

Conjugative DNA transfer into human cells by the VirB/VirD4 type IV secretion system of the bacterial pathogen *Bartonella henselae*

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Bacterial type IV secretion systems (T4SS) mediate interbacterial conjugative DNA transfer and transkingdom protein transfer into eukaryotic host cells in bacterial pathogenesis. The sole bacterium known to naturally transfer DNA into eukaryotic host cells via a T4SS is the plant pathogen *Agrobacterium tumefaciens*. Here we demonstrate T4SS-mediated DNA transfer from a human bacterial pathogen into human cells. We show that the zoonotic pathogen *Bartonella henselae* can transfer a cryptic plasmid occurring in the bartonellae into the human endothelial cell line EA.hy926 via its T4SS VirB/VirD4. DNA transfer into EA.hy926 cells was demonstrated by using a reporter derivative of this *Bartonella*-specific mobilizable plasmid generated by insertion of a eukaryotic *egfp*-expression cassette. Fusion of the C-terminal secretion signal of the endogenous VirB/VirD4 protein substrate BepD with the plasmid-encoded DNA-transport protein Mob resulted in a 100-fold increased DNA transfer rate. Expression of the delivered *egfp* gene in EA.hy926 cells required cell division, suggesting that nuclear envelope breakdown may facilitate passive entry of the transferred ssDNA into the nucleus as prerequisite for complementary strand synthesis and transcription of the *egfp* gene. Addition of an eukaryotic neomycin phosphotransferase expression cassette to the reporter plasmid facilitated selection of stable transgenic EA.hy926 cell lines that display chromosomal integration of the transferred plasmid DNA. Our data suggest that T4SS-dependent DNA transfer into host cells may occur naturally during human infection with *Bartonella* and that these chronically infecting pathogens have potential for the engineering of in vivo gene-delivery vectors with applications in DNA vaccination and therapeutic gene therapy.

bacterial conjugation | transformation | bacterial infection | relaxase | *oriT*

Bacterial type IV secretion systems (T4SS) are membrane-spanning supramolecular protein assemblies that represent versatile transporters facilitating the transfer of different macromolecular substrates (protein or protein/DNA complexes) from the bacterial cytoplasm into various target cells of bacterial, mammalian, or plant origin or directly into the extracellular milieu (1). The ancestral T4SS has probably evolved to facilitate conjugative DNA transfer between bacteria (2). Conjugative plasmids encode such bacterial conjugation systems and thus promote their own spread or that of coresiding mobilizable plasmids among bacteria. Conjugation systems encode a nicking enzyme (relaxase, Mob protein) recognizing the origin of transfer (*oriT*) of the conjugative/mobilizable plasmid. Upon binding to *oriT* the relaxase nicks one strand of the dsDNA and covalently attaches to the 5' end of the resulting ssDNA. Concomitant to the elongation of the ssDNA at its 3' end by rolling-cycle replication, the relaxase then serves as a pilot protein for directing 5'-to-3' transfer of the attached linear ssDNA into the recipient cell. Recircularization of the transferred ssDNA and second strand synthesis then results in the generation of a circular dsDNA plasmid (3). Such conjugation systems not only mediate DNA transfer between bacteria; some of them have also been shown to be capable of mediating transkingdom DNA transfer into yeast (4, 5) or mammalian cells (6).

Several pathogenic bacteria have adopted bacterial conjugation systems for interkingdom transfer of macromolecular substrates into eukaryotic target cells or the extracellular milieu. These pathogenesis-associated T4SSs transport protein/ssDNA complexes like in bacterial conjugation or are reduced to protein transfer systems (1). They even may function in bacterial pathogenesis independently of any noticeable substrate transfer (7, 8).

The VirB/VirD4 system of the plant pathogen *Agrobacterium tumefaciens* is the only known pathogenesis-associated T4SS that naturally facilitates conjugation-like DNA transfer to eukaryotic host cells (9). This T4SS mediates the transfer of a complex of the VirD2 relaxase and a covalently attached single stranded transfer DNA (T-DNA) derived from the tumor-inducing plasmid into infected plant cells. With the aid of additional virulence proteins, i.e., the cotransferred ssDNA-binding protein VirE2, the T-DNA is protected and targeted to the nucleus, where it integrates into the plant genome, leading to the expression of T-DNA encoded genes, which ultimately results in tumor formation. In addition to numerous reports on structure/function analysis of this paradigmatic T4SS machinery and the development of an efficient gene delivery system for plant genetic engineering, the *A. tumefaciens* VirB/VirD4 T4SS was also reported to be capable of transforming human cells under non-physiological laboratory conditions (10). However, this plant cell-targeting DNA-delivery system is adapted to function at ambient temperatures but is entirely nonfunctional at human body temperature and therefore hitherto has failed to find any application in DNA vaccination or gene therapy.

The majority of the pathogenesis-associated T4SSs translocate bacterial effector proteins into mammalian host cells that subvert cellular functions to promote the host-associated lifestyle of the pathogen (11). Prominent examples are the Cag T4SS of *Helicobacter pylori*, which transfers the CagA effector protein into gastric epithelial cells, thereby mediating various cellular changes related to gastric disease (12); and the Dot/Icm T4SS of *Legionella pneumonia*, which transfers numerous effector proteins into amoebae or mammalian macrophages that facilitate the establishment of an intracellular bacterial replication niche (13). Of note, it has been demonstrated that some of these pathogenesis-associated T4SSs maintained their ancestral

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conjugative function; i.e., plasmid mobilization was shown for the *L. pneumoniae* Dot/Icm system between bacteria (14) or the *A. tumefaciens* VirB/VirD4 T4SS between bacteria (15) and from *Agrobacterium* to plants (16).

T4SS also play prominent roles in the pathogenesis of species of the genus *Bartonella* (7, 17). The zoonotic pathogen *Bartonella henselae* causes an asymptomatic blood infection in its cat reservoir host, whereas incidental transmission to humans causes a benign inflammation of lymph nodes in immunocompetent patients known as cat-scratch disease or a vascular proliferative disease in immunocompromised patients known as bacillary angiomatosis (18). *B. henselae* uses a VirB/VirD4 T4SS to translocate a mixture of seven *Bartonella* effector proteins (Beps) into human endothelial cells (19). These Beps contain a conserved Bep intracellular delivery (BID) domain close to the C terminus, which, together with a C-terminal positively charged tail sequence, constitutes a bipartite signal for T4SS-mediated protein translocation. Importantly, BID domains are also present in the relaxases of a subset of bacterial conjugation systems, and the BID domain of the TraA relaxase encoded by the cryptic plasmid pATC58 of *A. tumefaciens* was shown to be functional as secretion signal for the *B. henselae* VirB/VirD4 T4SS (19). This finding prompted us to test whether, in addition to its well characterized role in intracellular protein delivery, the VirB/VirD4 T4SS of *B. henselae* may be capable of mobilizing DNA into human cells. Based on derivatives of the *Bartonella*-specific mobilizable plasmid pBGR1, which encodes a relaxase (Mob) and an *oriT* (20), we demonstrate DNA transfer into human cells in a VirB/VirD4-dependent and relaxase-dependent manner, and show that fusion of the secretion signal of the native VirB/VirD4 protein substrate BepD to the relaxase greatly increases the DNA transfer efficiency. We further provide evidence indicating that breakdown of the nuclear envelope and/or active replication of the host DNA is required for expression of the eukaryotic *egfp* cassette encoded by the transferred plasmid. Finally, we demonstrate that transgenic cell lines stably expressing the transferred plasmid DNA can be selected as a result of recombination with the host cell genome.

Results

***B. henselae* Translocates Plasmid DNA into Human Cells via the VirB/VirD4 T4SS.** To test whether the VirB/VirD4 T4SS of *B. henselae* may—in addition to its role in intracellular protein delivery—be capable of mobilizing DNA into human cells, we took advantage of the *Bartonella*-specific cryptic plasmid pBGR1, which, as its only discernable genetic elements, encodes a replication protein (Rep) and respective origin of replication (*oriV*), as well as a relaxase (Mob) and respective *oriT* (20). Although the Mob relaxase of pBGR1 does not contain a recognizable BID-like domain, we wondered whether this naturally occurring mobilizable plasmid might transfer into human cells during the course of infection. To monitor plasmid transfer, we introduced into *B. henselae* WT the plasmid pRS117, which is a pBGR1 derivative carrying an *egfp* reporter cassette under control of the CMV promoter that would mediate eukaryote-specific expression of EGFP (Fig. 1A). As negative controls, we included the T4SS-deficient $\Delta virB4$ strain carrying pRS117, as well as the WT strain carrying a derivative of pRS117 in which the *mob* gene is disrupted (pRGS06). Noteworthy, despite the deficiency for effector protein secretion, the $\Delta virB4$ strain does not display reduced viability during infection of the human endothelial cell line Ea.hy926 (21). After 3-d infections of Ea.hy926 cells with the different *B. henselae* strains, the cell populations were analyzed by flow cytometry to determine the percentages of GFP-positive cells (gpc) as functional readout for gene transfer and expression of the eukaryote-specific *egfp* reporter cassette (Fig. 1B and C). DNA transfer was detectable in cells infected with the WT strain carrying pRS117 (0.02% gpc), whereas infection with both con-

trol strains did not give rise to any detectable transfer event (detection limit, 0.003%). These data clearly demonstrate the transfer of the reporter plasmid pRS117 by *B. henselae* into human cells, and that this DNA transfer process is dependent on a functional VirB/VirD4 T4SS as well as the Mob relaxase.

Fusion of Type IV Secretion Signal from Native VirB/VirD4 Substrate to Mob Dramatically Increases Efficiency of DNA Transfer into Human Cells.

In an effort to increase transfer efficiency, we fused to the C terminus of Mob the previously characterized C-terminal secretion signal of the VirB/VirD4 effector protein BepD (19), which consists of a BID-domain and a positively charged C-terminal tail. Strikingly, infections with WT *B. henselae* carrying pRS122 encoding this Mob/BID fusion protein (Fig. 1A) showed an approximately 100-fold-increased transfer rate to EaHy.926 cells compared with the pRS117 reference plasmid encoding the native Mob relaxase (Fig. 1B–D). These results support a correlation between Mob transfer rates and gene transfer rates and confirm the role of the relaxase as a pilot protein for the translocation of the plasmid DNA via the VirB/VirD4 T4SS (Fig. 1E), similarly as already demonstrated for the VirD2 protein during the delivery of the VirD2/T-DNA complex into plant cells (22).

Integration of Translocated Plasmid DNA into Host Cell Chromosome.

To test whether the DNA transferred by this VirB/VirD4-dependent mechanism can integrate into the EaHy.926 chromosome, we included a resistance marker (*neo*) to facilitate geneticin selection of stable transfected cell lines, resulting in plasmid pRS130. Southern blot analysis of the genomic DNA of eight different geneticin-resistant cell lines (A–H) revealed that the entire pRS130 plasmid or parts of it had integrated into the genome (Fig. S1). Mapping of the transferred DNA with plasmid specific probes followed by PCR-based refinement revealed heterogeneity in the DNA integration patterns (Fig. 2A, Table S1, and SI Results). In four cases, the integration was largely restricted to the plasmid region encoding the *neo* resistance marker (cell lines D, E, G, and H). In two established cell lines, evidence was found that a plasmid fragment spanning the *oriT* region had been integrated (cell lines B and F), which could reflect the transfer and integration of a plasmid concatemer, as results from rolling-cycle replication or the integration of the plasmid DNA subsequent to precise recircularization at the *oriT*. To investigate whether, as in the case of VirD2 and the bound 5' end of the *A. tumefaciens* T-DNA, the Mob relaxase can protect the plasmid DNA proximal to the *oriT* (23, 24), we mapped some representative integration borders by using thermal asymmetric interlaced (TAIL)-PCR (10, 25). Sequence analysis of the 5' integration border in cell line C revealed that the junction to chromosomal DNA has occurred next to the *oriT* of pRS130 (Fig. 2B). This junction is reminiscent of an illegitimate recombination event, as it contains a 47-bp filler DNA (24) that consists of an inverted repeat in the 22 bp upstream of the *oriT*, followed by 25 bp showing short stretches of homology to both pRS130 and the human chromosomal insertion site. The three other integration sites (cell lines A, D, and E) presented in Fig. S2 do not share these characteristics, as no sequence similarity to the *oriT* has been detected. In the four cell lines (A, C, D, and E) for which the junctions were determined, the integrations lie in unrelated sequences of chromosomes 22, 4, 2, and 7, respectively, and thus no preference for specific integration sites could be distinguished. Taken together, our results show that the Mob protein can protect the 5' end of the transferred DNA and suggest a mechanism of integration similar to what has been reported for the VirD2-bound T-DNA of *A. tumefaciens* (23, 24).

Expression of a Delivered *egfp* Gene Requires Cell Division. Several processes may interfere with the expression of ssDNA delivered into the cytoplasm of EaHy.926 cells (Fig. 1E). In the case of the

A. tumefaciens T-DNA transfer system for plant cells, the cotransferred ssDNA-binding protein VirE2 protects the naked T-DNA from degradation and assists passage of membranes by its pore-forming activity (26). Additionally, VirE2 and the 5'-bound VirD2 carry nuclear localization signals (NLSs), which facilitate import through the nuclear pore complex (27–29). In case of *B. henselae* plasmid transfer to EA.hy.926 cells, there is no evidence for the existence of such helping factors, and the Mob relaxase does not contain any noticeable NLSs. Thus, nuclear uptake of the ssDNA may exclusively occur upon disassembly of the nuclear envelope during mitotic cell division. As cell division of the Ea.hy926 cell line is contact-inhibited, we measured the percentage of gpc of cell monolayers infected at different stages of confluence. Our results indicate that the expression of the translocated DNA inversely correlated with the cell density and thus appears to depend on cell division (Fig. 3A). In contrast, protein transfer as measured by the previously reported Cre recombinase assay for translocation (19) positively correlates with cell densities and is thus independent of cell division (Fig. 3A). Time-course experiments support these notions: whereas protein transfer rates constantly increased over a period of 6 d of infection, the rates of DNA transfer/expression reached a steady

state at day 3 or 4 when the monolayers reached confluence and cell divisions ceased (Fig. 3B). Together these results suggest that the transferred DNA enters the nucleus passively after disassembly of the nuclear envelope, although we cannot exclude the sole or additional requirement for active DNA replication—which occurs during cell division—to allow recombination/integration to happen.

Discussion

T4SSs are known to transport different macromolecular substrates into diverse target cell types and thus are considered to represent the most versatile class of bacterial secretion systems (11). However, naturally occurring transkingdom DNA transfer has been described only for the pathogenic *A. tumefaciens*–plant interaction. In this report, we describe T4SS-mediated DNA transfer from a human pathogenic bacterium into cultured human cells that possibly may occur naturally during human infection. We demonstrate here that a derivative of the *Bartonella*-specific cryptic plasmid pBGR1 (20) is transferred into human cells in dependency of a functional VirB/VirD4 T4SS and the plasmid encoded relaxase. This pathogenesis-associated T4SS has most likely evolved from an ancestral bacterial conjugation

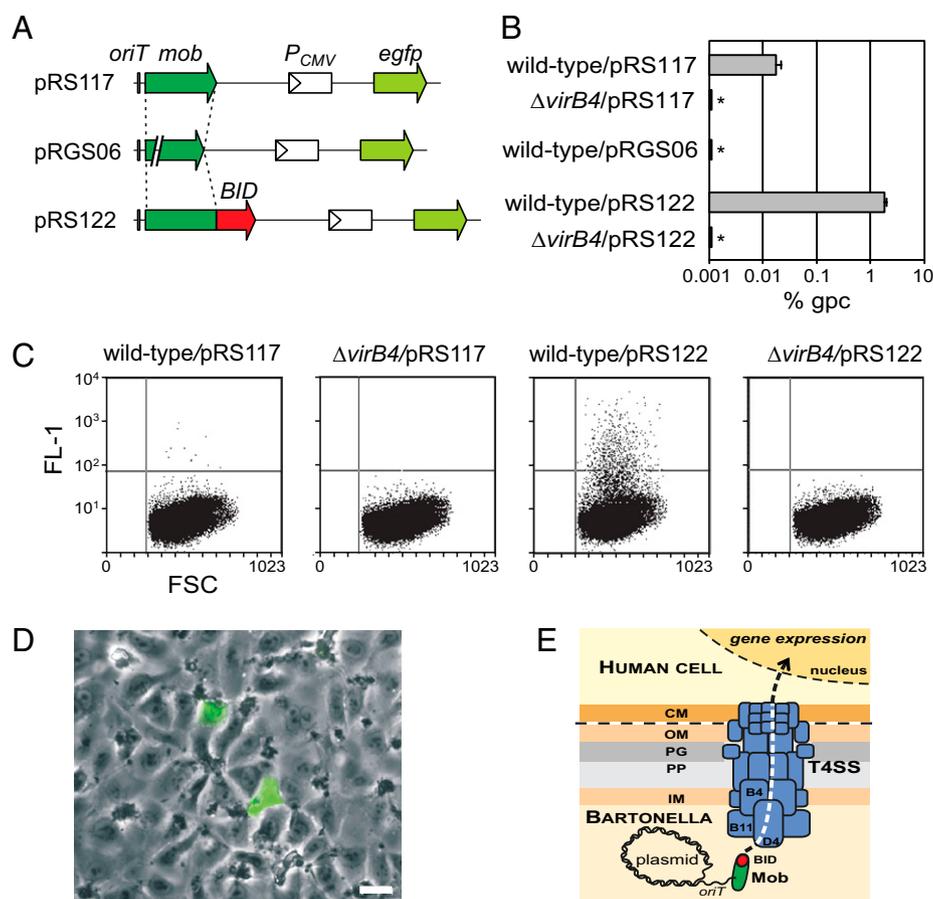


Fig. 1. *B. henselae* translocates plasmid DNA into human cells via the VirB/VirD4 T4SS. Ea.hy926 cells were infected with *B. henselae* WT or $\Delta virB4$ strains harboring the indicated plasmids. Plasmid transfer was monitored by measuring GFP fluorescence at 72 h of infection. (A) Schematic representation of the reporter plasmid pRS117 carrying an eukaryotic *gfp*-expression cassette (P_{CMV} , *egfp*) plus plasmid mobilization factors (*oriT*, *mob*). Derivatives of this plasmid include a disruption of *mob* (pRGS06) and a fusion of the C-terminal BID domain of BepD to Mob (pRS122). (B) Percentage of gpc of infected cell populations, monitored by FACS analysis. The values indicated by an asterisk are below the detection limit (<0.003%). (C) Representative FACS profiles. FL-1, relative GFP fluorescence intensity; FSC, forward scatter. (D) Fluorescence microscopic picture (GFP channel) of a cell preparation infected with *B. henselae* WT carrying pRS122. The image was overlaid with a phase contrast picture of the same microscopic field. (Scale bar: 10 μ m.) (E) Model view of the *B. henselae* VirB/VirD4 T4SS apparatus transporting plasmid DNA into a human host cell. Eleven components, including the ATPases VirB4, VirB11, and VirD4 (indicated as B4, B11, and D4, respectively), form a channel-spanning inner membrane (IM), periplasm (PP), peptidoglycan (PG), and outer membrane (OM) of the bacterium, plus the cellular membrane (CM) of the human host cell.

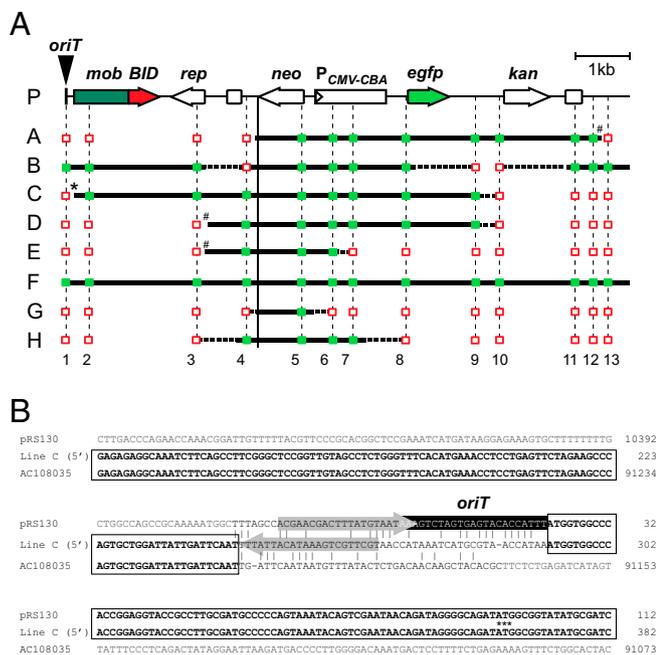


Fig. 2. Integration of translocated plasmid DNA into the host cell chromosome. Following infection of *Ea.hy926* cells with *B. henselae* strain RSE581 carrying the reporter plasmid pRS130, eight individual cell lines (named A–H) where isolated. (A) Schematic representation shows which parts of plasmid pRS130 have integrated into the genome of recombinant cell lines A–H, based on Southern blot analysis (Fig. S1 and S1 Results) and PCR analysis (Table S1). A total of 13 different primer pairs (numbered 1–13) were tested on each cell line for presence (green box) or absence (red open box) of the targeted sequence. Note that the leftmost amplicon spans the *oriT* of pRS130. The border presented in B is indicated by an asterisk and the borders presented in Fig. S2 are marked by a number sign (#). (B) Nucleotide sequence alignment of the *oriT* region of pRS130, the isolated integration junction of the transferred DNA [line C (5')], and the human DNA sequence (accession no. AC108035). The *oriT* of pRS130 [as defined in the parental plasmid pBGR1 (20)] is highlighted in black, and the start codon of the *mob* gene is marked by three asterisks. Homology of the junction fragment to pRS130 or AC108035 is indicated by open boxes. In the 47-bp spacer region that does not show colinearity to either donor or recipient sequence, the 22 nucleotides upstream from the *oriT* are found as an inverted element (gray arrow). Homologies to pRS130 or AC108035 within these 47 bp are indicated; 240 bp of the isolated fragment, centered around the integration site, are displayed.

system into an efficient device for effector protein delivery into mammalian host cells (2). Considering the evolutionary origin of this secretion system, the capacity to transfer DNA may not necessarily serve a particular virulence function like in *A. tumefaciens*, but may rather represent a relic of its ancestral role as conjugation system. This is also suggested by the notion that the cryptic plasmid pBGR1 encodes proteins with exclusive functions in plasmid replication and mobilization, i.e., the replication protein Rep and the relaxase Mob, respectively. Moreover, the lack of an efficient ssDNA protection and nuclear delivery system in the recipient host cells supports this assumption. Finally, our analysis demonstrated that the Mob relaxase can protect the 5' end of the transferred DNA during the transformation process, as described for the VirD2 protein of *A. tumefaciens* (30, 31).

The adaptation of the *B. henselae* VirB/VirD4 T4SS to human cells and its versatility for DNA transfer increases its potential for an application not only for protein therapy as described previously (19), but also for gene therapy and vaccination. In contrast to most other established gene delivery systems (32–34), conjugative DNA transfer may allow the transfer of very large

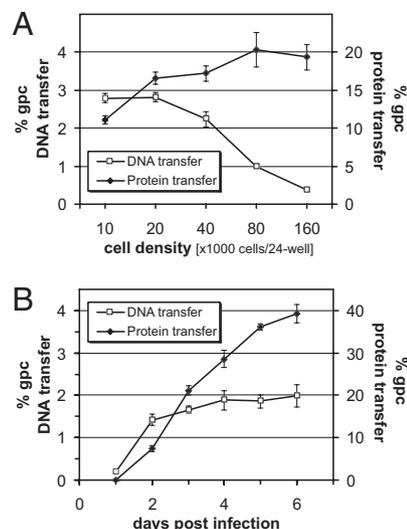


Fig. 3. Expression of a delivered *egfp* gene requires cell division. GFP expression resulting from DNA transfer or protein transfer was measured as a function of initial cell density or as a function of time post infection as percent gpc. (A) Host cells were seeded into 24-well plates at increasing cell densities (indicated in cells per well) to obtain different degrees of confluence. Bacterial strains RSE556 (DNA transfer) or RSE308 (protein transfer by Cre recombinase assay for translocation) were added at a multiplicity of infection of 200 and percent gpc levels were measured after 3 d. (B) Host cells (80,000 cells per well) were infected as before and percent gpc levels were monitored between day 1 and day 6 after infection.

DNA segments such as bacterial artificial chromosomes. By drastically increasing DNA transfer efficiency via fusion of the C-terminal secretion signal of BepD to the Mob relaxase, we have made a step toward the development of a potential tool for in vivo gene therapy in humans. Improvements of this system by assisting the steps following gene delivery, for increasing the efficiency of nuclear targeting and genomic integration, would need to follow.

Materials and Methods

Bacterial Strains, Cell Lines, and Growth Conditions. *B. henselae* and *Escherichia coli* strains were grown as described (21, 35). Bacterial strains used in this study and their origins are listed in Table S2. The endothelial cell line *Ea.hy926*, generated by fusion of human umbilical vein endothelial cells and the lung carcinoma cell line A549 was cultured as described previously (36). The cell line *Ea.hy926/pRS56-c#B1* was cultured as described previously (19).

Plasmid Constructions. DNA manipulations were carried out according to standard procedures (37). The plasmids used in this study are listed in Table 1. Plasmid DNA isolation and PCR purification were performed with Qiagen or Macherey and Nagel columns according to the manufacturer's instructions. *E. coli* NovaBlue was used for cloning steps and the *dap⁻* *E. coli* strain β 2150 for plasmid mobilization to *B. henselae* (38). For construction of pRS117, the 1861 bp *HincII*-*AflIII* fragment of pWay19 (gift from the Molecular Motion Laboratory, Montana State University, Bozeman, MT) containing the *egfp* gene under the control of the CMV promoter was blunt-ended by using Klenow polymerase and ligated with *HincII* cleaved pBGR-K18, a derivative of pBGR1 (20). The plasmid containing the *egfp* gene and the *kan* gene in the same orientation was selected, yielding pRS117. For construction of pRS122, a 567-bp fragment encoding the secretion signal of *B. henselae* BepD flanked by two *AgeI* restriction sites, obtained by using pRS51 as template and the oligonucleotides pRS350 and pRS351 (Table S3), was ligated with pRS117, and the plasmid containing the fragment in the correct orientation resulting in a fusion with the *mob* gene was selected. For construction of pRS506, pCHF01 (20) was digested with *Sall*/*XmaI*/*NcoI* and the 2,541-bp *Sall*-*XmaI* fragment containing the disrupted *mob* gene was ligated with pRS122 (*Sall*/*XmaI*). pRS130 was obtained by introducing the 1514 bp *SnaBI*-*EcoRV* fragment of pRS56 (19) containing the *neo* gene into *HincII*-cleaved pRS129. pRS129, in turn, was constructed by ligating a 2,990-bp *Sall*-

Table 1. Plasmids used in this study

Plasmid	Description	Source
pBGR1	Cryptic plasmid isolated from <i>Bartonella grahamii</i>	20
pBGR-K18	Derivative of pBGR1 endowed with the <i>kan</i> resistance gene and the ColE1 replicon of pK18	20
pRS117	Derivative of pBGR1 containing P _{CMV} / <i>egfp</i> for expression of GFP in eukaryotes	Present study
pRS122	Derivative of pRS117 wherein the secretion signal of <i>bepD</i> (aa 352–534) is fused to the 3' end of <i>mob</i>	Present study
pRGS06	Derivative of pRS122 wherein the <i>mob</i> gene is disrupted	Present study
pRS130	Derivative of pRS122 containing a <i>egfp</i> reporter gene and a <i>neo</i> gene conferring G418 resistance in transformed cell lines	Present study
pRS51	Cre vector encoding NLS::Cre::BepD (aa 352–534) reporter protein	19

PstI fragment of pCX-eGFP (39) containing an *egfp* expression cassette including the chicken β -actin promoter and the CMV-IE enhancer sequence, with pLR5122. The primer sequences are provided in Table S3.

Detection of DNA or Protein Transfer from *B. henselae* into Human Endothelial Cells. Infections of Ea.hy926 (to measure DNA transfer) and Ea.hy926/pRS56-c#B1 cells (to measure protein transfer) with *B. henselae* strains were performed as described (19, 21), and the multiplicity of infection (34) and duration of infection were as indicated in the text and figure legends. The higher protein transfer rates obtained in the present work versus the rates obtained previously (19) are related to the use of M199/10% FCS growth medium instead of DMEM/10% FCS during the infection, resulting in a higher activation of the VirB/VirD T45S (40). After infections, cells were trypsinized, recovered in growth medium, and 4×10^4 to 1×10^5 cells were analyzed by using a FACScalibur flow cytometer (Becton Dickinson).

Establishment of Stably Transformed Cell Lines. After initial infection of Ea.hy926 cells with *B. henselae* carrying pRS130 (strain RSE581), the cell growth medium was supplemented with gentamicin (20 mg/L) to kill the bacteria. Serial dilutions of the cells were then cultured in cell growth medium containing G418 (500 mg/L), and clonal colonies were propagated in this medium for isolation of individual cell lines.

PCR Analysis of the Transgenic Cell Lines. Genomic DNA isolated from the eight cell lines analyzed by Southern blot hybridization (Fig. S1 and *SI Materials and Methods*) was used for PCR detection of 13 different probes scattered across the entire sequence of pRS130. Genomic DNA from untransformed Ea.hy926 cells and the same DNA spiked with purified pRS130 were used as negative and positive control, respectively. PCR was carried out in 96-well format in 25 μ L volume per reaction containing 2.5 ng genomic DNA, 30 nM of each primer, and 1 \times Power SYBR Green PCR Mix (Applied Biosystems). Amplifications were carried out according to the manufacturer's instruction by using a StepOnePlus instrument (Applied Biosystems) and were analyzed for presence/absence of the targeted sequence by using StepOne software (Applied Biosystems). Sequences of the primers used for the PCR analysis are provided in Table S3.

Amplification of Integration Site by TAIL-PCR. TAIL-PCR was performed as described for mapping the genomic integration site of T-DNA insertions in *Arabidopsis* (25) or in HeLa cells (10). In brief, a set of three adjacent gene-specific primers (GSPs) facing the predicted DNA breakpoint of the integrated pRS130 were used in combination with two different arbitrary degenerated (AD) primers in three consecutive PCR reactions on genomic DNA isolated from the stable Ea.hy926 cell lines. The oligonucleotide sequences are provided in Table S3. The first PCR contained 100 μ g of genomic DNA, 400 μ M of dNTPs, 0.2 μ M of the first GSP, 1 μ M of either AD primer, and 2 U of FastStart Taq DNA polymerase (Roche) in 1 \times reaction buffer supplemented with GC-RICH resolution solution. PCR amplification was performed as follows: 6 min at 95 $^{\circ}$ C, followed by 10 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 63 $^{\circ}$ C, and 3 min at 72 $^{\circ}$ C, followed by one cycle at 95 $^{\circ}$ C for 30 s, 25 $^{\circ}$ C for 3 min, 72 $^{\circ}$ C for 2.5 min, and 15 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 63 $^{\circ}$ C, 2.5 min at 72 $^{\circ}$ C, 30 s at 95 $^{\circ}$ C, 30 s at 63 $^{\circ}$ C, 2.5 min at 72 $^{\circ}$ C, 30 s at 95 $^{\circ}$ C, 1 min at 44 $^{\circ}$ C, and 2.5 min at 72 $^{\circ}$ C. One microliter of the resulting reaction was used as template for the second PCR with the same reaction condition except the use of the second nested GSP, and incubated for 6 min at 95 $^{\circ}$ C followed by 15 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 63 $^{\circ}$ C, 2.5 min at 72 $^{\circ}$ C, 30 s at 95 $^{\circ}$ C, 30 s at 63 $^{\circ}$ C, 2.5 min at 72 $^{\circ}$ C, 30 s at 95 $^{\circ}$ C, 30 s at 44 $^{\circ}$ C, and 2.5 min at 72 $^{\circ}$ C. Finally, 1 μ L of the secondary reaction was used as template to set up the tertiary amplification by using a third nested GSP and incubated for 6 min at 95 $^{\circ}$ C followed by 12 cycles of 30 s at 95 $^{\circ}$ C, 30 s at 44 $^{\circ}$ C, and 2.5 min at 72 $^{\circ}$ C. The resulting amplified PCR product was cloned into a pGEM-T Easy plasmid (Promega) and analyzed by sequencing. The obtained integration site was verified by independent PCR amplification by using a primer annealing to the human genome (designed based on the obtained TAIL-PCR sequence information) and a second primer annealing on pRS130, followed by cloning and sequence analysis.

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