Improved expression vector for *Brucella* species

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We constructed an improved vector pNSGroE for gene expression studies in *Brucella* spp. It is derived from the broad host range cloning vector pBBR1MCS (1). This new plasmid has several advantages over pBBR1MCS or its derivatives pBBGroE (2): (i) it is smaller in size, 2.9 kb; (ii) it expresses proteins as His-tagged fusion for easy detection and purification; (iii) it carries the *groE* promoter for constitutive expression that is enhanced under conditions of stress in vitro and in vivo; and (iv) it has higher levels of heterologous protein expression. Our expression studies in *Brucella 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.

The smaller 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 (MCS)] and removing all nonessential elements in the original vector pBBR1MCS or pBBGroE (*lacZ* gene, mobilization gene, and 1 kb upstream of the *groE* promoter). The vector was constructed by excision of a 185-bp fragment containing the MCS of pRSETA vector (Invitrogen, Carlsbad, CA, USA) using *Xba*I and *Hind*III. This fragment was cloned into *Xba*I and *Hind*III sites of pGEM11 vector (Promega, Madison, WI, USA) to form construct A. The *groE* promoter of *Brucella* spp. was amplified from the

genomic DNA of *B. abortus* strain 2308 using Platinum[®] PCR SuperMix High Fidelity kit (Invitrogen). In the reverse primer (Table 1), six histidine and one glycine residues were engineered after the translational start codon to facilitate the epitope tagging at the amino terminus of any expressed protein. The amplified *groE* 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 plasmid (3) was amplified along with its own promoter from the pBBR1MCS vector using a primer pair with *Sal*I and *Xba*I sites. The chloramphenicol resistance gene (*cat*), along with its own promoter, was amplified from the pBBR1MCS using a primer pair with *Spe*I and *Hind*III sites. An *Xba*I site was engineered into the reverse primer of the *cat* gene to add an extra unique restriction site within the MCS of the new plasmid followed by a transcriptional stop codon.

The *groE* promoter along with the downstream MCS was excised from construct B using *Sal*I and *Hind*III sites. The *rep* gene was digested with *Sal*I and *Xba*I, and the *cat* gene product was digested with *Spe*I and *Hind*III. The three fragments were purified and ligated to form plasmid pNSGroE (Figure 1) (GenBank[®] accession no. AY576605). Strain RB51 was transformed by electroporation according

Figure 1. Plasmid map. The engineered pNSGroE expression vector with *Brucella groE* 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.

Table 1. PCR Primers Used in the Amplification Procedure

Primer	Sequence
groE promoter Forward	5'- CCCGT CGAC GCTGTTGCGCGAAAAACGCCA-3'
groE promoter Reverse	5'- CCGGATCC ACC ATGATGATGATGATGATG CATGGTATAACCCTTGGTGTATAGACGTT-3'
rep gene Forward	5'- CCCGT CGAC GCCTGCCCTCCCTTTTGGTG-3'
rep gene Reverse	5'-CCCT CTAGA ATAGTCTGGAACAGCGCACTT-3'
cat Forward	5'- CCC ACTAGT GCTGCATTAATGAATCGGCCA-3'
cat Reverse	5'- CCC AAGCTT CCCT CTAG AGAATAAATACCTGTGACGGAAGATCACTTC-3'

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

to a previously described method (4). With the minimization of the size, the transformation efficiency of the pNSGroE vector in *B. abortus* strain RB51 increased 452 times compared to the pBBGroE vector. Both plasmids were found to be stable after eight serial passages over 24 days on tryptic soy agar (TSA) plates in the absence of chloramphenicol selection.

Western blot analysis was performed by loading the same amount of protein extracted from strain RB51pNSGroE/GFP and RB51pBBGroE/GFP, and membranes were developed with Anti-HisG-HRP Antibody (1:5000; Invitrogen) for detection of recombinant green fluorescent protein (GFP) containing a polyhistidine sequence and monoclonal anti-GFP antibodies (1:1000; BD Biosciences Clontech, Palo Alto, CA, USA). The analyses of the band intensities were accomplished with a Kodak Digital-Science Image Station 440CF (Eastman Kodak, Rochester, NY, USA). The expression of GFP from strain RB51pNSGroE/GFP was 1.7-fold more than that of strain RB51pBBGroE/GFP as estimated by densitometry of Western blot analyses visualized using monoclonal anti-GFP antibodies (data not shown). 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 (Figure 2A), which also can be exploited for affinity purification as well as for posttranslational modification studies of the proteins expressed in *Brucella* spp.

We studied the activity of the new vector and compared the level of expression in vivo. A promoterless GFP gene was excised from pGFPuv vector (BD Biosciences Clontech) and cloned in frame downstream of the promoter in the MCS of pNSGroE and

pBBGroE and used as a visual marker of gene expression and promoter activity inside the macrophage cell line J774.A1 (5). Fluorescence microscopic examination and expression of the GFP in the macrophage cell line infected with RB51pNSGroE/GFP were visual-

ized after 4 and 24 h using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Thornwood, NY, USA). The fluorescence observed clearly demonstrates the ability of pNSGroE vector to express GFP intracellularly inside macrophages (Figure 2B). Fluorescence

Figure 2. Green fluorescent protein (GFP) expression encoded by pNSGroE in vivo and in vitro. (A) Western blot analysis showing the expression of GFP in *Brucella abortus* RB51 and the specificity and sensitivity of detection of the expressed fusion protein using anti-His antibodies. Lane 1, pNSGroE/GFP that expresses genes as a His-tag fusion; lane 2, pBBGroE/GFP does not express genes as a His fusion protein and cannot be recognized by the anti-His antibodies. (B) 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. (C) Western blot analysis showing the expression of pNSGroE vector using anti-His antibodies. Lane 1, expression of GFP in *B. abortus* strain RB51; lane 2, expression of red fluorescence protein (RFP) in *B. abortus* strain 2308; lane 3, expression of GFP in *Ochrobactrum anthropi* strain 49237.

intensity measured by flow cytometry of the infected macrophages was 2-fold higher after 24 h of macrophage infection with strain RB51pNSGroE/GFP relative to those infected with strain RB51pBBGroE/GFP (data not shown).

In order to study the expression of *Escherichia coli* β -galactosidase in *B. abortus* strain RB51, a 2.9-kb insert containing the *lacZ* gene was excised from pRSETB/ β -gal (Invitrogen) and cloned into pNSGroE and pBBGroE. The expression of β -galactosidase and enzyme activity, as calculated in modified Miller units, was compared between strain RB51pNSGroE/LacZ and RB51pBBGroE/LacZ. Without any heat-shock stimulus, the enzyme activity in strain RB51pNSGroE/LacZ was approximately twice compared to that expressed by strain RB51pBBGroE/LacZ. The β -galactosidase activity in both strains increased by 25% after heat-shock treatment for 20 min, reflecting a characteristic feature of the *groE* promoter.

Previous studies have shown that pBBR1MCS replicates and is stably maintained in all *Brucella* spp. (6). Therefore, the 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* strain 49237 (Figure 2C).

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 groE* promoter. The difference may be attributable to the increased copy number due to decreased size of the pNSGroE plasmid.

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COMPETING INTERESTS STATEMENT

The authors declare no conflicts of interest.

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Method based on electrophoresis and gel extraction for obtaining genomic DNA-free cDNA without DNase treatment

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For studying differential gene expression by reverse-transcriptase PCR (RT-PCR) applications, eliminating genomic DNA contamination from the cDNA is essential prior to amplification of the specific gene sequence. Residual genomic DNA can disturb the amplification of the target gene from cDNA and lead to false-positive results. DNase I treatment is generally used to remove genomic DNA from RNA samples. However, RNA can degrade totally or partially during the DNase treatment. It has also been shown that some contamination of genomic DNA can remain

after the DNase treatment, even after an overnight incubation (1). Certain tissue types can be a problem because some contain elevated levels of RNases (2,3) or a limited amount of RNA (4). Fruit tissues are regarded as particularly problematic because of high amounts of secondary metabolites, polysaccharides, and elevated levels of RNases (5). In our experiments with RNA from different plant tissues, especially bilberry (*Vaccinium myrtillus*) fruit and leaf, we found that RNA degraded during the DNase treatment. We also noticed that despite the DNase treatment, some