Constitutive and Inducible Green Fluorescent Protein Expression in Bartonella henselae

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Received 18 March 1998/Returned for modification 23 April 1998/Accepted 12 May 1998

The green fluorescent protein (GFP) gene was expressed on a plasmid in B. henselae, and GFP-expressing bacteria were visualized by fluorescence microscopy. HEp-2 cells infected with GFP-expressing bacteria were separated from uninfected cells with a fluorescence activated cell sorter. Promoter fusions of B. henselae chromosomal DNA to gfp were examined by flow cytometry, and a B. henselae groEL promoter fusion which induced expression at 37°C was isolated.

Bartonella henselae, a gram-negative, fastidious, rod-shaped bacterium, is the infectious agent responsible for several human diseases including cat scratch disease in immunocompetent hosts and bacillary angiomatosis and bacillary peliosis hepatitis in immunocompromised hosts (13, 15). The domestic cat is the primary reservoir of B. henselae (9, 11, 14), and the cat flea, Ctenocephalides felis, has been shown experimentally to be the vector in cat-to-cat transmission (2, 10). Transmission to humans is associated with traumatic contact with an infected cat (15, 19).

B. henselae interacts with human cells has been investigated in vitro with HEp-2 and HUVEC cell lines (1, 3, 6). Specific bacterial virulence genes have yet to be identified for this organism due to a lack of genetic tools. Introduction into this organism was the first important step toward developing genetic tools (5). Here we describe a green fluorescent protein (GFP) reporter system that has been instrumental in the study of several bacterial pathogens (20, 22).

A promoterless gfp gene, gfpmut3 (4), was cloned into the BamHI and SphI sites downstream of the ptac promoter in pCom100, making pANT4 (see Table 1 for descriptions of all strains and plasmids). GFP was expressed constitutively because a kanamycin cassette disrupted the lacP repressor gene. pANT4 in SM10pir was transferred to 882str via bacterial conjugation. Our conjugal transfer technique was similar to that described previously (5) with these exceptions: cultures were pelleted and washed twice in Luria-Bertani broth for conjugation. Our conjugal transfer technique was similar to that described previously (5) with these exceptions: cultures were pelleted and washed twice in Luria-Bertani broth for conjugal transfer. Our conjugal transfer technique was similar to that described previously (5) with these exceptions: cultures were pelleted and washed twice in Luria-Bertani broth for conjugation. Our conjugal transfer technique was similar to that described previously (5) with these exceptions: cultures were pelleted and washed twice in Luria-Bertani broth for conjugal transfer.

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>E. coli&lt;sup&gt;a&lt;/sup&gt;</td>
<td>end-1 hsdR17 (rK&lt;sup&gt;−&lt;/sup&gt;) supE44 thi-1 x&lt;sup&gt;−&lt;/sup&gt; recA1 gyrA96 relA1 Δ(lacIZY40-arf)U169 deoR F&lt;sup&gt;R&lt;/sup&gt;[F&lt;sup&gt;−&lt;/sup&gt; proA endA1 &lt;br&gt;lacI&lt;sup&gt;q&lt;/sup&gt;lacZ&lt;sup&gt;ΔM15&lt;/sup&gt; Δ(groES-groEL) &lt;br&gt;(lac-proAB)]</td>
<td>8</td>
</tr>
<tr>
<td>SM10pir</td>
<td>thi-1 the leu&lt;sup&gt;+&lt;/sup&gt; lac&lt;sup&gt;−&lt;/sup&gt; supE&lt;sup&gt;−&lt;/sup&gt; recA1 &lt;br&gt;Rp4-2-24 &lt;br&gt;M&lt;sup&gt;−&lt;/sup&gt; K&lt;sup&gt;12&lt;/sup&gt; apr&lt;sup&gt;+&lt;/sup&gt;</td>
<td>16</td>
</tr>
<tr>
<td>B. henselae&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Str&lt;sup&gt;+&lt;/sup&gt; derivative of ATCC 49882 selected with 200 μg of streptomycin per ml</td>
<td>J. Peck</td>
</tr>
<tr>
<td>882str</td>
<td>First 882str strain carrying a GFP plasmid; neither stably expresses GFP nor maintains plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>882-ANT4</td>
<td>Selected 882-GFP that stably expresses GFP and stably maintains plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>882-ANT5</td>
<td>J. Peek</td>
<td></td>
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<tr>
<td>JK13</td>
<td>Human isolate from cutaneous BA lesion</td>
<td>J. Kochler</td>
</tr>
<tr>
<td>JK13str</td>
<td>Str&lt;sup&gt;+&lt;/sup&gt; strain of JK13 selected with 200 μg of streptomycin per ml</td>
<td>G. Murakawa</td>
</tr>
<tr>
<td>JK13-ANT5</td>
<td>JK13str carrying pANT5; stably expresses GFP and maintains plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>BtgroEL</td>
<td>JK13str carrying pANT-3 with B. henselae groEL promoter cloned upstream of gfpmut3</td>
<td>This study</td>
</tr>
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<td>Btcon-1, Btcon-2, Btcon-3</td>
<td>DNA fragment containing a constitutive promoter cloned upstream of gfpmut3</td>
<td>This study</td>
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<td>Plasmids</td>
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<td>pCom100</td>
<td>onRSF1010 bla&lt;sup&gt;−&lt;/sup&gt; mob&lt;sup&gt;−&lt;/sup&gt; lacP : pir K&lt;sup&gt;−&lt;/sup&gt;</td>
<td>R. Valdivia</td>
</tr>
<tr>
<td>pANT4</td>
<td>pCom100 with gfpmut3 under ptac control</td>
<td>This study</td>
</tr>
<tr>
<td>pANT5</td>
<td>pANT4 selected to be stable in B. henselae</td>
<td>This study</td>
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<tr>
<td>pRS201</td>
<td>onRSF1010 mob&lt;sup&gt;−&lt;/sup&gt; kan&lt;sup&gt;−&lt;/sup&gt; strep&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>pRK600</td>
<td>onRSF30108 cam&lt;sup&gt;−&lt;/sup&gt; tra&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>pANT4</td>
<td>onRSF30108 mob&lt;sup&gt;−&lt;/sup&gt; kan&lt;sup&gt;−&lt;/sup&gt; strep&lt;sup&gt;−&lt;/sup&gt;; promoterless gfpmut3</td>
<td>This study</td>
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<sup>b</sup> All E. coli strains were grown in Luria-Bertani media supplemented with appropriate antibiotics at 37°C.

<sup>c</sup> B. henselae strains were grown on heart infusion agar supplemented with 5% RB and appropriate antibiotics at 37 or 26°C and 5% CO<sub>2</sub>.

<sup>d</sup> Str<sup>+</sup>, streptomycin resistant; BA, bacillary angiomatisis.
pression in the absence of antibiotic selection. After three consecutive passages on nonselective media, only 5% of the 882-ANT4 population had a mean fluorescence above 700. From this minority population, a single, highly fluorescent clone, 882-ANT5, was selected. After five serial passages on nonselective media, the 882-ANT5 population maintained consistently a mean fluorescence of about 700. Sequence analysis comparing the $\text{ptac}$ promoter region in pCom100 to the $\text{ptac}$ promoter region in pANT5 (the plasmid isolated from 882-ANT5) revealed a single-base-pair transition in the $-35$ consensus sequence from TTGACA to TTGGCA. The $\text{gfpmut3}$ gene sequence in pANT5 was unchanged compared to the original $\text{gfpmut3}$ sequence (4). pANT5 was conjugated into strain JK13str, a more invasive strain for HEp-2 cells (17), creating JK13-ANT5, which constitutively and stably expresses GFP after two passages on nonselective media. By flow cytometry, we could distinguish and separate JK13-ANT5 from nonfluorescent $B.~\text{henselae}$ (Fig. 1b).

$B.~\text{henselae}$ was reported to enter HEp-2 cells (1). GFP-expressing $B.~\text{henselae}$ was used to visualize intracellular bacteria. HEp-2 cells seeded onto glass coverslips were infected with JK13-ANT5 for 2 h at a multiplicity of infection (MOI) of 10, and then the cells were fixed with 3.7% formaldehyde. Polyclonal rabbit anti-$B.~\text{henselae}$ antibody was added for 30 min, and the cells were washed with phosphate-buffered saline. Then, tetramethyl rhodamine B isothiocyanate (TRITC)-conjugated anti-rabbit antibody was added for 30 min, and the cells were again washed. Only extracellular bacteria were labeled with antibody because the cells were not permeabilized. JK13-ANT5 bacteria inside cells were visualized easily by fluorescence microscopy (Fig. 1c).

GFP-expressing $B.~\text{henselae}$ could be used as a fluorescent marker for separating infected cells from noninfected cells with a fluorescence-activated cell sorter (FACS). HEp-2 cells were infected with JK13-ANT5 at a MOI of 10 for 2 h. Whole cells were resuspended in RPMI (5% fetal calf serum), and infected cells were separated from uninfected cells with the FACStar cytometer (Becton Dickinson) (Fig. 1d).

Little is known about $B.~\text{henselae}$ gene regulation and expression. To identify constitutive and inducible $B.~\text{henselae}$ promoters, a promoterless GFP vector, pANT3, was constructed from pRS201. A promoterless $\text{gfpmut3}$ gene was

**FIG. 1.** (a) $B.~\text{henselae}$ strain 882-ANT4 expresses GFP. Bacteria were visualized with an epifluorescence microscope with a fluorescein isothiocyanate (FITC) filter. (b) JK13-ANT5 has about a 475-fold-higher mean fluorescence intensity ($x$) than non-GFP-expressing bacteria. Fluorescence levels were examined with a FACS calibur scanner (Becton Dickinson). Cell Quest software (Becton Dickinson) was used for the analysis and quantitation of fluorescence. (c) Intracellular bacteria can be distinguished from extracellular bacteria by antibody labeling. All bacteria express GFP. However, only extracellular bacteria are labeled red. Colocalization of GFP and TRITC markers renders extracellular bacteria yellow, while intracellular bacteria remain green. Cells were visualized with an epifluorescence microscope with FITC and TRITC filters to visualize green and red fluorescence, respectively. Images were superimposed with Adobe Photoshop 3.0.5 software. (d) HEp-2 cells infected with JK13-ANT5 can be distinguished from uninfected cells with the FACStar cytometer (Becton Dickinson). Cells within the gated region have cell-associated, GFP-expressing $B.~\text{henselae}$ and were separated from uninfected cells.
cloned into the PstI and EcoRI sites of pRS201, and the streptomycin resistance gene was partially removed by exonuclease deletion. A *B. henselae* promoter library was generated by cloning Sau3AI fragments (0.4 to 1.4 kb) of JK13 chromosomal DNA into the BamHI site immediately upstream of the promoterless *gfpmut3*. This library, in DH5α, was conjugated into JK13str as described above, with the addition of the helper plasmid pRK600 in DH5α. By FACS analysis, 1.33% of the library was found to be fluorescent, indicating that some DNA fragments contained constitutive promoters. Three clones, Bhcon-1, Bhcon-2, and Bhcon-3, expressed GFP at varying levels of fluorescence, as measured by flow cytometry (Fig. 2). Bhcon-1, Bhcon-2, and Bhcon-3 maintained their fluorescence levels of fluorescence, as measured by flow cytometry (Fig. 2). Bhcon-1, Bhcon-2, and Bhcon-3 maintained their fluorescence when passaged on nonselective media. A sequence analysis of these three fragments showed no homology to sequences in the GenBank database.

We then used the *B. henselae* promoter library to identify inducible promoters by differential fluorescence induction (21). In accordance with this technique, promoters that were activated at high temperature were selected as follows. The library population was grown at 26°C for 4 weeks and shifted to 37°C for 24 h, and bacteria expressing GFP were sorted by flow cytometry and plated on RB agar. This sequence was repeated to amplify clones carrying promoters that were activated only at high temperature. Most clones expressed GFP constitutively. However, one clone, BhGroEL, had a low level of GFP expression at 26°C but when shifted to 37°C experienced a fivefold increase in mean fluorescence (Fig. 3). A sequence analysis of the 500- bp insertion fragment showed that a 117-bp partial open reading frame adjacent to the GFP junction was 99% identical to the 5′ region of the gene encoding *Bartonella* heat shock protein GroEL (7).

The stable expression of GFP in *B. henselae* provides a useful method for studying the interactions of *B. henselae* with different cell types in vitro and in vivo. Animal studies, especially, will benefit from the stable GFP-expressing *B. henselae*. In addition, we have been able to identify a differentially expressed *B. henselae* gene by the differential fluorescence induction technique (21). We hope that these new genetic tools will help identify *B. henselae* virulence genes and that the pathogenesis of the organism can be studied more easily.

We are grateful to Lusijah Rott and Tim Knaak for their technical expertise with the FACS machines. We would like to extend special thanks to Joan Meccas and Lalita Ramakrishnan for critical readings of the manuscript and to Denise Monack for graphics assistance. JK13 was a generous gift from Jane E. Koehler, and Gerhard Miksch provided pRS201.

Anthea Lee was funded by NIH training grants ST32 GM07276-22 and ST32 GM07276-23.

**REFERENCES**


Editor: P. E. Orndorff