Green fluorescent protein as a detection marker for Coxiella burnetii transformation

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Received 20 February 1999; received in revised form 22 April 1999; accepted 23 April 1999

Abstract

The molecular biological study of the obligate intracellular bacterium Coxiella burnetii is hampered because of the lack of an efficient DNA transformation system. We used expression of the green fluorescent protein (GFP) in addition to ampicillin resistance as a selection marker for detection of transformed C. burnetii cells. Fluorescent microscopy studies revealed that transformed C. burnetii cells can be detected easily inside the host cell line. A high level of GFP expression was reached with the strong Escherichia coli trc (trp/lac) promoter. The use of GFP not only provides a convenient marker for transformation of C. burnetii, but also allows detection of this obligate intracellular pathogen inside host eukaryotic cells. Possible applications for GFP in the study of host-pathogen interactions are discussed. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Coxiella burnetii; Green fluorescent protein; DNA transformation

1. Introduction

Coxiella burnetii is an obligate intracellular pathogen that grows, reproduces and differentiates within an acidic phagolysosome compartment of eukaryotic host cells and was recognized as the etiologic agent of Q fever in 1937. There are many restrictions to the study of this organism since no axenic growth medium has been developed. C. burnetii isolates normally are grown in embryonated chicken eggs and it is also possible to obtain relatively high titers in L929 fibroblast cell culture [1].

Although there is little information concerning gene expression in this microorganism, it is clear that some C. burnetii genes can be expressed in Escherichia coli. Recently, an autonomous replication sequence (ars) was isolated as a 5.8-kb fragment from C. burnetii chromosomal DNA; it is able to initiate plasmid replication in E. coli [2]. This ars, located on the pSKO(+)1000 plasmid, was used in the first successful transformation of C. burnetii by electroporation [3]. However, this approach is labor intensive and time consuming. To make the transformation of C. burnetii suitable for general use fast-
er detection of transformants is required. This study describes the construction of plasmid pGFP-CB with a reporter gene (gfp) encoding the green fluorescent protein which allows easier detection of C. burnetii transformants by fluorescent microscopy.

2. Materials and methods

2.1. Bacterial strains, cell line and culture media

Bacterial strains and plasmids used are listed in Table 1. E. coli strains were grown in L broth. The Nine Mile strain of C. burnetii phase II (164 egg passages) was propagated in chick embryo yolk sacs. C. burnetii was purified in a sucrose gradient as described previously [4]. After purification, the cell density was determined by the method described by Ormsbee et al. [5]. The usual yield of C. burnetii was 2.5 x 10^10 cells from one yolk sac. These cells were stored in PBS buffer (136.9 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 2.4 mM KH_2PO_4, pH 7.2) with 10% glycerol at -70°C prior to use for electroporation.

Mouse L929 fibroblasts were grown in minimum essential medium (MEM) with 10% heat-inactivated fetal calf serum (FCS) and 0.06% glutamine at 37°C.

2.2. Electroporation of C. burnetii and infection of L929 cells

C. burnetii strain Nine Mile phase II was electroporated using BTX 820 Electroporator at 3 kV, 99 μs and 10 impulses. Plasmid pGFP-CB (0.5 μg) was used to transform 100 μl of C. burnetii cell suspension with a cell density of 2.5 x 10^10 cfu ml^-1. The cell suspension was pelleted, washed and resuspended in deionized water before electroporation.

L929 monolayers (in 75-ml flasks) were overlaid with 90 μl of electroporated C. burnetii cells. The cells were allowed to absorb at 37°C for 2 h, after which monolayers were fed with 10 ml of MEM with 10% CFS, 0.06% glutamine and 0.6 mg ampicillin. Infected L929 cells were cultivated in monolayers for approximately 60 days in the presence of ampicillin at which time persistent infection was established. Infected cells were passaged up to 20 times, and medium was changed about every 3 days.

2.3. Isolation of C. burnetii cells from L929 fibroblasts

The tissue culture medium with infected L929 cells was harvested after trypsinization regularly following each passage of infected cells (each passage was separately stored at -70°C). Infected cell suspensions from some early and late passages were lysed by freezing and thawing and disrupted in a homogenizer [6]. The lysate was centrifuged at 3000 x g for 10 min to separate the rickettsial suspension and the cell debris. C. burnetii cells from supernatant were pelleted by centrifugation of at 30,000 x g for 30 min at 4°C. The pellet was saved after washing with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2). Cell debris that still contained C. burnetii particles, determined by Gimenez staining [7], was subjected to five vortex-lysing-centrifugation cycles in lysis buffer (22.3 mM K_2HPO_4, 135.7 mM KCl, 13.4 mM NaCl, 89 mM glycine, 10 mM MgCl_2, 1 mM glucose, 1 mM glutamate, 250 mM sucrose, pH 7.0). After centrifugation at 3000 x g for 10 min the supernatants were pooled and centrifuged at 30,000 x g for 30 min at 4°C. Rickettsial pellets were combined and stored in preservation buffer (PB) [8] at -20°C prior to analysis by fluorescent microscopy.

2.4. Light and fluorescent microscopy

A fluorescent microscope was employed for detection of expressed GFP in cell line L929 infected with electroporated C. burnetii cells, in C. burnetii cells alone and in E. coli cells. E. coli strain IB419 containing plasmid pGFP-CB was cultured in 10 ml of LB medium at 37°C. The cell culture was collected 3 h after induction of gfp transcription by IPTG. 400-μl culture samples were transferred to 1.5-ml Eppendorf tubes, collected by centrifugation and the pellet resuspended in PB. Epifluorescence micrographs were recorded on Kodak transparency film with a Nikon E 400 microscope using a filter set (420–480 excitation, DM500 dichroic reflector, and 515 emission filters). Film transparencies were digitized and the epifluorescence micrographs were prepared for printing using CorelDraw 6.0 software.
2.5. Genetic techniques

Methods for transformation of E. coli strains, selection of antibiotic resistance markers and other standard genetic techniques were carried out as described previously [9]. Amplification of DNA by PCR was carried out using standard methods [10]. The total DNA from infected L929 cell line was isolated after disruption of cells as described above [6].

As a source of cycle 3 gfp gene, we used the plasmid pGFP-N1uv which is a derivative of pGFP-N1 from Clontech, in which the BsrGI-XbaI fragment from wild-type gfp was replaced by the cycle 3 variant of gfp from pBAD-GFPuv [11].

PCR amplification of the probe for Southern blot hybridization was accomplished using the primers 5'-ATGGGTAAAGGAGAAAGA-3' (located at position 1–18 bp of the gfp coding region) and 5'-GTA-GAGCTCATCCATGC-3' (located at position 713–696 bp of the gfp coding region). After cleaning with Magic PCR (kit supplied by Promega), this specific probe was labeled by the incorporation of [α-32P]-dCTP (Amersham) into DNA by using an oligomer random priming kit (Boehringer). Southern blot hybridization was carried out at high stringency conditions as described previously [9].

3. Results and discussion

3.1. Construction of pGFP-CB vector and its introduction into C. burnetii

We constructed the expression vector pKK-GFP by cloning a 1.8-kb NcoI fragment, containing the cycle 3 variant of gfp, into the NcoI backbone of pKK233-2 plasmid. The gfp in this case was obtained from the partial digest of pGFP-N1uv. This reconstruction placed the gfp gene under the control of the strong IPTG-inducible promoter trc (a trp-lac fusion promoter). Even a very small colony (less than 0.5 mm in diameter) of the strain expressing gfp from the trc promoter was detected easily on LB agar plates using a standard UV light box, 365 nm (not shown).

We cloned a 5.8-kb EcoRI fragment from pSKO(+)1000, containing an ars from C. burnetii, into the EcoRI partially cut backbone of pKK-GFP, creating our the gfp reporter vector pGFP-CB (Fig. 1). It has been shown that plasmid pSKO(+)1000 can be transformed into C. burnetii and selection for ampicillin resistance can be used to detect transformants [3]. These authors showed that stable transformants maintained this plasmid in the chromosome as a result of homologous recombination and that plasmid sequence was also located in an episome. pGFP-CB was introduced into C. burnetii by electroporation as described in Section 2. In contrast to the first described transformation of C. burnetii [3] we used the less virulent phase II instead of phase I.

It was shown that long-term ampicillin treatment results in a group of antibiotic-resistant C. burnetii cells, which do not maintain the bla gene [3]. Therefore, additional reference markers, for instance GFP, are necessary for fast, reliable and inexpensive detection of true transformants.

3.2. Visualization of transformed C. burnetii in the L929 cell line by fluorescent microscopy

We detected C. burnetii transformants containing the gfp sequence by fluorescent microscopy. We were

Fig. 1. The physical map of pGFP-CB plasmid. Ptrc represents the trp-lac fusion promoter; 5S RNA, T1 and T2 terminators are part of the rrnB region; origin of E. coli replication is marked ori E. coli; ampicillin resistance gene and gfp coding region are marked bla and gfp.
Fig. 2. Fluorescent microscopy of GFP-associated fluorescence in *E. coli* (A), in *C. burnetii* cells (B) and in *C. burnetii* cells inside L929 cell line (C and D). All figures (A–D) are processed as described in Section 2 and the scale bar represents 2 μm.

Table 1
Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype, phenotype and relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><em>E. coli</em></td>
<td><em>endE thiA hsdR17 supE44</em></td>
<td>Backman et al. [12]</td>
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<tr>
<td>JM109</td>
<td><em>recA1 endA1 gyrA496 thi hsdR17Δlac-proAB</em></td>
<td>Yanisch-Perron et al., 1985</td>
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<td>IB501</td>
<td>JM109 strain transformed with pKK-GFP plasmid</td>
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</tr>
<tr>
<td>IB502</td>
<td>JM109 strain transformed with pGFP-CB plasmid</td>
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<tr>
<td>Nine Mile</td>
<td>phase II</td>
<td>This work</td>
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<td><em>C. burnetii</em></td>
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<td>J. Kormanec</td>
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<td>Promega</td>
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able to visualize the GFP signal as whole cell *C. burnetii* fluorescence inside the L929 cell within 10 days (3–5 passages) after electroporation of pGFP-CB (Fig. 2C). In this case we were able to detect *C. burnetii* as individual cells inside the host cell. After more passages the infection of L929 by transformed *C. burnetii* cells was more evident and in many cases we detected L929 cells with strong whole cell fluorescence, as it is likely that these cells are fully filled with parasite cells expressing GFP just before lysing (Fig. 2D). To compare the expression of GFP in both *E. coli* and *C. burnetii* we scanned the fluorescence signal of IB502 cells after induction with IPTG and the GFP signal from isolated *C. burnetii* transformed cells (Fig. 2A,B). Although Fig. 2A,B shows a substantial difference in intensity of scanned fluorescence signals in both microorganisms, it does not mean that expression of GFP from the promoter $P_{trc}$ in *E. coli* is higher than in *C. burnetii*. The plasmid pGFP-CB has the *E. coli* origin of replication from pBR322 and it should have about 15 copies of plasmid per cell in *E. coli* [12]. However, we have no precise information about its copy number in *C. burnetii* but it should be rather low [3].

3.3. Verification of transformants by PCR and Southern blot hybridization

To further confirm that the pGFP-CB plasmid DNA was transformed into *C. burnetii* we used PCR with total extracted DNA of infected L929 cell line. We obtained a specific PCR product 0.7 kb in length repeatedly using template DNA from transformed *C. burnetii* that was collected after four or more passages in the L929 cell line. As a negative control we used the total DNA from similarly treated cell line samples that were mixed with *C. burnetii* cells and the pGFP-CB plasmid (not electroporated into *C. burnetii*). We obtained no visible band from this control PCR. Only a 0.7-kb PCR product from transformed *C. burnetii* cells hybridized with a labelled gfp DNA probe (Section 2) using Southern blot (results not shown).

3.4. Future applications of GFP in study of *C. burnetii*-host interaction

Fig. 2 shows that *C. burnetii* expressing gfp is easily visualized within live cell lines with epifluorescence microscopy. Intracellular *C. burnetii* cells producing GFP were clearly detectable during many passages and thus this method should allow us to image long-term interactions between live pathogen and live host cells. *C. burnetii* cells transformed with plasmid pGFP-CB can be used for the study of colonization, multiplication and transmission of this pathogen in its hosts. The use of GFP for *C. burnetii* will not only allow easier detection of transformants in classical genetic methods not yet existing for this intracellular pathogen, but GFP can also be a good detection marker for study of expression levels directly in this microorganism.

Acknowledgments

The authors would like to thank Herbert A. Thompson for supplying plasmid pSKO(+)-1000, for comments on the manuscript and for encouragement. We also thank DeEtte Walker for comments on the manuscript. In addition, we thank Ján Kormanec for supplying plasmid pGFP-N1uv, Eva Závodská for use of the fluorescent microscope and Eva Gajdošová for supplying the L929 cell line and technical advice. This work was supported in part by Grants VEGA 3002 and VEGA 5025, both from the Slovak Academy of Sciences.

References

ceedings of the IIIrd International Symposium, Bratislava (Kazár, J. and Toman, R., Eds.), pp. 146–149.


