomic DNA can drive the expression of *luxAB* in *E. coli*. Among the two clones sequenced, pJD6 was found to contain a fragment almost same as the 5' uncoding region of *fliC* gene of *B. abortus* 544 (GenBank accession number: AF019251) which encodes flagellin (78). But the *fliC* is in the opposite orientation (Figure 4). If the expression of *luxAB* is due to the promoter of *fliC*, we have another example that *B. abortus* promoters can be transcribed backward in *E. coli*. This does not rule out the possibility that another promoter lies in this region and drives a gene away from *fliC*, but the sequence is not long enough to reveal that gene and no match was found through a BLASTX search. The other clone sequenced, pJD9, had no match through a BLAST search. Neither was any long open reading frame found. The nucleotide sequence of pJD9 insert is shown in Figure 5.

Promoter-probe vector pJD27

pJD27 employed the replication machinery of broad-host-range plasmid pBBR1 and contained the promoterless *V. harveyi* luciferase gene *luxAB* in the opposite orientation to the ampicillin resistance gene *bla* and the *rep* gene which is required for replication of the plasmid (Figure 6).

JD27 was tested for background expression of luciferase in a luminometer against blank media TB and XL-1 Blue. Data suggested that there was weak expression of

luciferase (Figure 7). The expression can be driven by a weak promoter in the pBBR1 portion. It is possible that a promoter in *luxA* gene coding region is driving expression of *luxB* which can form an active homodimer (79). Furthermore, a curved DNA can form in the first 200 bp of the *luxA* coding region containing tracts of A residues (80). The curved DNA can influence the expression of *luxAB* under certain conditions (81).

B. abortus 2308 genomic DNA library in pJD27

A library was constructed by insertion of *Sau*3AI partially digested genomic DNA into *Bam*HI site of pJD27. The insertion efficiency was checked in *E. coli* due to its easy growth and manipulation. All 26 clones checked had inserts (Figure 8), indicating a greater than 96.2% efficiency. Because some library constructions in *B. abortus* 2308 were grown on tryptose plates without ampicillin overnight for recovery before selection, the number of independent clones was unable be counted. The estimation was about five thousand.

Screening of promoters in _B. abortus_ in _E. coli_

One hundred one colonies of *E. coli* from the insertion efficiency check were screened for expression of luciferase. One strongly luminous colony was found and designated as JD35. Some other less luminous colonies were picked for future study (Figure 9). The plasmid from JD35, pJD35, was sequenced and searched in BLAST. No meaningful match was found. But the consensus -35 and -10 regions were found in the insert of pJD35 (Figure 10). Another colony in the right end of the lower-right white strike was thought to be luminous and designated as JD36. But JD36 was later found actually not to express luciferase (Figure 11).

Luminescence of JD35, JD36, BJD35, and BJD36

pJD35 and pJD36 were electroporated into *B. abortus* 2308 resulting in BJD35 BJD36. Luminescence of JD35, JD36, BJD35,and BJD36 was examined by exposure to X-ray film for 15 min. JD35 strongly expressed luciferase, but JD36 did not at all. On tryptose plate neither BJD35 nor BJD36 had luciferase activity detected (Figure 11). This result along with that from study of *glk* promoter indicates the significant difference between *E. coli* promoters and *B. abortus* ones which may be due to the difference between their RNA polymerases. The expression difference between JD35 and BJD35 may also be due to the existence of different repression systems in *E. coli* and *B. abortus*.

As the negative control, the JD27 and BJD27 background was not detected in this experimental condition (Figure 11).

Thermal stability of *V. harveyi* luciferase *in vivo*

In order to screen heat-shock inducible promoters using pJD27, the thermal stability of the *V. harveyi* luciferase had to be tested first. Thus JD35 was used to test the activity of *V. harveyi* luciferase at 42 °C. The result showed the luciferase was active at 42 °C in *E. coli* (Figure 12) and able to monitor gene expression under heat shock condition.

Screening of promoters in *B. abortus*

Twenty eight clones were randomly selected from the *B. abortus* 2308 genomic DNA library in *B. abortus* 2308 and grown on a tryptose plate with ampicillin. Luminescence was detected from 18 clones by X-ray film (Figure 13). The *B. abortus* strains were designated as BJD45, BJD46, and BJD48-64. The corresponding plasmids were designated as pJD45, pJD46, and pJD48-64.

Expression of *luxAB* of BJD45, BJD46, and BJD48-64 on different carbon source

BJD45, BJD46, and BJD48-64 were transferred to minimal media with erythritol, glucose, galactose, succinate, and glycerol as single carbon source. Different expression patterns of *luxAB* were found (Figure 14). On the tryptose medium, the most luminous colony was BJD60, and the second was BJD55 followed by BJD45, BJD48, BJD52, BJD57, and BJD59, and then BJD53, BJD54, BJD58, and BJD61. BJD49, BJD51, BJD56, BJD46, and BJD62 were the least luminous. On the minimal media with erythritol, 12 luminous colonies were found. They are BJD45, BJD48, BJD49, BJD51, BJD52, BJD53, BJD54, BJD55, BJD57, BJD57, BJD58, BJD59, and BJD60. Among them the luminescence from BJD48 and BJD60 was the strongest. But it was only comparable to that from BJD51 on tryptose. The second luminous colonies were BJD45 and BJD55. Only BJD45, BJD48, and BJD52 had active luciferase expressed on the minimal medium with glucose and their luminescence was similar to BJD46 in tryptose medium. BJD57, BJD59, and BJD55 were the only luminous colonies on the minimal medium with galactose and BJD57 and BJD59 were more luminous than BJD55. Five colonies emitted light on the minimal medium with succinate. The luminescence intensity of BJD60 and BJD54 was greater than that of BJD48, BJD55, and BJD59. The minimal medium with glycerol was completely dark probably due to the very slow growth on this medium. The greatest luminescence intensity on these minimal media was close to that of BJD56 on tryptose medium. The luciferase expression pattern is summarized on Table 4.

Inserts of pJD45, pJD46, and pJD48-63 were sequenced and submitted to GenBank (Table 5). Though most had no meaningful matches through BLAST search, some sequences were worth mentioning.

pJD48 has an insert of 1449 bp. Except for a few mismatches, the 175 bp at 5' end of the pJD48 insert is the 1838 to 2009 bp at 3' end uncoding region of *B. abortus* 2308 *htrA* (GenBank accession number: U07352) which stops at 1816 bp (39). The 178 to 273 bp of pJD48 insert was aligned to an *Agrobacterium tumefaciens* receiver module of putative response regulator gene (GenBank accession number: U82574) (Figure 15). ORF1, the 119 AA open reading frame in this region, was found in the pJD48 insert. It starts at 73 bp and ends at 432 bp. The ORF1 has 31% identity and 53% similarity to *E. coli pcoR* protein (GenBank accession number: S70164) which is a transcriptional regulator responding to copper sensor *pcoS* and *pcoS** (82) and 38% identity and 54% similarity to *B. suis* FeuP protein (Figure 16). These alignments suggested the putative peptide was very possibly a transcriptional regulator of a two-component system. Its close downstream location from *htrA* suggests it might be in the same operon as *htrA* and involved in the heat shock response if it does not carry its own promoter, which is still hard to identify at this point since the *B. abortus* promoters can vary from those consensus ones in *E. coli*. There is another open reading frame, ORF2, starting at 1186 bp and truncated at the 3' end of pJD48 insert. The ORF2 was found through BLASTX search to align to the N-terminal 87 AA in KefB (GenBank accession number: P45522), the N-terminal 86 AA KefC (GenBank accession number: P03819) in *E. coli*, the N-terminal 84 AA in KefC of *Myxococcus xanthus* (GenBank accession number: U37008), and the N-terminal 89 AA in KefX of *Haemophilus influenzae* strain Rd KW20 (GenBank accession number: P44933) (Figure 17). All these proteins function as K^+/H^+ antiporters in glutathione-regulated potassium-efflux systems which detoxify the endogenous electrophile methylglyoxal when bacteria are grown on a poor carbon source

or exogenous electrophiles. It will be very interesting to study whether this gene has a detoxification function in *B. abortus* or is involved in adaptation to the low pH environment inside the macrophage. Because of their distances to the *luxAB*, the expression of luciferase is more possibly driven by the promoter of the putative *kef* homologue then by the promoter of the putative transcriptional regulator.

The insert in pJD49 has 106 bp and was aligned to the antisense strand of the *Pseudomonas aeruginosa oprC* gene (GenBank accession number: D28119) coding region for the mature polypeptide (Figure 18). The identity is 77%. *oprC* encodes protein C of the outer membrane which functions as channel-forming and copper-binding (83).

The 64 bp insert in pJD53 is exactly same as 1278 to 1215 bp of *B. abortus* 544 flagellar hook *flgE* gene (GenBank accession number: AF019252) whose coding region is from 175 bp to 1365 bp (78). The alignment is inverse. In this study, fragments of both *fliC* and *flgE* were found in *B. abortus 2308* and were same as those reported in *B. abortus* 544. The existence of flagella genes in the non-motile *B. abortus* is interesting. Further study will reveal whether the flagella are lost during evolution or *B. abortus* is actually motile in certain environment. It is also possible that the flagella genes are related to virulence since some flagellar proteins are exported by a type III secretion pathway which can export some other virulence factors as well (78).

pJD56 contains 337 bp insert which is made up of by 2 separate *Sau*3AI fragment. The 1 to 186 bp fragment has no match in BLAST while the 187 to 337 bp is same as 4055 to 4205 bp of the coding region of an ATP-coupled transporter gene from *B. abortus* 19 (GenBank accession number: U21919) (70). The two fragments were ligated

at their *Sau*3AI sites. Which fragment actually drove the expression of luciferase needs to be further identified.

The 119 bp insert in pJD60 was aligned to the antisense strand of the coding region of *A. tumefaciens miaA* gene (GenBank accession number: M83532) encoding tRNA: 2-isopentenyltransferase. The identity is 71%. tRNA: 2-isopentenyltransferase specifically modifies the A-37 residue in UNN codon tRNA species, and a *miaA* mutant of *A. tumefaciens* has reduced expression of *vir* genes probably due to a reduced translation efficiency (84).

In this study, several sequences containing promoter activity were aligned to the antisense strand of some genes in the coding region, it is possible that the promoters function to transcribe an antisense mRNA to regulate the expression of these genes. To prove this, further study is needed.

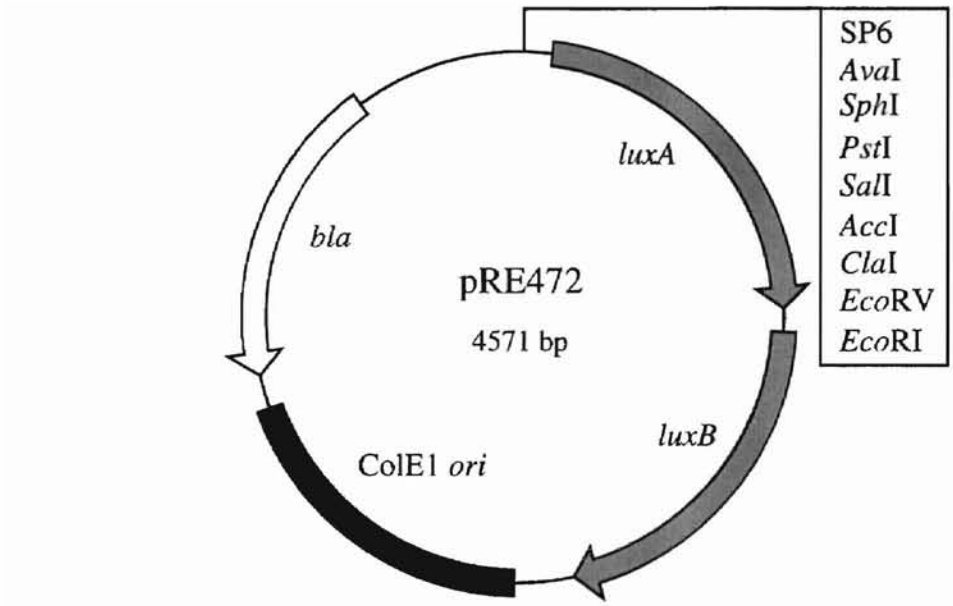

Figure 2. Promoter-probe vector pRE472.

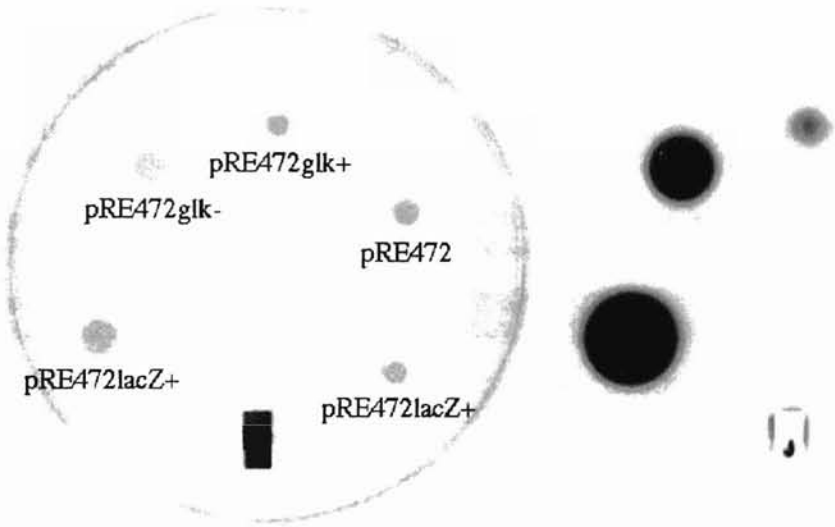

Figure 3. Luminescence of *glk* promoter. The left is the X-ray film with 12 hr 20 min exposure. The right is the picture of the plate.

```
AF019251    521                    CATAGTTTTTTGCCCCTTGGGAAAAACAATCTG
AF019251    488 AGGGACAGGCCGGATTTCAGATTACCGGCATGGTGGAGCGGCATCATGCC
AF019251    438 TGTCGCCGCGAACACACGGGACCCACCATATCGCCATTCCTAAGGCTATA
AF019251    388 AATATTAAGAAAGCCCTAATCGAGGAAGTGCAGAAATTTCGAAATTTCGT
AF019251    338 TTTCAGCAAGACATGACGGGAAACATGTATATTTAGTATATTTTAATGTA
AF019251    288 AACATATAATAATATAGAACAATTTTATTATAAAATTACTTTCTTATAGA

pJD6insert    1 CGTCAAAATAGACGCATAACTATTAGTCTTTAAATTATTCAAAATTTGTT
AF019251    238 CGTCAAAATAGACGCATAACTATTAGTCTTTAAATTAT-CAAAATTTGTT

pJD6insert   51 GTAATATATTCAGGCGAATCATTTTGGCTTATGGGGATCGCATTTGTCTA
AF019251    189 GTAATATAT-CAGGCGAATCATTTTGGCTTATGGGGATCGCATTTGTCTA

pJD6insert  101 GGCGACGTTCTGGCGCAAGCTTGATCGGGACACGACGCTAATTTCCACCT
AF019251    140 GGCGACGTTCTGGCGCAA-CTTGATCGGGACACGACGCTAATTTCCACCT

pJD6insert  151 TAAGCATAGAAGAGATGCGTAC-GAGGGGCTTTGCTTAATTTATTGAAAT
AF019251     91 TAAGCATAGAAGAGATGCGTACTGAGGGGCTTTGCTTAATTTATTGAAAT

pJD6insert  200 ATGATGCGATCAATAACGATCACCTATAAACCTCTGGGATGATGTTGAAA
AF019251     41 ATGATGCGATCAATAACGATCACCTATAA-CCTCTGGGATGA 1

pJD6insert  250 TGCAGTTGCATCAACATGATCCCGGGCATTTTTTGCTTATGAATAACAGA
pJD6insert  300 ATACTCGTACCTCCCTTTTGAAATGACCGCGAATTGATGTGGTAAATGAC
pJD6insert  350 AGAGGCGGGGTTGAGATATGCCTAATTTGGCCAATCAGGATTTTACGCAG
pJD6insert  400 TTCAAGCGTGAGCAACAGACTGCTCCTGCATGGTTCCGCATCTTGCGCGG
pJD6insert  450 CGGGCGCATGACCAAATGGAAAGGCAAAGTTGCCAGCTATGCCCCGCATG
pJD6insert  500 TGGCACCGGCCATTGGTTGGGCATTTTCCCGTACACTGCAATTATCGCTT
pJD6insert  550 ATTTCTCTTGTGATGGCGGGAACCGCAGCAGCGTCTGATCGCTACTGGGA
pJD6insert  600 TTCCAATGGCACCGCTGTGGGGCGCGGAGGGTCTGGCGataagcttgata
pJD6            tcgaattccgtatg
```

Figure 4. Alignment of the insert of pJD6 to *B. abortus fliC* (GenBank accession number: AF019251). The sequence in low case are from pRE472. The 'atg' in bold is the start codon of *luxA*. The start codon of *fliC* was indicated by bold 'CAT' which is transcribed and translated backward. Identical bases are in gray boxes. Gaps are indicated by '-'.

```
1   CGCCGGATAG CGCTGTGCAT CGGCATTTGC CGAGAAAAGC GTCTTGCCGG
51  TGTTCGCATC AACGACGATC GCGGCATATC TGTTGTCGCC TTTCGCCATG
101 GCCGGTGCAA TGGAGGAAAC CGTGGAGTAA CCCGTCACGG CTGTTAGAAG
151 AAGAGAGACC TGTGCCGCTT TTTTCAAGGC ATATGCGACT TTACTGAATG
201 CGGTACGCAC GATGATGACC TGCCTGTATC CCGGAATTTC TTACTGCGGC
251 GGTGAAAGCA CACCGTAACC TAATTCGGAC ACCTTACGGC CATCAGCGTT
301 ACCAATCCGT TTATGGTAAC CGGCTTGTTC ATAATTTTCT GAAAAATTTG
351 GGAGGTGAAG TTTCGCCGGG CAGGGATATC GGGAGTGTCT CGCCCGCCGC
401 CCGGAGCGGG TTATGATCCG GGCGTTTCAT ATAGGCCCTG GTTTCACTGC
451 GGCCGGATTG TGCTCCGCCC GACGGATTGC TGGTGGATGA AATCCGAAAA
501 CTTGCTTGTG CAAGCCCGGC ATTGCCACTT TCCGGCGCGC GCGTTTGCTT
551 TCACATCTGC GATATAATTG TGCCCAGTGC GGCCCTTGCA ACAAGTCCCG
601 GTTGCAAGTG ATGGCGAAGG GCTTAAAATC CCCATATGAC G 641
```

Figure 5. Nucleotide sequence of pJD9 insert.

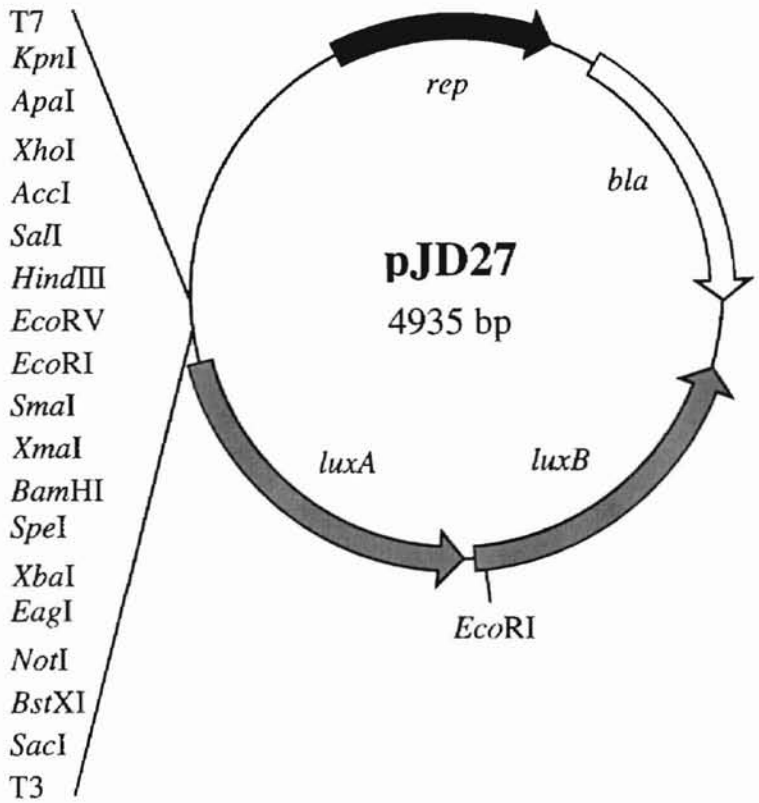

Figure 6. Promoter-probe vector pJD27.

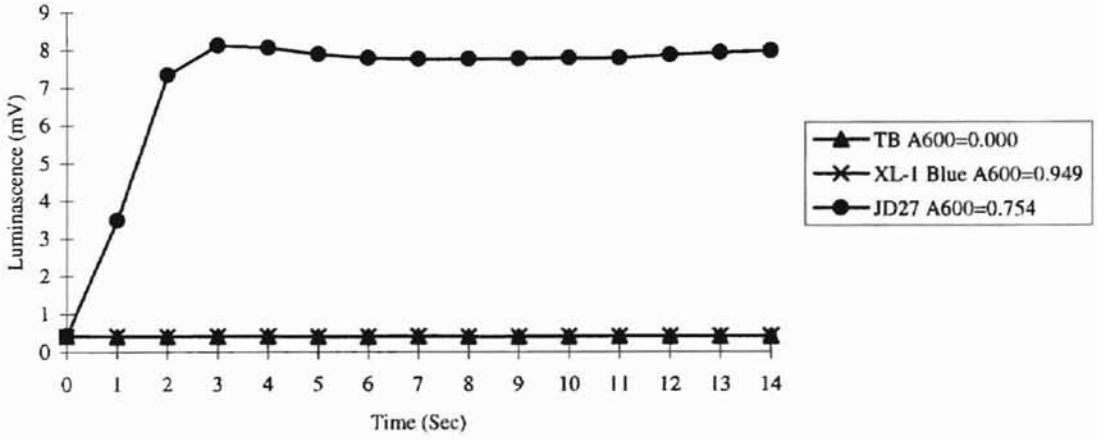

Figure 7. Background expression of luciferase in JD27. The cell concentrations are indicated with A600 readings shown in the legend box.

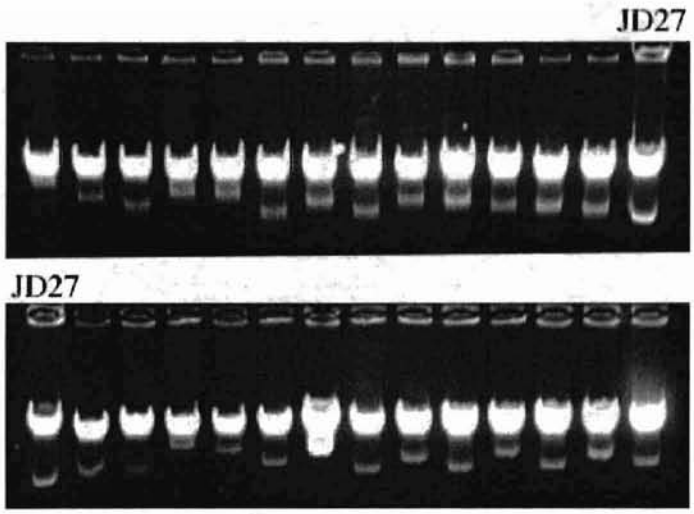

Figure 8. Insertion efficiency of *B. abortus* 2308 genomic DNA library. The upper thick bands are genomic DNA of E. coli XL-1 Blue. The lower bands are the supercoil plasmids.

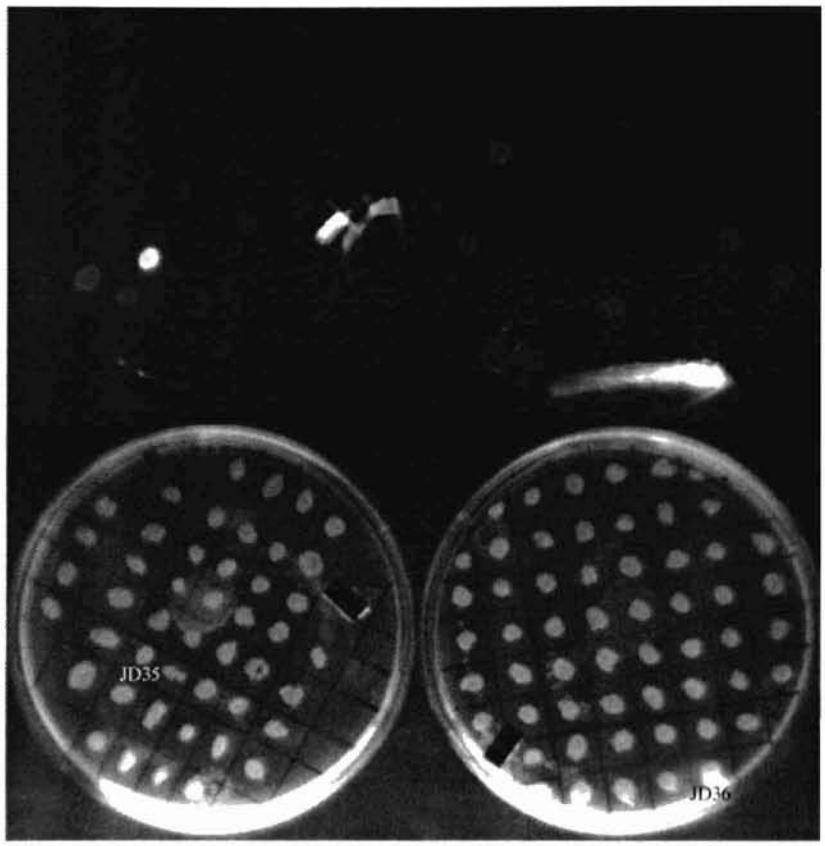

Figure 9. Screening for *B. abortus* 2308 promoters in *E. coli*. The upper part is picture of luminescence from Gel Doc 1000. The lower part is corresponding plates. JD35 is the brightest colony at the lower-right corner of the upper part. Two bright strikes are not due to the luminescence from bacteria.

```
0   GATCCAGAAA TCCCACGCTG CGCTCATCAT TGGCTCCCTC ATGCAAATTC
50  AACGGTGTTG CCCGAAATCT TCCCAGAATT TGATAGTTTC ATTTTGTTGA
100 CAAAATATCG ATAAGATGAT GACATAaAAT ACATATAATT GAGGACGCGG
150 CAAGAATGGG CAGAATGAGG AAAACGGGCG ATGGGCCGGA AGCGCCTGTT
200 GATC
```

Figure 10. Nucleotide sequence of the insert in pJD35. The possible -35 region is indicated with bold type. The -10 region is underlined. Though other -35 and -10 regions were found, they were separated by inappropriate distances. The lower case bases are mismatches with the consensus sequences. The *luxAB* genes downstream carry their own Shine-Dalgarno sequences and start codons.

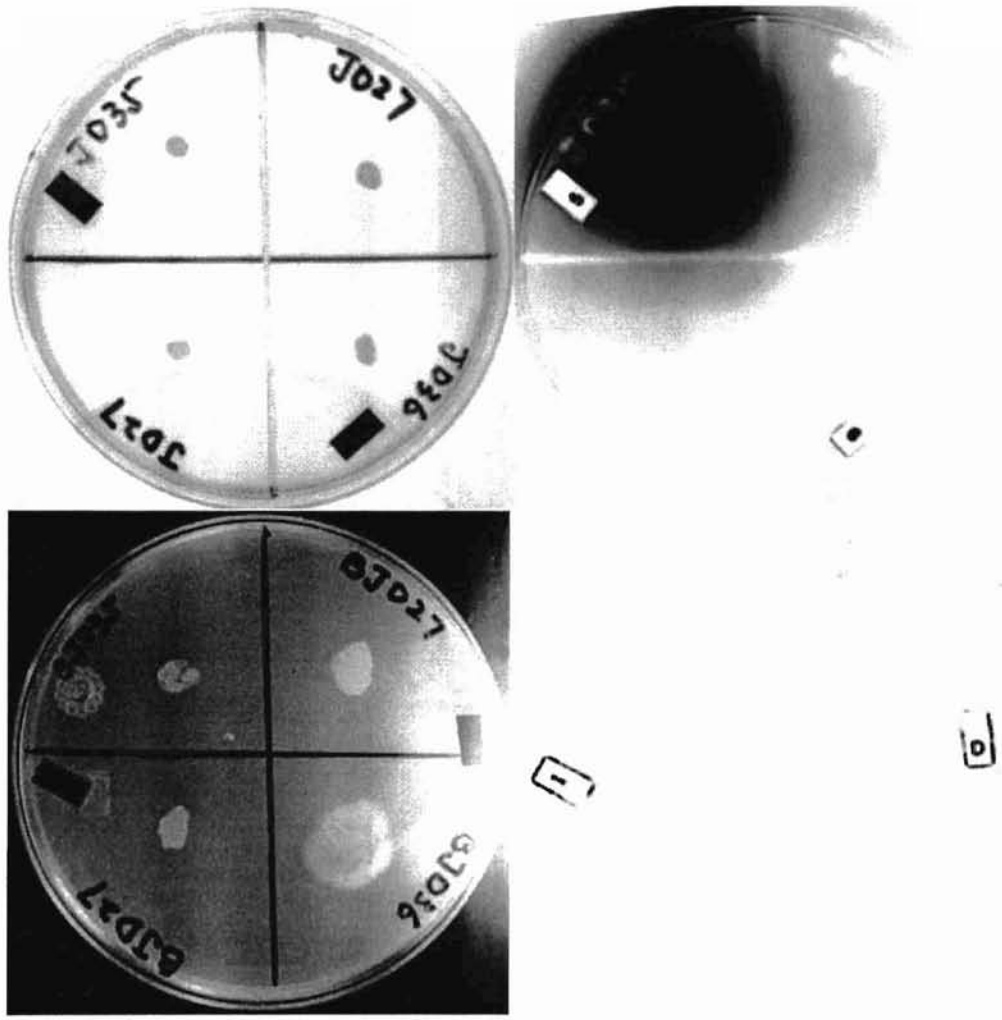

Figure 11. Luminescence of JD27, JD35, and JD36 on LM plate with ampicillin and BJD27, BJD35, and BJD36 on tryptose plate with ampicillin. *E. coli* indicated with strain names are in the upper plate with the corresponding X-ray film in the right of it. *B. abortus* indicated with strain names are in the lower plate with the corresponding X-ray film in the right of it.

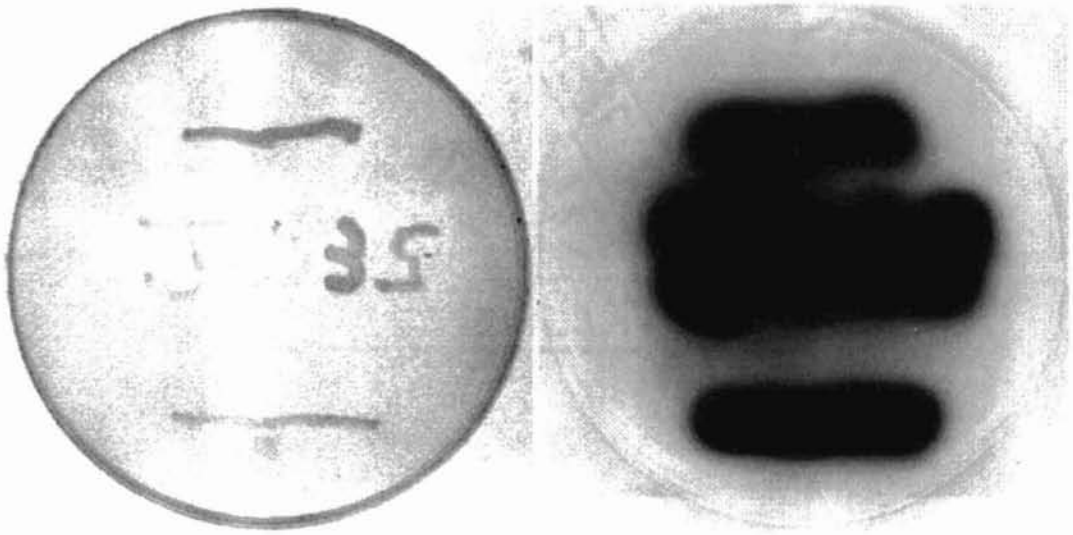

Figure 12. Activity of *V. harveyi* luciferase at 42 °C in *E. coli*. The plate is on the left and the corresponding X-ray film is on the right.

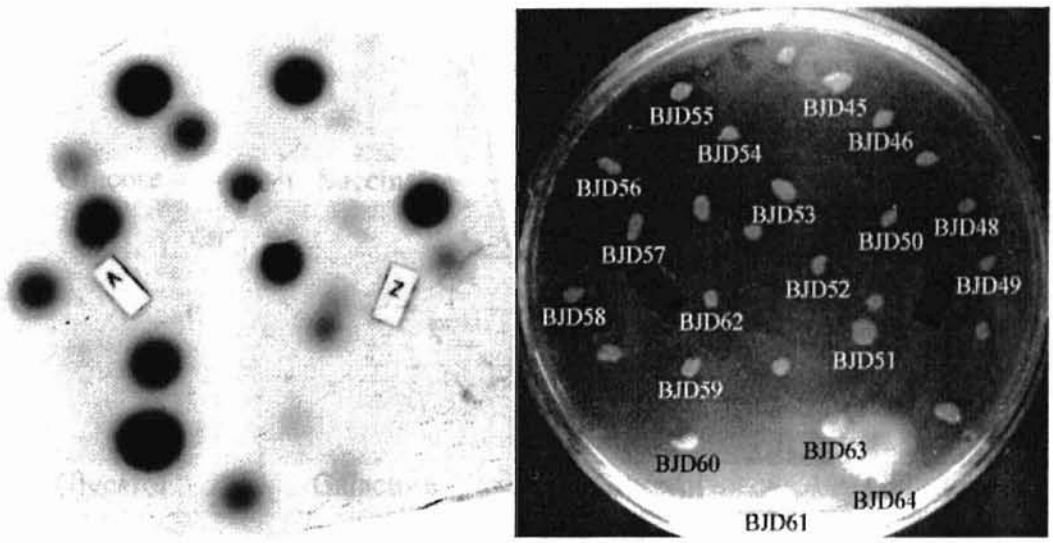

Figure 13. Screen for promoters of *B. abortus* 2308 on tryptose plate with ampicillin. The plate is no the right side and the corresponding X-ray film is on the left side. Colonies are labeled with the strain names under it on the plate.

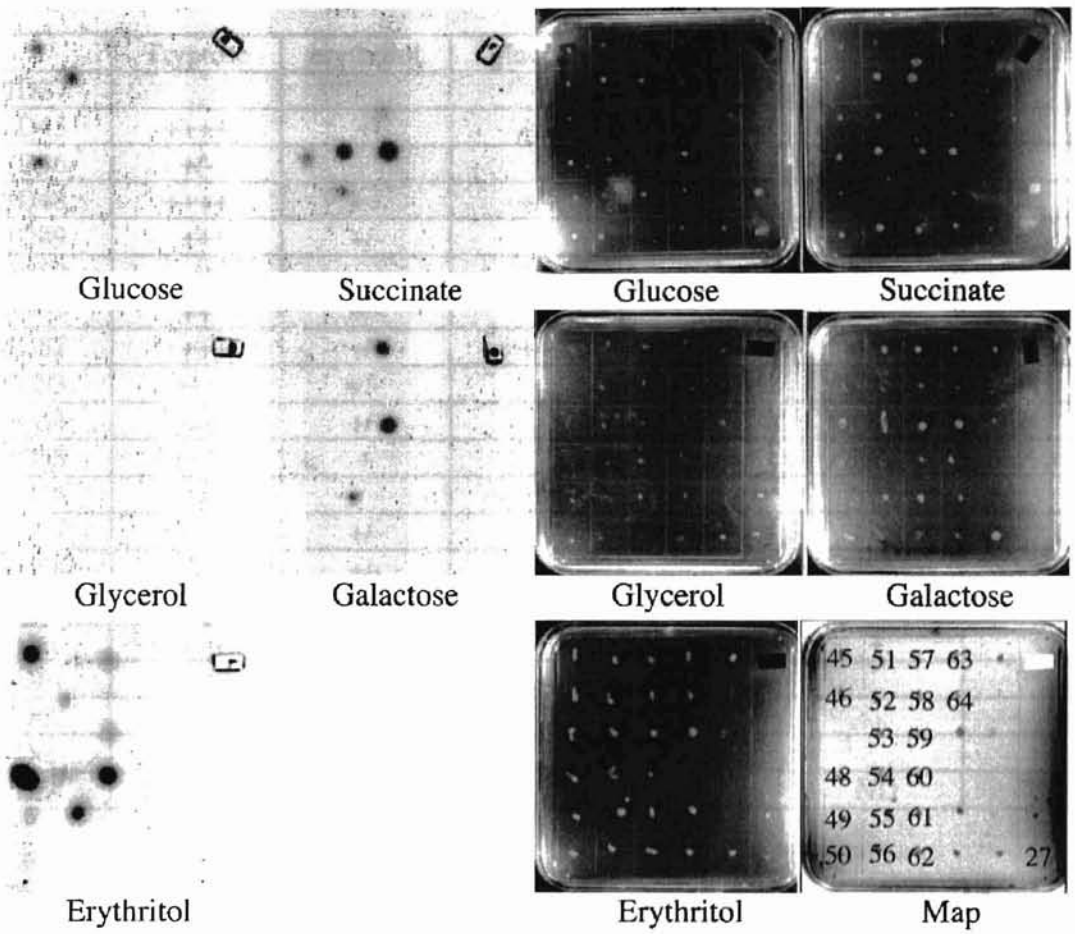

Figure 14. Luminescence of some *B. abortus* colonies on minimum media with different carbon sources. Plates are replicates and shown on the right side with their corresponding X-ray films on the left side. Map indicates the position of colonies on the plates and the X-ray film. Only the digits are shown.

	Tryptose	erythritol	glucose	galactose	succinate	glycerol
BJD27						
BJD45	++++	+	+/-			
BJD46	+/-					
BJD48	++++	++	+/-		+/-	
BJD49	++	+/-				
BJD50	+					
BJD51	++	+/-				
BJD52	++++	+/-	+/-			
BJD53	+++	+/-				
BJD54	+++	+/-			+	
BJD55	+++++	+		+/-	+/-	
BJD56	++					
BJD57	++++	+/-		+		
BJD58	+++	+/-				
BJD59	++++	+/-		+	+/-	
BJD60	++++++	++			+	
BJD61	+++					
BJD62	+/-					
BJD63	+					
BJD64	+	ND	ND	ND	ND	ND

Table 4. Luminescence of some *B. abortus* colonies on different media. Due to the contamination by mold, BJD64 had no data except for on tryptose media. The number of + indicates the intensity of luminescence. ND indicates no data. Blank indicates dark.

Figure 15. Sequence, alignments, and open reading frames of pJD48 insert. Two open reading frames are indicated with underlines. In the alignments to *B. abortus* 2308 *htrA* (GenBank accession number: U07352) and *A. tumefaciens* receiver module of putative response regulator gene (GenBank accession number: U82574) identical bases are in gray boxes and gaps are indicated by '-'.

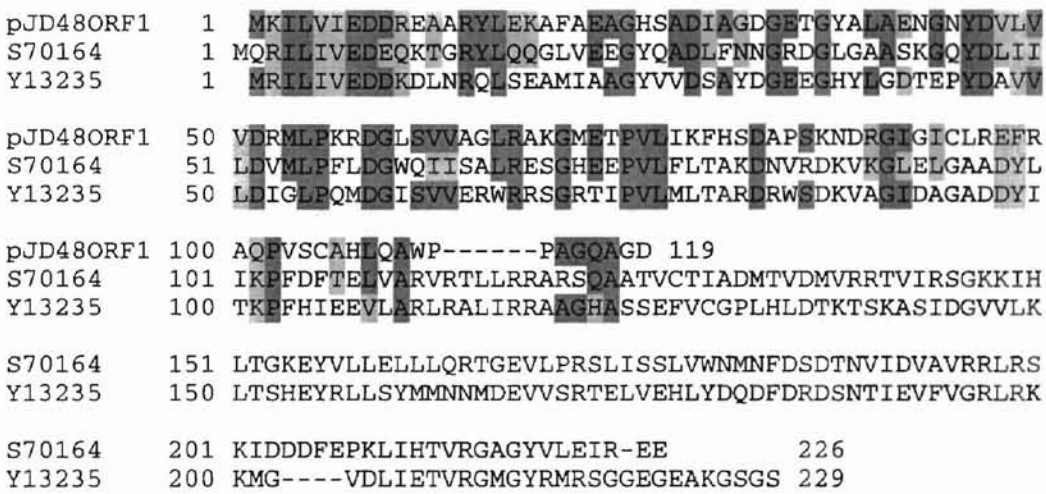

Figure 16. Alignment of pJD48 OFR1 to *E. coli* PcoR (GenBank accession number: S70164) and *B. suis* FeuP (GenBank accession number: Y13235). Identity is marked with dark gray. Similarity is marked with light gray. '-' indicates gap. Sequences are complete.

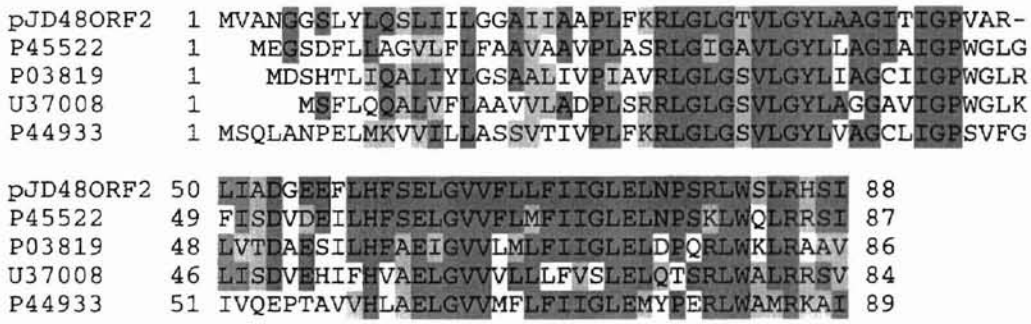

Figure 17. Alignment of pJD48 ORF2 to *E. coli* KefB (GenBank accession number: P45522), KefC (GenBank accession number: P03819), *M. xanthus* KefC (GenBank accession number: U37008), and *H. influenzae* KefX (GenBank accession number: P44933). Identity is marked with dark gray. Similarity is marked with light gray. '-' indicates gap. Sequences are truncated.

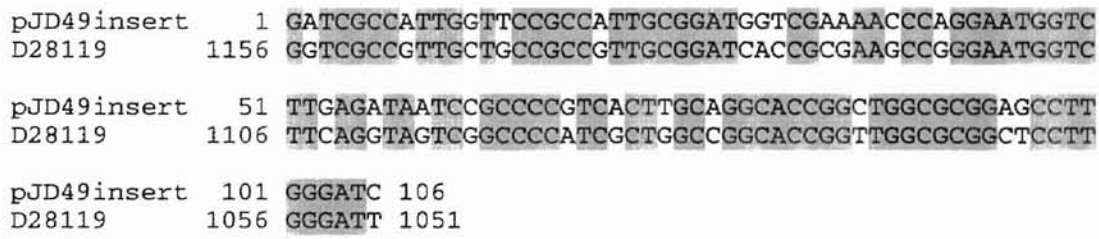

Figure 18. Alignment of pJD49 insert to *P. aeruginosa oprC* (GenBank accession: D28119). Identical bases are in gray boxes.

Figure 19. Alignment of pJD60 insert to *A. tumefaciens miaA* (GenBank accession: M83532). Identical bases are in gray boxes.

Insert of plasmid	GenBank accession number
pJD45	AF072119
pJD46	AF072120
pJD48	AF075168
pJD49	AF072121
pJD50	AF072569
pJD51	AF072570
pJD52	AF072571
pJD53	AF072572
pJD54	AF072573
pJD55	AF072574
pJD56	AF072575
pJD57	AF072576
pJD58	AF072577
pJD59	AF072578
pJD60	AF072579
pJD61	AF073884
pJD62	AF072580
pJD63	AF074323

Table 5. GenBank accession numbers for the inserts of pJD45, pJD46, and pJD48-pJD63.

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