

The Sequence of the Ribosomal Binding Site Controls the Gene Expression in Brucella

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Research

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Abstract

Background

Brucella is an important pathogen causing Brucellosis. Vaccine strains obtained by a single knockout cannot combine low virulence and immunogenicity. Our study modified the SD sequence and spacer sequence of the RBS of *Brucella* to affect its protein expression. We altered the RBS of LPS-associated genes to reduce LPS-associated protein expression while retaining LPS integrity.

Results

We first established an evaluation system based on the reporter gene red fluorescent protein mCherry. The mCherry expression could be changed by altering the Shine Dalgarno sequence and spacer sequence of RBS. After optimizing the Shine Dalgarno sequence, mCherry expression was increased 4-fold in *E. coli* and decreased by 1/4 in *Brucella*. The mCherry expression was increased 1.5-fold in *E. coli* and decreased to 1/2 in *Brucella* when the length of the spacer sequence was 0. When the spacer sequence was NA (N = 4, 8, 12nt) or NG (N = 4, 8, 12nt), mCherry expression was reduced in both *E. coli* and *Brucella*. Accordingly, two mutant strains were constructed in an attempt to decrease the expression of *LptA* and *LpxO*, *Brucella* LPS-related genes, by 1/4. Silver staining experiments of LPS SDS-PAGE revealed an alteration in the composition of LPS in the two mutant strains. Polymyxin B experiments revealed that both mutant strains were more sensitive to Polymyxin B resistance.

Conclusion: In *Brucella*, the expression of the target gene could be affected by changing the length or the composition of the RBS sequence. The LPS gene remained unchanged while reducing the expression of its associated protein, achieving the original goal of reducing bacterial virulence while retaining immunogenicity. It is a promising strategy to improve the safety and efficacy of vaccines.

Background

Brucella is a Gram-negative bacterium that causes brucellosis, a widespread zoonotic disease [1-5]. By expressing different virulence factors and using various strategies to escape the host's immune response, *Brucella* is able to survive and replicate in host cells without being completely wiped out by the host, allowing Brucellosis to progress from an acute stage to chronic infection [6].

Lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria, is an important virulence factor for *Brucella* [7]. LPS is involved in bacterial survival in many hosts, contributing to complement and bactericidal peptide resistance and to bacterial adherence and entry into cells [8]. LPS contains a marked pathogen associated molecular pattern (PAMP) that can be recognized by immune cells and body fluids. It also triggers specific antibody responses and protective immunity against pathogens [9]. At the same time, LPS provides a permeable barrier that makes an important contribution to the structural integrity of the bacteria, which is linked to their viability.

LPS is constituted by a hydrophobic lipid A linked to a short, usually negatively charged, oligosaccharide core followed (smooth LPS) or not (rough LPS) by a hydrophobic O-polysaccharide (O-chain or O-antigen) [7]. However, some species of *Brucella* are of weak virulence. Bacterial virulence can be altered by deleting certain genes involved in the LPS modification regulatory pathway [7, 10]. However, this knockdown approach also reduces the immunogenicity of the strain, thus weakening the protective effect of the vaccine. Therefore, reducing virulence and retaining some immunogenicity by decreasing the protein expression of LPS-related genes would be an effective vaccine design attempt.

Gene expression requires efficient translation initiation. The initiation process is characterized by the formation of the 30S initiation complex, including the 30S ribosomal subunit, three initiation factors, the fMet-tRNA, and the mRNA at the ribosomal binding site (RBS) [11]. In bacteria, proteins are synthesized by ribosomes according to instructions encoded by mRNA sequences [12-15]. RBS affects the translation process [16]. The non-coding regions of mRNA, especially the 5'-untranslated region (5'-UTR), contribute significantly to the efficiency of protein synthesis [17, 18]. The most important 5'-UTR sequence elements in bacterial mRNAs were Shine-Dalgarno (SD) sequences, the 16S rRNA binding sites, and the spacer between the target gene start codon and the SD sequence [19, 20]. The same RBS sequence in different genetic backgrounds can also lead to large differences in protein expression levels.

The folding of transcripts into secondary structures in the RBS region is critical for the initiation of translation. Stable structure in this region may prevent ribosomes from entering the transcripts and reduce the rate of translation initiation [21, 22]. The spacer between the start codon and the SD sequence is a component of the RBS and is therefore expected to affect the folding of transcripts in this region [23-27]. The optimum distance between the SD cassette and the start codon was 5-9 nt in *E. coli* [28, 29] and 7-8 nt in *B. subtilis* [30]. It is demonstrated that the composition of the spacer sequence has a significant effect on the expression yield. The expression level is determined not only by the length but also by the different nucleotide composition of the spacer bars [30].

Bacterial protein synthesis systems are one of the main targets of biotechnological modification. The functional evaluation of protein 5'-UTR sequence elements is usually performed by monitoring the expression of their downstream reporter gene [31]. In this study, the mCherry reporter gene was introduced into *Brucella* genome in order to analyze how much the spacer sequence of RBS affects gene expression in *Brucella*, based on our experience of *E. coli*. It was confirmed that the number of bases and base composition of the RBS spacer in *Brucella* affects the expression of the downstream reporter gene mCherry. The patterns were applied to the RBS of *Brucella* LPS-related genes. They caused a reduction in the expression level of the *Brucella* LPS-related genes and thus hampered the integrity of LPS.

Results And Discussion

Construction of standard expression plasmids

SecE is a non-essential protein translocase from *Brucella abortus* 2308 vaccine strain 104M. The expression of *SecE* is controlled by a strong *Brucella* spp. promoter called P*sojA* [32, 33]. The RBS

sequence of *SecE* gene is GAGGTTGTGCCTGATCAGA constituted by the SD sequence GAGGTTGTGCCTGAT and the spacer sequence CAGA. The mCherry protein is a red fluorescent protein[34]. Accordingly, an RBS alteration evaluation system based on mCherry red fluorescent protein was established to determine the effect of RBS alteration on *Brucella* gene expression. Sequentially ligate the mCherry gene, the homologous arm of *SecE* and Kan resistance by Overlap PCR. Clone the ligation onto the pBM16A T vector and the acquired new plasmid was named as pMCH. The 5'-flanking region of *SecE* gene contained *PsojA* and RBS sequence of *SecE* gene. The pMCH is a shuttle plasmid. It not only contains the origin of *E. coli*, but also is able to recombine in *Brucella*. Transform pMCH into *E. coli*, and the new strain was named E. MCH. Integrate pMCH into the genome of *Brucella* 104M to replace the *SecE* gene, and the new strain was named B. MCH (Fig. 1a). The SD sequence of mCherry is GAGGTTGTGCCTGAT and the spacer sequence is CAGA in E. MCH and B. MCH. Due to the expression of mCherry protein, E. MCH and B. MCH were observed to be red by naked-eye, fluorescence microscope and multi-function imager. Red arrow indicates one colony. (Fig. 2). B. MCH and E. MCH transformants emitted bright red fluorescence under laser confocal microscopy. These fluorescence were distributed throughout the cells as small red dots, whose position and size were consistent with the strain. It is noteworthy that the red fluorescence of the wild strain of *E. coli* is brighter than that of *Brucella*. This may be due to the fact that mCherry is present in *E. coli* as a multi-copy plasmid, while in *Brucella* it is present as a single copy integrated into the genome.

SD sequences influence the expression of mCherry

Replace the SD sequence GAGGTTGTGCCTGAT of mCherry with AAAGGAGGT, and the new plasmid was named as pMCH1. Transform pMCH1 into *E. coli*, and the new strain was named E. MCH1. Integrate pMCH1 into the genome of *Brucella* 104M to replace the *SecE* gene, and the new strain was named B. MCH1 (Fig. 1b).

We first observed qualitatively the intensity of fluorescence by confocal microscopy. The red fluorescence intensity of E. MCH1 was increased compared to E. MCH (Fig. 3a). On the other hand, B. MCH1 caused a decrease in the fluorescence intensity compared to B. MCH (Fig. 3b). Then the fluorescence intensity of mCherry was quantitatively analyzed by a multi-functional microplate reader. The fluorescence intensity of E. MCH1 increased to 432.235% of E. MCH's and B. MCH1 decreased to 74.964% of B. MCH's, respectively (Fig. 3c, 3d). This may be due to the different nature in translation levels between *E. coli* and *Brucella*. The differences of red fluorescence intensity indicated the differential expression of mCherry protein. Thus, the changes of SD sequence influenced the expression of mCherry.

Verification of the effect of RBS with different spacer length on gene expression

It was shown that the expression level is determined not only by the length of the spacer sequence, but also by the different nucleotide composition of the spacer sequence [35]. The preliminary results of this experiment showed that the expression level of mCherry gene was not upregulated in *Brucella* after changing the optimal SD sequence. To determine the effect of spacer sequence of gene expression in

Brucella, subsequent experiments were designed to modify the spacer sequence of RBS. The fluorescence intensities of B. MCH and E. MCH were used as negative controls.

We constructed and screened mCherry RBS for different spacer sequences NA (N = 0, 4, 8, 12nt) or NG (N = 0, 4, 8, 12nt). For this purpose, plasmids containing different spacer sequences were introduced into *E. coli* and *Brucella*. The new *E. coli* strains were named as E. MCH0, E. MCH4A, E. MCH4G, E. MCH8A, E. MCH8G, E. MCH12A, and E. MCH12G. The recombination *Brucella* strains were named as B. MCH0, B. MCH4A, B. MCH4G, B. MCH8A, B. MCH8G, B. MCH12A, and B. MCH12G (Fig. 1b). Their red fluorescence intensities were observed qualitatively and quantitatively.

The differences in fluorescence intensity were first observed by confocal microscopy. E. MCH4G caused a decrease in the fluorescence intensity and E. MCH0 was increased compared to E. MCH. Meanwhile, B. MCH0 caused a decrease in the fluorescence intensity compared to B. MCH. The fluorescence of other strains was not obvious (Fig. 4a, 4b).

Then the fluorescence intensities of mCherry were analyzed by a multi-functional microplate reader. The fluorescence intensity of E. MCH0 increased to 156.693% of E. MCH's. But E. MCH4G, E. MCH8A, and E. MCH12A showed decreased to 82.572%, 7.772%, and 5.879% of E. MCH's, respectively. And the fluorescence intensities of the other strains were reduced to 0 (Fig. 4c). What's more, the fluorescence intensity of B. MCH0 and B. MCH4G decreased to 52.532% and 25.135% of B. MCH's. The fluorescence intensities of the other strains were reduced to 0 (Fig. 4d).

The results of fluorescence intensity observed by laser confocal microscopy and microplate reader were consistent. These experiments demonstrated that the composition and length of RBS spacer sequence had an influence on gene expression in *Brucella*.

RBS influence the expression of *Brucella* LPS

After obtaining the relationship between RBS and gene expression in *Brucella abortus* strain 104M, to further explore the role of RBS alterations in different *Brucella* species, the experiment was subsequently validated in a *Brucella Melitensis* 16M wild strain. Previous studies on *Brucella* related genes were performed by knockout mutations of a single gene. The strategy reduced the virulence of *Brucella*, but also reduced the immunoprotective effect as well. In this study, the genetic integrity of the strain was preserved, and in order to retain some immunoprotective effects while reducing some virulence, the RBS designing which retains 3/4 of protein expression, was chosen to modify *Brucella* LPS-related genes.

LptA and *LpxO*, were closely related to the synthesis of LPS lipid A. It was shown that when two *Brucella* LPS genes, *LptA* and *LpxO*, were knocked out separately, the mutant strain was less virulent and more sensitive to Polymyxin B as LPS integrity reduced [10]. Therefore, *LptA* and *LpxO* were chosen for this study, and the obtained mutant strains could be tested by Polymyxin B.

The gene *LptA* and *LpxO* were changed by an overlap extension PCR in the same way as described for pMCH. The RBS of the *LptA* gene was altered and inserted kan^r and the plasmid was named pA1 (Fig. 1c). The RBS of the *LpxO* gene was altered and inserted Gm^r and the plasmid was named pO1 (Fig. 1d). Since LPS is the product of multiple genes, we designed single-gene mutations and double-gene combination mutations in order to study the effect of RBS alteration of genes on LPS. After the transformation of *Brucella* 16M, two clones were screened on resistance plates, named B. A1 and B. A101.

To better investigate the relationship between the LPS content of *Brucella* and the RBS of the gene. The LPS of the mutant strains were extracted to conduct SDS-PAGE and gel silver staining. LPS consists of O-antigen, Core, and Lipid A. The breadth and color shades of the LPS bands were used as a measure of the integrity of the LPS. The O-chain was related to the type of LPS of the strain, with more pronounced bands in the smooth strain and almost blank bands in the rough strain. The difference between the LPS bands of the mutant strains and the wild strain was observed (Fig. 5). The breadth and color of O-antigen, Core and Lipid A of the LPS of B. A1 and B. A101 were weaker than 16 M. This result corroborated the RBS sequence had an impact on the integrity of *Brucella* LPS.

When it comes to the function of LPS, an intact LPS gene contributed to Polymyxin B resistance in *Brucella*. The killing efficacies of Polymyxin B on the mutant strain were examined by survival at concentrations of 50, 100, 200, and 500 $\mu\text{g}/\text{mL}$ for 1 to 5 h. The results showed that the survival of *Brucella* gradually decreased as the increase of drug concentration and treatment time. The survival of mutant strains was decreased compared with that of *Brucella* wild strain 16M (Fig. 6). The mutant strains were more sensitive to Polymyxin B than wild strain *B. melitensis* 16M.

In summary, the results of silver staining and Polymyxin B sensitivity test showed that B. A1 and B. A101 strains had weaker LPS integrity and drug resistance than wild strains. Preliminarily, the B. A1 and B. A101 mutant strains might be weak virulent strains of *Brucella* 16M strain. The virulence and immunogenicity of these three mutant strains will be further demonstrated by animal immunization experiments in future studies. This will further illustrate the relationship between RBS and bacterial virulence and immunogenicity.

Conclusions

Genetic modification is essential for *Brucella* studies. Modifying RBS is an effective way to alter gene expression. Our study evaluated the spacer sequence between SD sequence and the start codon to alter *Brucella* gene expression [21]. In this study, mCherry red fluorescent protein was used to exhibit visually the effect of RBS on *Brucella* gene expression. Accordingly, an RBS alteration evaluation system based on mCherry red fluorescent protein was established to determine the effect of RBS alteration on *Brucella* gene expression. *Brucella* strains with altered LPS gene expression was obtained after RBS transformation of *Brucella* LPS-related genes. In *Brucella*, the expression of the target gene could be affected by changing the length or the composition of the RBS sequence. The LPS gene remained

unchanged while reducing the expression of its associated protein, achieving the original goal of reducing bacterial virulence while retaining immunogenicity. It is a promising strategy to improve the safety and efficacy of vaccines.

Materials And Methods

Strains and plasmids

E. coli strains were routinely cultured in Luria Bertani (LB) broth or agar at 37°C. *B. abortus* vaccine strain 104M and *B. melitensis* strain 16M were cultured in trypticase soy agar (TSA) or trypticase soy broth (TSB) (BD, USA) at 37°C for 2-3 days. When mutation screening was required, Kan^r or Gm^r was added to the culture medium at a final concentration of 50 µg/ml. Other strains were obtained as shown in Table 1. Experimental operations related to *Brucella* 104M and 16M were performed in a Class II Biological Safety Cabinet (BSC). The plasmids used in this paper were shown in Table 1.

Prediction of RBS sequence using Bioinformatics analysis

The Ribosome Binding Site (RBS) Calculator can predict RBS sequences of a protein coding sequence in bacteria to rationally control the translation initiation rate. This tool was used to analysis the RBS sequence of *Brucella*. It is available at <https://www.denovodna.com/software/> [36].

Construction of standard expression plasmids

To generate the standard expression plasmids, approximately 1 kbp of 5'-flanking region and 3'-flanking region of the *SecE* gene were amplified by PCR using the *Brucella* 104M genomic DNA as the template. The Kanamycin resistance gene (Kan^r) cassette was PCR amplified from pMAZ-SK plasmid[37]. The red fluorescence protein (mCherry) cassette was PCR amplified from pTREX-mCherry plasmid [38, 39]. PCR was performed using PrimeSTAR high fidelity DNA polymerase (Takara, Japan). The PCR fragments were fused by overlap extension PCR and cloned into the pBM16A T vector (Biomed, China) for sequencing. The recombinant vectors were designated pMCH. The plasmids were verified by PCR and automated sequencing analysis. The primers are shown in Table S1. The plasmid pMCH1, pA1, and pA101 was constructed in the same way (primers are shown in Table S1).

Construction of different spacer sequence lengths of plasmids

The amplified fragment includes an RBS sequence that was amplified as AflIII / PstI fragments using the primers SP1 and SP2 (Table S1) from the pMCH1 plasmid. The spacer sequence CAGA of AflIII / PstI fragments was replaced by NA (N = 0, 4, 8, 12nt) or NG (N = 0, 4, 8, 12 nt)) using AflIII and MscI restriction enzymes with the oligos Sp3- Sp16 (Table S1) to obtain fragments with different spacer sequences. Subsequently, the pMCH1 plasmid was hydrolyzed using the restriction enzymes AflIII and PstI, and fragments with different spacer sequences were ligated into the pMCH1 plasmid. The acquired plasmids series contains different spacer sequences.

Transformation of *E. coli* and *Brucella*

The target fragments were cloned into a pBM16A T vector (Biomed, China). The plasmids were transformed into chemical competent DH5 α . Pick up the single colony for sequencing (Biomed, China) (The *E. coli* strain obtained is shown in Table 1 for comparison with *Brucella*). The plasmid DNA was isolated and transformed into *Brucella* 104M or 16M electro-transformed competent, and was screened for resistance. By using the homologous recombination function of *Brucella* itself, the homologous parts of the plasmid were integrated into the chromosome, and different mutants were constructed (The *Brucella* strain obtained is shown in Table 1).

Confocal microscope observation

Strains were harvested through centrifugation for 1 min at 12,000rpm. The sediment was suspended in 4% formaldehyde for immobilizing the cell for 20min. Subsequently, the sediment was resuspended in PBS. Preparing agarose pads and imaging bacteria using agarose pads [40, 41]. Image the fixed bacteria using agarose pads during the imaging process, allowing to perform color fluorescence microscopy experiments. A Zeiss LSM880 laser scanning confocal microscope (Zeiss, Germany) was used to examine the red fluorescent of the transformants.

Detection of fluorescence intensity

This approach was designed to investigate the effect of changing RBS on the fluorescence intensity of *Brucella*. The relative fluorescence units (RFU) of bacteria were measured using a multi-functional microplate reader (Omega FLUOstar). To reduce the error caused by the concentration of the bacterial solution, the OD₆₀₀ of the bacterial solution was measured. Accordingly, the relative fluorescence intensity was expressed as RFU/OD₆₀₀ for the bacterial solution. For samples, a PBS well was used as a control.

Silver staining analysis of *Brucella* LPS

LPS was obtained by LPS Extraction kit (iNtRON Biotechnology, South Korea) and was quantified by LPS ELISA kit (Beijing Yanbidin technology, China). Purified LPS was separated in 12.5% SDS-PAGE gels, and the gels were silver stained by Protein Stains Q (Sangon Biotech, China).

Sensitivity of *Brucella* to Polymyxin B

Strains were harvested through centrifugation for 1 min at 12,000rpm. The sediment was resuspended in PBS. The final concentrations of 50, 100, 200, and 500 μ g/mL Polymyxin B were added into bacteria liquid of *Brucella* cultured for 1 to 5 h at 37°C and 200 rpm. Then, 100 μ L of the diluted solution was plated on the TSA agar medium, and the plates were incubated at 37°C for 72 h. Calculate of the survival rates of mutant strains under the pressure of Polymyxin B.

Statistical analysis

All results were presented as mean \pm standard deviation (SD). Statistical analysis was performed using Prism 9.0 (GraphPad Software, CA, USA). T student test and one-way analysis of variance (ANOVA) with Dunnett's correction were applied. P-values were indicated as ns \geq 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 indicating the significant difference. All experiments were repeated at least three times.

Table 1. Strains and plasmids used in this work. The materials were stored in the laboratory.

Strain name	Description	Source
<i>E. coli</i> DH5α	General-purpose host strain for subcloning	Lab preservation
<i>Brucella</i> 104M	<i>B. abortus</i> vaccine strain, originally isolated from the fetus of an aborted cow in the former Soviet republic	Laboratory preservation
<i>Brucella</i> 16M	<i>B. melitensis</i> strain, is the virulent reference strain.	Laboratory preservation
E. MCH / B. MCH	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , RBS origin sequence (GAGGTTGTGCCTGATCAGA), mCherry protein, Kan ^r	This study
E. MCH1 / B. MCH1	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , RBS predict increased sequence (AAAGGAGGTCAGA), mCherry protein, Kan ^r	This study
E. MCH0 / B. MCH0	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), mCherry protein, Kan ^r	This study
E. MCH4A / B. MCH4A	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), spacer sequence (AAAA), mCherry protein, Kan ^r	This study
E. MCH4G / B. MCH4G	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), spacer sequence (GGGG), mCherry protein, Kan ^r	This study
E. MCH8A / B. MCH8A	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), spacer sequence (AAAAAAAAA), mCherry protein, Kan ^r	This study
E. MCH8G / B. MCH8G	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), spacer sequence (GGGGGGGG), mCherry protein, Kan ^r	This study
E. MCH12A / B. MCH12A	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), spacer sequence (AAAAAAAAAAAAA), mCherry protein, Kan ^r	This study
E. MCH12G / B. MCH12G	(<i>E. coli</i> DH5α / <i>Brucella</i> 104M) P _{sojA} , SD sequence (AAAGGAGGT), spacer sequence (GGGGGGGGGGGG), mCherry protein, Kan ^r	This study
B. A1	(<i>Brucella</i> 16M) RBS predict increased sequence (AAAGGAGGTAACC), <i>LptA</i> gene, Kan ^r	This study
B. A101	(<i>Brucella</i> 16M) RBS predict increased sequence (AAAGGAGGTAACC), <i>LptA</i> gene, Kan ^r , RBS predict increased sequence(AAAGGAGGTTGAG), <i>LpxO</i> gene, Gm ^r	This study

Plasmid name	Description	Source
pMAZ-SK	<i>E. coli</i> vector, mCherry protein, Kan ^r	Laboratory preservation[37]
pTREX-mCherry	mCherry protein	Laboratory preservation[38, 39]
pMCH	pBM16A T vector, P _{sojA} , RBS origin sequence (GAGGTTGTGCCTGATCAGA), mCherry protein, Kan ^r	This study
pMCH1	pBM16A T vector, P _{sojA} , RBS predict increased sequence (AAAGGAGGTCAGA), mCherry protein, Kan ^r	This study
pMCH0	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, mCherry protein, Kan ^r	This study
pMCH4A	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, spacer sequence (AAAA), mCherry protein, Kan ^r	This study
pMCH4G	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, spacer sequence (GGGG), mCherry protein, Kan ^r	This study
pMCH8A	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, spacer sequence (AAAAAAAA), mCherry protein, Kan ^r	This study
pMCH8G	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, spacer sequence (GGGGGGGG), mCherry protein, Kan ^r	This study
pMCH12A	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, spacer sequence (AAAAAAAAAAAA), mCherry protein, Kan ^r	This study
pMCH12G	pBM16A T vector, P _{sojA} , SD sequence AAAGGAGGT, spacer sequence (GGGGGGGGGGGG), mCherry protein, Kan ^r	This study
pA1	pBM16A T vector, RBS predict increased sequence (AAAGGAGGTAACC), LptA gene, Kan ^r	This study
pA101	pBM16A T vector, RBS predict increased sequence (AAAGGAGGTAACC), LptA gene, Kan ^r , RBS predict increased sequence (AAAGGAGGTTGAG), LpxO gene, Gm ^r	This study

Amp^r, Resistance to ampicillin; Kan^r, Resistance to kanamycin; Gm^r, Resistance to gentamicin

Abbreviations

LPS: Lipopolysaccharide; PAMP: Pathogen associated molecular pattern; mRNA: Messenger RNA; rRNA: Ribosomal RNA; RBS: Ribosomal binding site; SD sequence: Shine-Dalgarno sequence; *E. coli*:

Escherichia coli; *B. subtilis*: *Bacillus subtilis*; UTR: Untranslated region; LB: Luria bertani; *B. abortus*: *Brucella abortus*; *B. melitensis*: *Brucella melitensis*; TSA: Trypticase soy agar; TSB: Trypticase soy broth; Kan^r: Resistance to kanamycin; Gm^r: Resistance to gentamicin; Amp^r: Resistance to ampicillin; BSC: Biological safety cabinet; DNA: Deoxyribonucleic acid; PCR: Polymerase chain reaction; RFU: relative fluorescence units; OD: Optical density; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gelelectrophoresis; SD: standard deviation.

Declarations

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Not applicable.

Authors' contributions

Conceptualized the idea: LZ and SL. Experiments designed: LZ, SL, PW, SL. Experiments conducted: SL. Data analyzed and results discussed: SL, PW, HX. Manuscript written: SL, PW, YD. Edited, reviewed, and approved: LZ and YD. All the authors helped in reviewing the whole manuscript. All authors read and approved the final manuscript. SL and PW contributed equally to this article.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

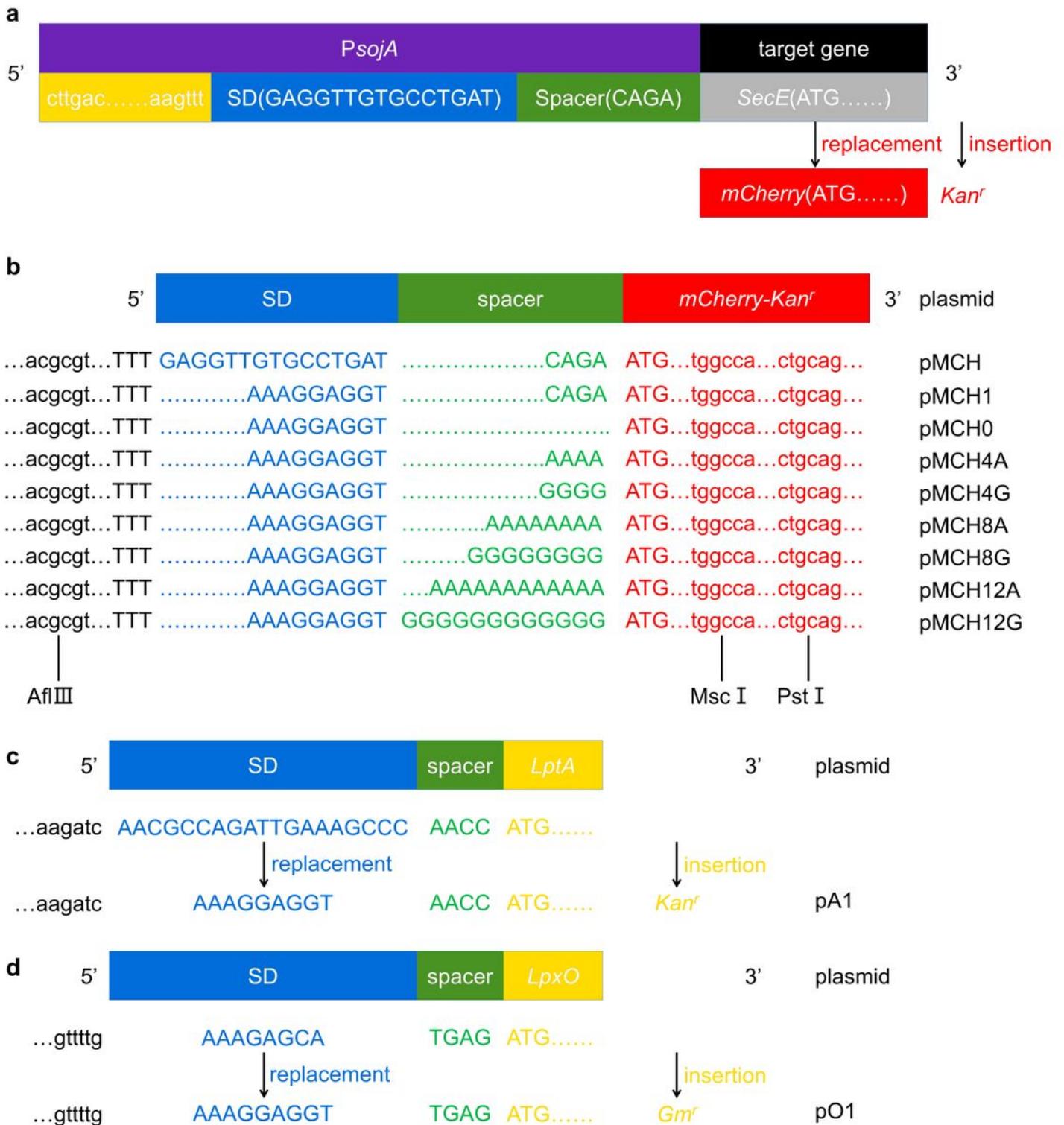


Figure 1

Nucleotide composition of RBS of plasmids. (a) The expression of target gene (black) was controlled by a strong *Brucella* spp. promoter called *PsojA* (purple). SD sequence (blue), spacer (green) and start of *SecE* (grey) were highlighted. The *mCherry* gene (red) was integrated into the genome of *Brucella* 104M to replace the *SecE* gene, and the new strain was named *B. MCH*; (b) Nucleotide composition of RBS of plasmids pMCH, pMCH1, pMCH0, pMCH4A, pMCH4G, pMCH8A, pMCH8G, pMCH12A, pMCH12G. SD

sequence (blue), spacer (green) and start of target gene (red) were highlighted. The cleavage sites of labeled AflIII, MscI, and PstI were used to construct RBS of different spacer sequences; (c) SD sequence (blue), spacer (green) and start of LptA (yellow) were highlighted. The SD sequence (blue) aacgccagattgaaagccc was replaced by AAAGGAGGT, and the new plasmid was named pA1; (d) SD sequence (blue), spacer (green) and start of LpxO (yellow) were highlighted. The SD sequence (blue) aaagagca was replaced by AAAGGAGGT, and the new plasmid was named pO1.

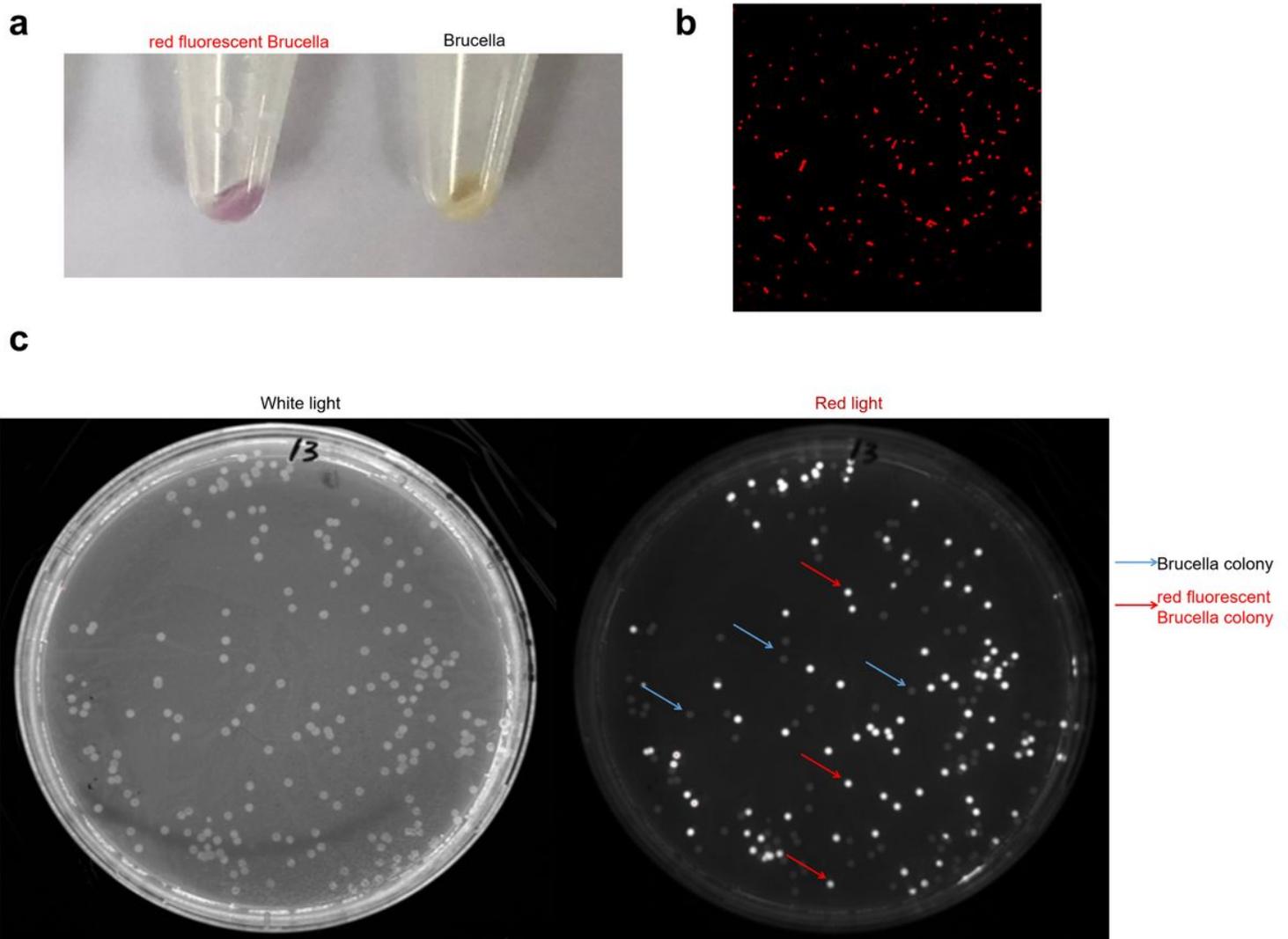


Figure 2

Images of strains with red fluorescent protein. (a) naked-eye; (b) fluorescence microscope; (c) multi-function imager.

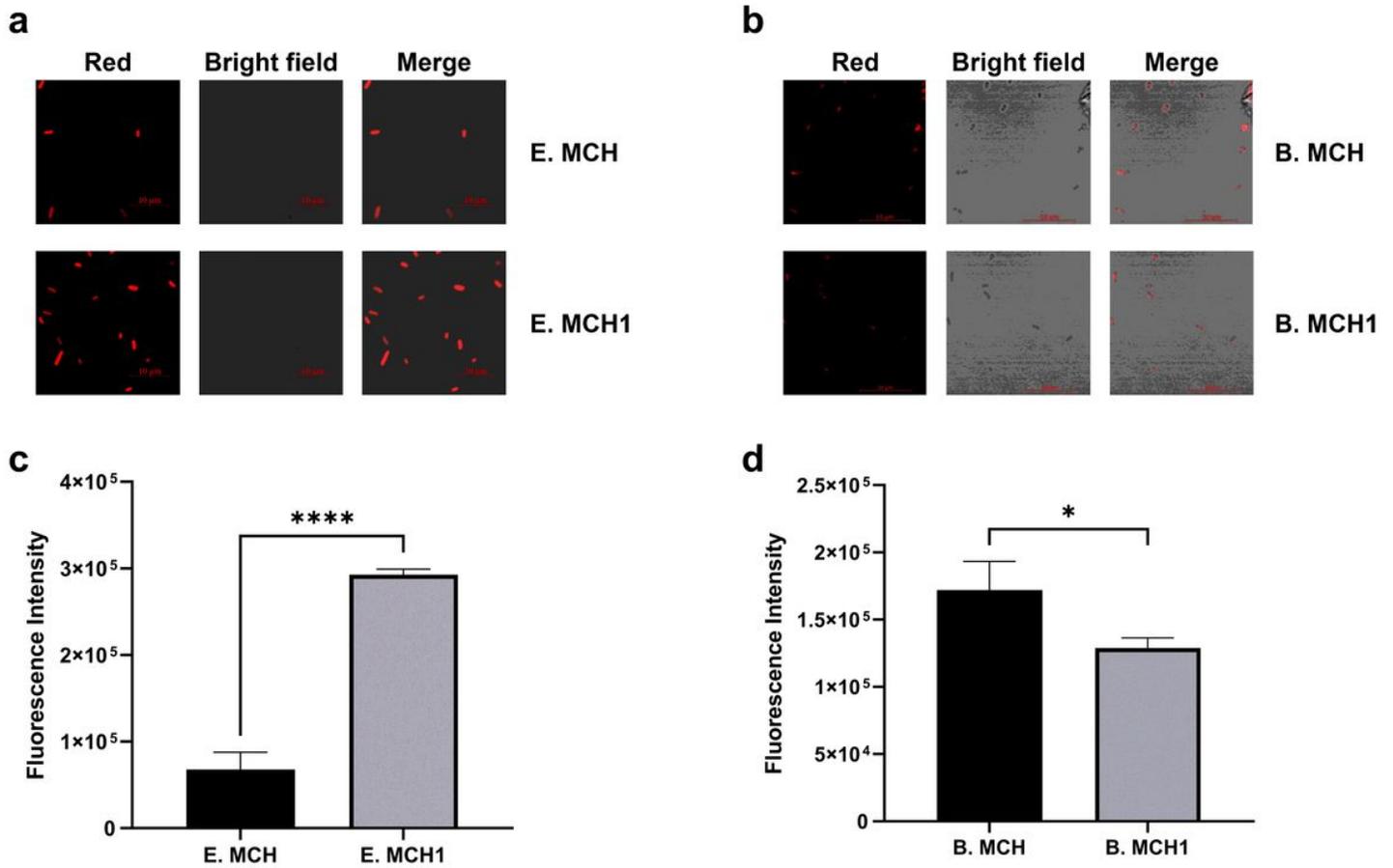


Figure 3

Effect of SD sequences on gene expression in *E. coli* and *Brucella*. (a) The difference of fluorescence intensity between E. MCH and E. MCH1. ****, $P < 0.0001$; (b) The difference of fluorescence intensity between B. MCH and B. MCH1. *, $P < 0.05$; (c) Images of confocal microscope showed red fluorescence of E. MCH and E. MCH1; (d) Images of confocal microscope showed red fluorescence of B. MCH and B. MCH1.

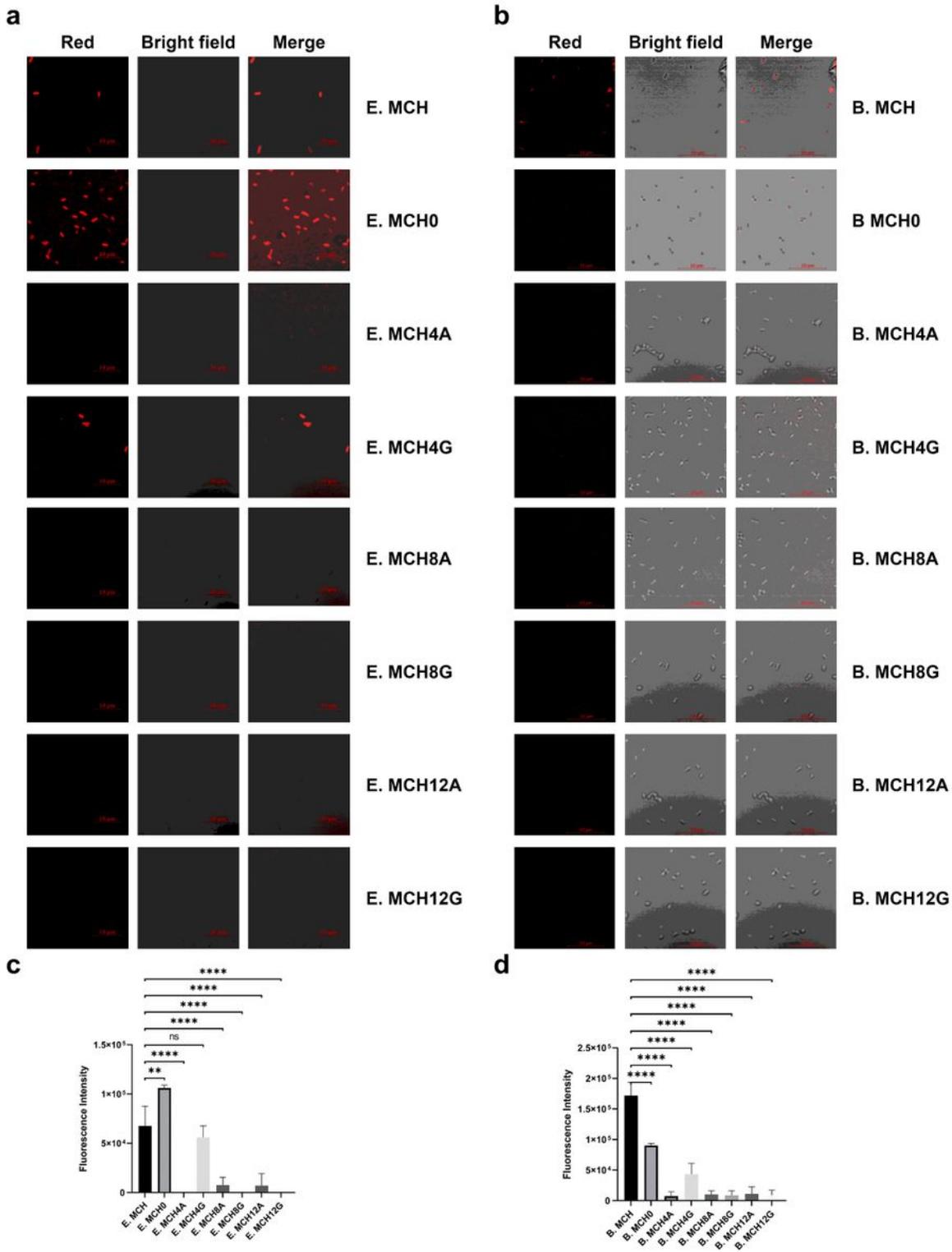


Figure 4

Effect of spacer sequences on gene expression in *E. coli* and *Brucella*. (a) The difference of fluorescence intensity between *E. MCH* and *E. MCH0*, *E. MCH4A*, *E. MCH4G*, *E. MCH8A*, *E. MCH8G*, *E. MCH12A*, *E. MCH12G*. ns, $P \geq 0.05$. **, $P < 0.01$. ****, $P < 0.0001$; (b) The difference of fluorescence intensity between *B. MCH* and *B. MCH0*, *B. MCH4A*, *B. MCH4G*, *B. MCH8A*, *B. MCH8G*, *B. MCH12A*, *B. MCH12G*. ****, $P < 0.0001$; (c) Images of confocal microscope showed red fluorescence of *E. MCH* and *E. MCH0*, *E. MCH4A*, *E.*

MCH4G, E. MCH8A, E. MCH8G, E. MCH12A, E. MCH12G; (d) Images of confocal microscope showed red fluorescence of B. MCH and B. MCH0, B. MCH4A, B. MCH4G, B. MCH8A, B. MCH8G, B. MCH12A, B. MCH12G.

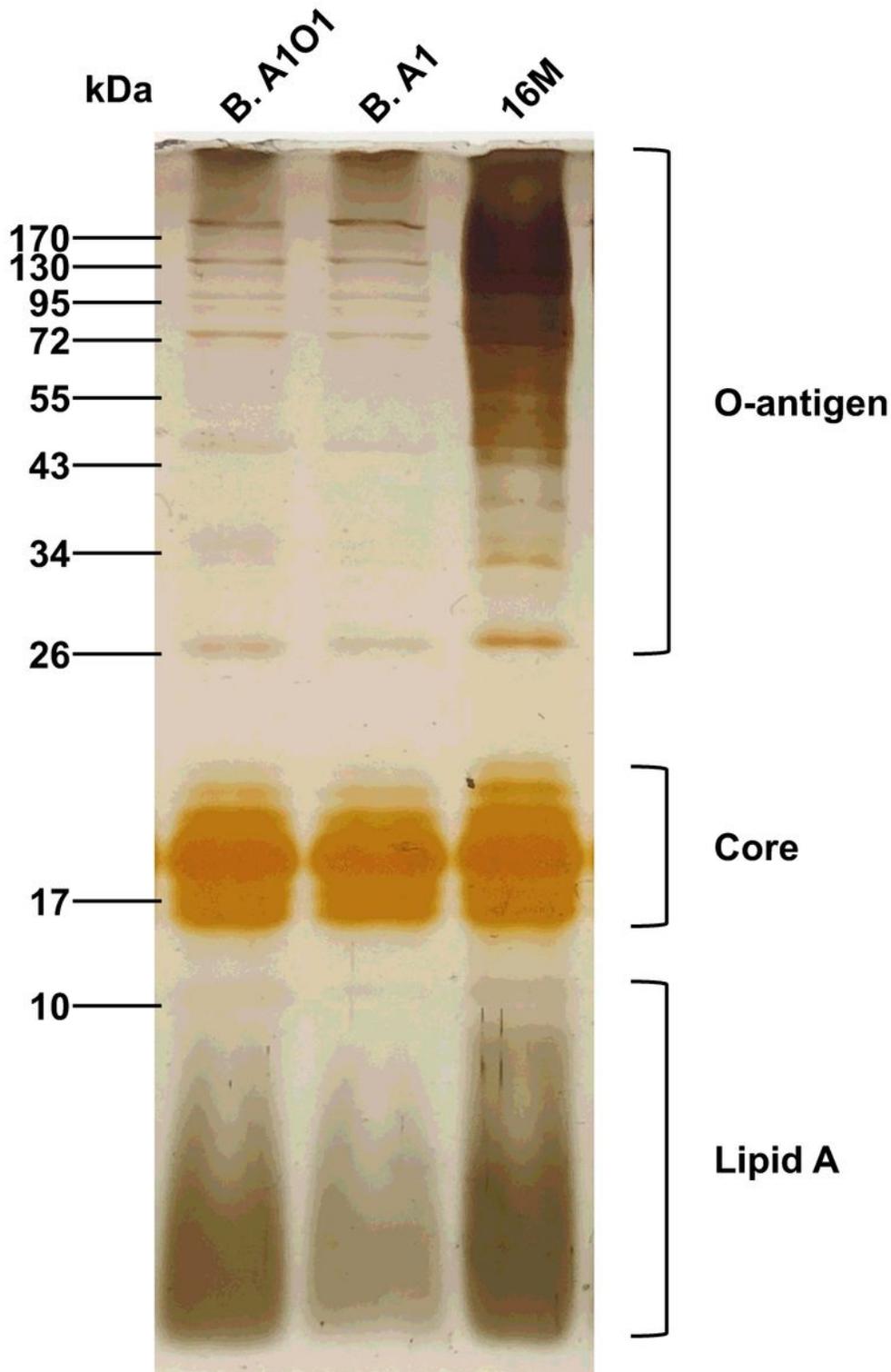


Figure 5

The picture of SDS-PAGE gel of silver staining. The LPS, after extracted from the Brucella mutant strains, was visualized by silver staining. LPS consists of O-antigen, Core, and Lipid A. The breadth and color of

O-antigen, Core and Lipid A of the LPS of B. A1 and B. A101 were weaker than 16 M.

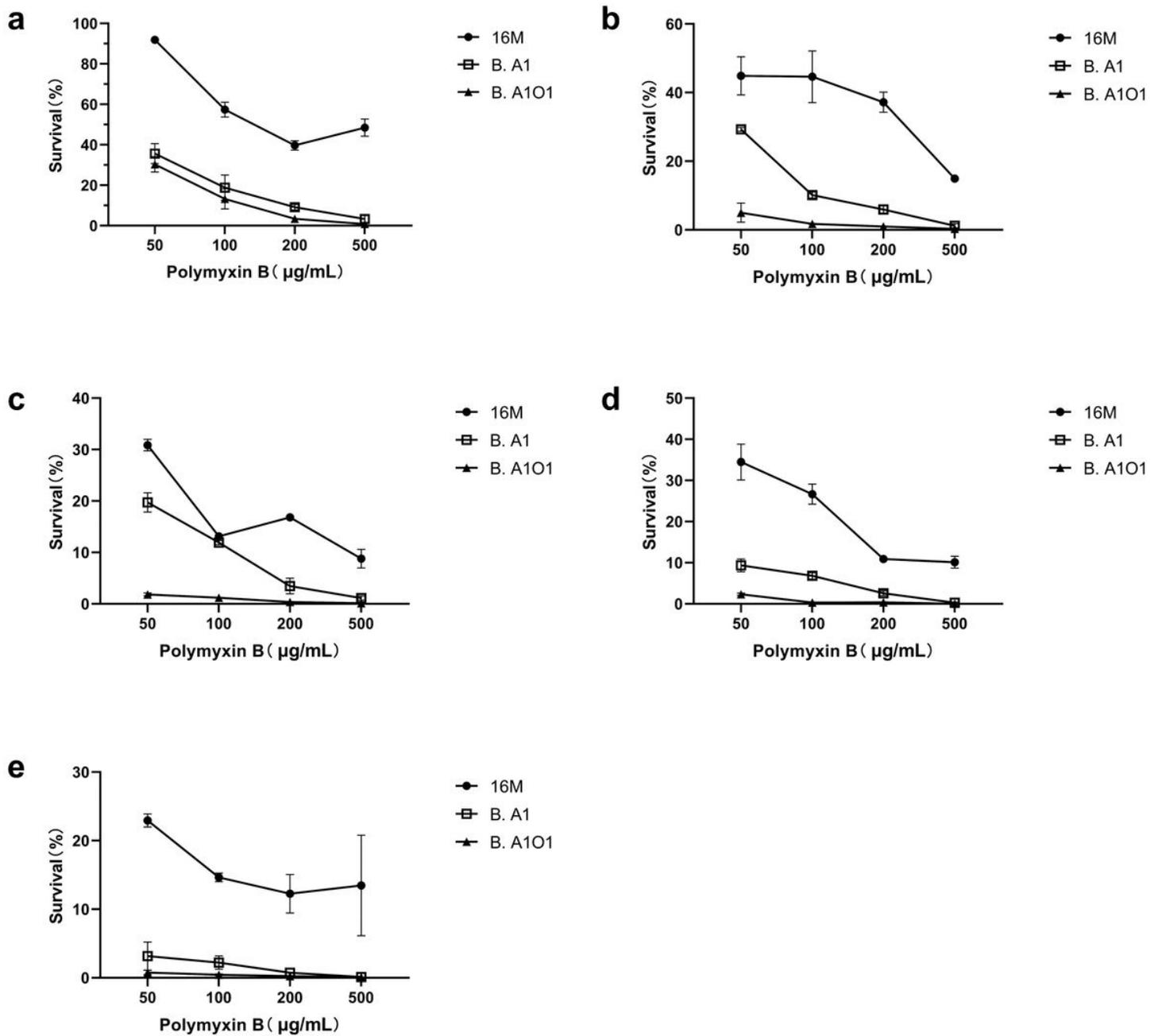


Figure 6

Survival curves of *Brucella* under the treatment of Polymyxin B at concentrations of 50, 100, 200, and 500 µg/mL for 1h (a), 2h (b), 3h (c), 4h (d) and 5h (e).

Supplementary Files

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