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Plasmid-Based System for High-Level Gene Expression and Antisense Gene Knockdown in *Bartonella henselae*▽†

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Six broad-host-range plasmid vectors were developed to study gene expression in *Bartonella henselae*. The vectors were used to express a β-galactosidase reporter gene in *B. henselae* and to generate antisense RNA for gene knockdown. When applied to *ompR*, a putative transcription response regulator of *B. henselae*, this antisense RNA gene knockdown strategy reduced bacterial invasion of human endothelial cells by over 60%.

The genus *Bartonella* consists of an expanding number of species, all of which are gram-negative fastidious bacilli and one of which, *B. henselae*, is the causative agent of cat scratch disease, chronic bacteremia, and, in some infected patients, highly vascularized lesions of the skin and organs called bacillary angiomatosis (4). Several virulence factors have been identified in *B. henselae* that are important in bacterial attachment, invasion, and ultimately proliferation of endothelial cells, leading to the angiogenesis seen in patients with bacillary angiomatosis. However, very little is known about how *B. henselae* regulates the genes encoding these virulence factors. Toward this end, we have identified a two-component regulatory system locus, annotated in the *B. henselae* genome as *ompR-envZ*, that when overexpressed results in bacteria that are more efficient at invading endothelial cells (B. Anderson, A. Hinchey, and A. McCord, presented at the Joint 20th National Meeting of the American Society for Rickettsiology and the 5th International Conference on Bartonella as Emerging Pathogens, Pacific Grove, CA, 2 to 7 September 2006). To further assess the role of OmpR/EnvZ in controlling virulence genes, we utilized a novel plasmid-based system to knock down *ompR* expression by means of antisense RNA (as-RNA).

The bacterial strains and plasmids and the oligonucleotides used in this study are listed in Tables S1 and S2 in the supplemental material. Kanamycin was used at a final concentration of 50 μg/ml for the selection of *B. henselae*-containing plasmids. *B. henselae* Houston-1 (ATCC 49882) (11) was grown on chocolate agar prepared with heart infusion agar base (Difco, Detroit, MI) at 37°C with 5% CO_2 and humidity to saturation. The series of six vectors reported in this study were constructed using the backbone of the broad-host-range vector pNSGroE (12), with only the promoter region replaced (Table 1). Each of the various promoters was amplified by PCR and ligated into pNS2 (see the supplemental material for details). To ensure transcription and to gauge promoter strength, a *lacZ* reporter gene was amplified and cloned into all six vectors downstream of the various promoters, resulting in His_6-tagged fusion proteins. The *lacZ* expression constructs were transformed into *Bartonella* via electroporation, and β-galactosidase assays were performed with the transformant cells as previously described (9). All amplified promoters were able to initiate transcription inside *B. henselae* greater than that seen in the wild-type *B. henselae* Houston-1 strain, with no promoter transcribing *lacZ* in pNS2. The P_{trc} hybrid promoter found in pNS2Trc (Fig. 1) generated the highest β-galactosidase activity (Table 1). Low-level activity was noted in the promoterless vector, possibly due to readthrough from the kanamycin resistance gene or other cryptic promoters. Immunoblot analysis was also performed, indicating levels of β-galactosidase protein proportional to the enzymatic activity (see Fig. S1 in the supplemental material). The results indicate that all the promoters described here may be used to express an exogenous gene in *B. henselae*; however, it is clear that some (P_{T5} and P_{trc}) are much more efficient in terms of reporter gene activity.

In order to produce various amounts of *ompR* as-mRNA, the entire coding region of the gene was directionally cloned behind the promoters in each of the vectors in the reverse orientation such that the antisense strand was transcribed. The PCR product (corresponding to the genome sequence from bases 1307363 to 1308067; http://www.ncbi.nlm.nih.gov/nuccore/NC_005956) was amplified from *B. henselae* Houston-1 genomic DNA by using primer asOmpR-F, containing a BamHI site, and primer asOmpR-R, containing an XbaI restriction site. After ligation into the vectors, the presence of the correct inserts was confirmed by sequencing and the resultant plasmids were electroporated into *B. henselae* Houston-1. The electrotransformants were used to infect the immortalized human microvascular cell line HMEC-1 (1) at a multiplicity of infection of 100 as previously described (8). The medium was aspirated, and the cells were washed three times and then lysed by the addition of 0.1% saponin. The lysates were plated to quantify intracellular bacteria. Assays were performed in trip-

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TABLE 1. Descriptions of the plasmids used in this study and the β-galactosidase activities resulting from transcription of *lacZ* directionally cloned immediately downstream of the vector-specific promoter

Plasmid	Size (bp)	Promoter	GenBank accession no.	Mean β-galactosidase activity ± SD (Miller units)
pNS2Trc	3,109	P_{trc}	EU600162	36,531 ± 3,326
pNS2T5	3,000	P_{T5}	EU600163	1,204 ± 162.5
pNS2Ch	2,917	P_{Ch}	EU600164	1,166 ± 161.4
pNS2Kan	2,823	P_{kan}	EU600165	687 ± 70.1
pNs2Amp	2,905	P_{amp}	EU600169	394 ± 67.2
pNS2GroE	2,877	P_{groE}	EU600166	273 ± 56.0
pNS2	2,680	None	NA[a]	153 ± 43.4

[a] NA, not applicable.

licate, and the mean and standard deviation were calculated for each data set. The *B. henselae* strain, with P_{trc} transcribing *ompR* as-RNA, demonstrated invasion reduced to 37% of that of the Houston-1 wild type and 40% of that of the control, with no promoter transcribing *ompR* as-RNA (Fig. 2). The P_{Ch} (46%) and P_{T5} (53%) promoters resulted in intermediate levels of *ompR* gene knockdown, with the P_{kan} (62%) and P_{amp} (70%) promoters showing the least reduction in the number of intracellular bacteria and the P_{groE} promoter showing no significant effect. It is also interesting to note that constitutive overexpression of *ompR* alone in our control strain results in a more invasive phenotype (Fig. 2).

The study of pathogenesis and gene function in *Bartonella* spp. has been limited by its slow-growing and fastidious nature as well as the limited availability of genetic tools for this bacterium. The construction of targeted mutations in *B. henselae* has been restricted to the use of strains with a streptomycin-resistant background; however, recently, knockout mutants of *B. quintana* have been made from a wild-type strain (7). A strategy such as the interference of gene expression by as-RNA may prove to be a useful alternative. To demonstrate the utility of as-RNA, we knocked down the expression of the putative transcriptional regulator *ompR* of *B. henselae* to test the hypothesis that *ompR* is necessary for cell invasion. This appears to be the case, with over a 60% reduction in the numbers of intracellular bacteria of the knockdown strains.

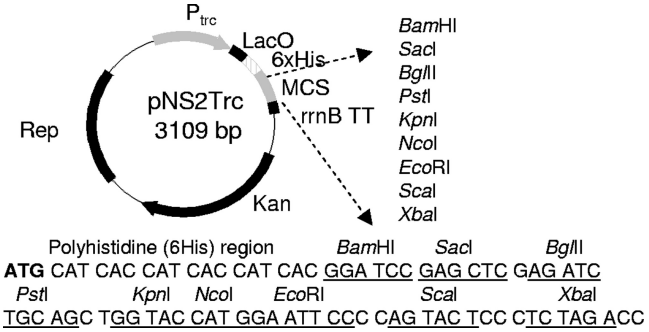

FIG. 1. Map of pNS2Trc used for gene expression and gene knockdown in *Bartonella*. P_{trc}, *trc* hybrid promoter; MCS, multiple-cloning site; Kan, kanamycin resistance gene; Rep, gene required for plasmid replication; 6xHis, His_6 tag fusion; LacO, *lac* operator; rrnB TT, T1 and T2 transcription terminators derived from the *rrnB* rRNA operon.

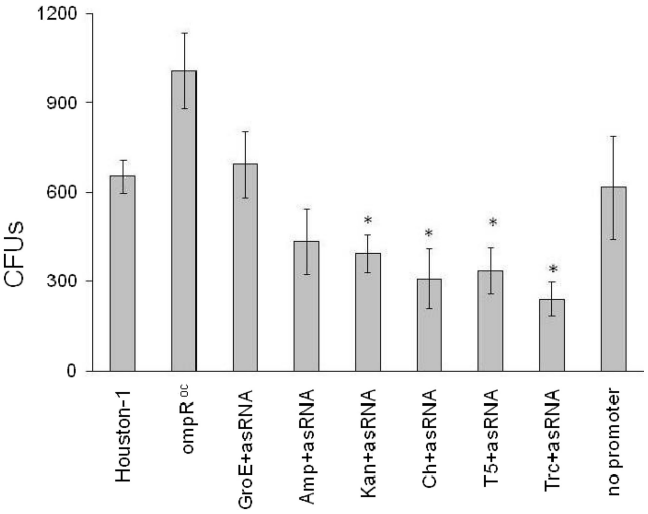

FIG. 2. Invasion of HMEC-1 cells by *B. henselae* strains harboring plasmids with various promoters transcribing *ompR* as-RNA. Infected cells were treated with gentamicin (200 μg/ml for 1 hour) to kill extracellular bacteria, and the lysate was plated to determine the number of intracellular bacteria as CFU per well for each promoter transcribing plasmid-borne *ompR*. Wild-type *B. henselae* Houston-1 with and without ("no promoter") the promoterless plasmid (pNS2) served as controls. *B. henselae* ($ompR^{OC}$) overexpressing *ompR* from the T5 promoter in pNS2T5 is also shown. $P < 0.05$ compared to Houston-1.

The interference of mRNA expression with as-RNA has been widely used in eukaryotes. as-RNA knockdown of gene expression has also proven useful for the study of gene expression and function in a limited group of bacteria (2, 3, 5, 6, 10, 13). The use of as-RNA has proven particularly useful in studying the function of genes that are lethal if completely knocked out in a given bacterium, as is the case for the RNase E (*rne*) gene of *Escherichia coli* (6) and the alkyl hydroperoxide reductase (*ahpC*) gene of *Helicobacter pylori* (2). For this reason, an as-RNA approach to diminish gene expression but not completely abolish it may be desirable for some genes. In our case, the *ompR* as-RNA corresponds precisely to the full-length *ompR* located on a plasmid, a strategy to generate as-RNA that has proven optimal for other bacteria (2).

The genetic techniques described herein should be applicable to the study of other significant organisms because the pNS2 series of vectors is known to replicate in many bacteria (12). The small size of the pNS2 vectors allows these vectors to be introduced via transformation and electroporation at greater frequencies. It is also thought that the small size of these vectors may be responsible for the increase in copy number compared to that of larger vectors with the same origin of replication (12). In addition, affinity purification of the His_6-tagged fusion protein directly from *Bartonella* should be possible. Furthermore, the use of episomally encoded as-RNA offers a simple and practical means to generate interfering as-RNA for gene function studies.

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