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Dendrimer-enabled transformation of *Anaplasma phagocytophilum*

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Abstract

Anaplasma phagocytophilum is an obligate intracellular bacterium that causes the emerging infection, granulocytic anaplasmosis. While electroporation can transform *A. phagocytophilum* isolated from host cells, no method has been developed to transform it while growing inside the ApV (*A. phagocytophilum*-occupied vacuole). Polyamidoamine (PAMAM) dendrimers, well-defined tree-branched macromolecules used for gene therapy and nucleic acid delivery into mammalian cells, were recently shown to be effective in transforming *Chlamydia* spp. actively growing in host cells. We determined if we could adapt a similar system to transform *A. phagocytophilum*. Incubating fluorescently labeled PAMAM dendrimers with infected host cells resulted in fluorescein-positive ApVs. Incubating infected host cells or host cell-free *A. phagocytophilum* organisms with dendrimers complexed with pCis GFPuv-SS Himar A7 plasmid, which carries a Himar1 transposon cassette encoding GFPuv and spectinomycin/streptomycin resistance plus the Himar1 transposase itself, resulted in GFP-positive, antibiotic resistant bacteria. Yet, transformation efficiencies were low. The transformed bacterial populations could only be maintained for a few passages, likely due to random Himar1 cassette-mediated disruption of *A. phagocytophilum* genes required for fitness. Nonetheless, these results provide proof of principle that dendrimers can deliver exogenous DNA into *A. phagocytophilum*, both inside and outside of host cells.

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Conflict of interest
None declared.

Keywords

Anaplasmataceae; *Rickettsiales*; dendrimers; intracellular bacteria; pathogen-occupied vacuole

1. Introduction

A conundrum to studying obligate intracellular bacterial pathogens is that inactivating a gene that is critical for invasion or intracellular survival prevents recovery of the mutant. With the exception of *Coxiella burnetii*, for which axenic cultivation and knock out-complementation are possible [1, 20], approaches developed to genetically manipulate these bacteria are largely inefficient [25]. Most involve isolating the organisms from their host cells, transforming them, and then reincubating them with naïve host cells to recover the transformants or chemically mutagenizing them.

Anaplasma phagocytophilum is an obligate intracellular bacterium of the Family *Anaplasmataceae* that causes granulocytic anaplasmosis in animals and humans, the latter of which is an emerging and potentially fatal infection in Europe, Asia, and the United States [24]. The *A. phagocytophilum* infection cycle initiates when an infectious dense-cored form (DC) binds to a host cell and induces its uptake into a host cell-derived vacuole, the ApV (*A. phagocytophilum*-occupied vacuole). Within the ApV, the DC transitions to the replicative reticulate cell form (RC). After the replication phase, RCs covert back to DCs, which are released as infectious progeny [23]. Munderloh and colleagues developed a method for transforming *Anaplasmataceae* species that is based on electroporation of constructs that facilitate HimarI random transposition of cassettes expressing fluorescent reporter and antibiotic resistance genes into the bacterial chromosome [9, 10]. This pioneering work paved the way for gene functional studies of multiple *Anaplasma* species as well as gene-specific disruption in *Ehrlichia chaffeensis* [5-8]. While an important step forward, this method must be performed on host cell-free bacteria. A system that circumvents the need to purify bacteria from host cells would potentially foster development of improved transformation protocols and gene functional studies in *A. phagocytophilum* and other obligate intracellular bacteria.

Polyamidoamine (PAMAM) dendrimers (dendrimers) are highly branched macromolecules that are used for gene therapy and drug delivery into mammalian cells [2, 16, 12, 19, 26]. Dendrimers are endocytosed and can be modified to improve transfection efficiency and targeting of specific cell types [22, 29]. Dendrimer-DNA complexes were used to transform *Chlamydia* spp. growing inside host cells [11, 15, 18], highlighting their potential to target vacuole-adapted bacteria. Herein, we evaluated the hypothesis that dendrimers can be used to transform intracellular and host cell-free *A. phagocytophilum*.

2. Materials and methods

2.1. Cultivation of uninfected and *A. phagocytophilum* infected host cell lines

Uninfected and *A. phagocytophilum* (strain NCH-1) infected human promyelocytic leukemic HL-60 cells and RF/6A (rhesus monkey choroidal endothelial cells) were cultured as described [3, 13].

2.2. Preparation of fluorescein isothiocyanate (FITC)-labeled dendrimers

PAMAM dendrimer generation 4.0 (1,4-diaminobutane core, amine terminated) (NanoSynthons, Mt. Pleasant, MI) was FITC labeled at a dendrimer:FITC molar ratio of 1:5. Conjugation was confirmed by proton nuclear magnetic resonance spectroscopy and light absorbance ($\lambda=490$ nm) [26]. FITC-dendrimer conjugates were lyophilized followed by reconstitution in methanol and storage at -20°C .

2.3. Monitoring uptake of FITC-labeled dendrimers into the ApV

RF/6A cells grown on coverslips in 24-well plates were infected with *A. phagocytophilum* as described [13]. Twenty-four h later, the cells were washed with PBS followed by a two-h incubation with $1\text{ }\mu\text{g}$ of FITC-conjugated dendrimers in Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich, St. Louis, MO) lacking FBS or glutamine at 37°C in a CO_2 incubator. The medium was removed, the monolayers were washed with PBS, Dulbecco's modified Eagle's medium containing 10% FBS, 15 mM HEPES, MEM non-essential amino acids (Gibco, Grand Island, NY), and 4 mM glutamine (DMEM-10) (Gibco) was added, and the cells were incubated for up to 6 h at 37°C in a CO_2 incubator. The cells were subjected to indirect immunofluorescence analysis using rabbit polyclonal antibody against the *A. phagocytophilum* major surface protein, P44 [24], and 4',6-diamidino-2-phenylindole (DAPI) as described [3]. Images were obtained using an Olympus BX-51 fluorescence microscope affixed with a disk-spinning unit or a Zeiss 700 laser-scanning confocal microscope.

2.4. Preparation of dendrimer-plasmid complexes

pCis GFPuv-SS Himar A7 [6] was kindly provided by Ulrike Munderloh (University of Minnesota). G4 dendrimers were complexed with plasmid DNA by vortexing both components in sterile water followed by incubation at room temperature for 30 min. Zeta potential and particle size were determined as described [18]. G4 dendrimers have 64 primary amine (N) groups that can form complexes through electrostatic interactions with DNA phosphate (P) groups [11]. Similar to results reported for *Chlamydia* spp. [11, 15, 18], dendrimer-DNA complexes with an N/P ratio of 8 provided superior results to those with an N/P ratio of 4. Data reported were obtained using complexes having an N/P ratio of 8.

2.5. Dendrimer-mediated transformation of *A. phagocytophilum* and analyses of transformants

For transformation of *A. phagocytophilum* growing in host cells, dendrimer-pCis GFPuv-SS Himar A7 complexes ($0.5\text{ }\mu\text{g}$ of DNA per well) or dendrimers alone were resuspended in medium lacking FBS or glutamine and incubated with either *A. phagocytophilum* infected

RF/6A or HL-60 cells in 24-well plates. Infected cells incubated with media alone were a mock transformation control. After 2 h, the cells were washed and DMEM-10 or IMDM-10 was added to RF/6A or HL-60 cell cultures, respectively. Also, host cell-free *A. phagocytophilum* DC organisms were isolated [14], washed with PBS, incubated with dendrimer-DNA complexes or controls for 2 h, resuspended in IMDM-10, and used to infect naïve HL-60 cells [23]. Beginning at 24 h post transformation, the infected cultures were maintained in the presence of spectinomycin or spectinomycin plus streptomycin (100 mg/ml each). Media that contained antibiotic was replaced three times per week and the cultures were split as needed to prevent host cell overgrowth. Cultures were monitored daily for GFP-positive *A. phagocytophilum* organisms via live cell imaging or indirect immunofluorescence analysis of fixed cells. For immunofluorescence analyses, RF/6A cells grown on coverslips or HL-60 cells cytocentrifuged onto glass slides were fixed in 4% (vol/vol) paraformaldehyde in PBS followed by permeabilization with 0.5% (vol/vol) Triton X-100 in PBS. To determine the percentage of ApVs that contained GFP-positive bacteria, the cells were screened with antibodies against GFP (Life Technologies, Eugene, OR) and P44 (the latter of which would label all *A. phagocytophilum* organisms), appropriate secondary antibodies, and stained with DAPI [4]. The number of ApVs containing GFP- and P44-positive bacteria was divided by the number of all (P44-positive) ApVs, and the quotient was multiplied by 100. For gene expression analyses, total RNA was extracted from dendrimer:DNA complex treated infected RF/6A cells and subjected to RT-PCR [4] or RT-qPCR [21] using primers listed in Table 1.

3. Results

3.1. FITC-labeled dendrimers are delivered into the ApV

To determine if dendrimers traffic into the ApV, FITC-labeled dendrimers were incubated with *A. phagocytophilum* infected RF/6A cells. Approximately $4.4 \pm 1.9\%$ of ApVs contained FITC at 0 h and $18.2 \pm 5.9\%$ 6 h post incubation. The percentage of FITC-positive ApVs did not increase beyond that observed at 6 h (data not shown). Thus, exogenously added dendrimers traverse the ApV membrane into the ApV lumen.

3.2. Dendrimer-enabled transformation of *A. phagocytophilum* growing in host cells

It was next determined if dendrimers could be used to transform *A. phagocytophilum* growing in host cells. The Himar1 transposase mediates random insertion into AT-rich sites [17, 32]. The plasmid, pCis GFPuv-SS Himar A7, carries a Himar1 transposon cassette encoding GFPuv and spectinomycin/streptomycin resistance (conferred by the aminoglycoside adenyltransferase [*aadA*] gene) plus the Himar1 transposase itself, both of which are under control of the *A. marginale* transcriptional regulator (Am-Tr) promoter [6, 8]. This construct was complexed with dendrimers and subsequently incubated with *A. phagocytophilum* infected RF/6A or HL-60 cells. The earliest post transformation time point at which GFP-positive *A. phagocytophilum* organisms were detected within either host cell type was 72 h (Fig. 2). Less than 1% of host cells contained GFP-positive bacteria. Also starting at 72 h, the cultures were maintained under antibiotic selection. RT-PCR and RT-qPCR analyses conducted on total RNA isolated from RF/6A cells that had been incubated with dendrimer-plasmid conjugates detected abundant levels of transcripts encoding GFP

and spectinomycin/streptomycin resistance at 72 and 144 h, but barely detected transcript for either target at 240 h (Fig. 3). Continued monitoring of both cultures by immunofluorescence microscopy infrequently detected GFP-positive ApVs for up to 4 weeks in RF/6A cells and 3 weeks in HL-60 cells.

3.3. Dendrimer-enabled transformation of host cell-free *A. phagocytophilum* organisms

Considering that the dendrimer-DNA complexes would have to cross the plasma membrane, ApV membrane, and bacterial cell wall to transform *A. phagocytophilum*, we assessed the ability of the complexes to transform extracellular bacteria. DC organisms isolated from infected HL-60 cells were incubated with the complexes and added to naïve HL-60 cells. ApVs harboring GFP-positive *A. phagocytophilum* organisms were detected beginning at 72 h (Fig. 4). Results regarding the transformation efficiency and period during which GFP-positive *A. phagocytophilum* organisms were detectable in culture were highly similar to those obtained by transforming the bacteria in host cells.

4. Discussion

The limited options of genetic tools available for obligate intracellular bacteria have restricted investigation of these organisms' molecular pathogenesis. Electroporation is the only method reported to transform *Anaplasma* spp [9, 10]. This method requires the isolation of bacteria from host cells, which likely contributes to its inefficiency, as only DCs are infectious [23] and the relative amenability of DCs versus RCs to electroporation is unknown. We demonstrated the applicability of PAMAM dendrimers for transforming *A. phagocytophilum* growing inside the ApV, an achievement similar to that for *Chlamydia* spp [11, 15, 18]. PAMAM dendrimers are worth considering as a tool for transforming other obligate intracellular bacteria that reside inside pathogen-occupied vacuoles. Given that gene-specific disruption methods for obligate intracellular bacteria are sub-optimal [25], dendrimers could be used to deliver asDNA oligos, as already explored for *Chlamydia trachomatis* [18], or constructs that encode asDNA against a pathogen gene of interest. Dendrimers can transform not only actively growing, intracellular non-infectious RCs but also metabolically less active, extracellular infectious DCs, making them worth considering as a means for transforming other intracellular bacteria that undergo biphasic development, such as *Ehrlichia* spp.

While dendrimers traverse into approximately 18% of ApVs, recovery of transformants was less than 1%, and recovered transformants could not be maintained beyond several passages. These results were consistent regardless of whether spectinomycin or spectinomycin/streptomycin selection was used for selection and are likely due to random Himar1 cassette disruption of *A. phagocytophilum* genes that are critical for intracellular fitness or infectivity. One way that the recovery process could be improved is by increasing the numbers of ApVs that dendrimers reach, which would potentially lead to more bacteria being transformed. Given that dendrimers have been functionally modified to improve their targeting of specific mammalian cell types and to enter via specific uptake pathways [26-31], it is reasonable to envision modification of dendrimers to enhance their specific

targeting of the ApV. The work presented herein represents an essential first step in the path of developing dendrimers as a tool for effectively transforming *A. phagocytophilum*.

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Figure 1.

FITC-conjugated dendrimers traffic into the ApV. *A. phagocytophilum* infected RF/6A cells that had been incubated with FITC-conjugated dendrimers were screened with P44 antibody and examined by immunofluorescence microscopy (A). Host cell and bacterial DNA were stained with DAPI. An arrow denotes a representative FITC-positive ApV. (B) Percentages of FITC positive ApVs at 0 and 6 h post incubation. Results are representative of two experiments with similar results. DIC, differential interference contrast.

Figure 2.

Dendrimers enable transformation of *A. phagocytophilum* inside host cells. Infected RF/6A or HL-60 cells were incubated with dendrimer-pCis GFPuv-SS Himar A7 plasmid complexes. At 72 h, the cells were fixed and analyzed by immunofluorescent microscopy to screen for GFP-expressing *A. phagocytophilum* organisms. Host cell and bacterial DNA was labeled with DAPI. Arrows indicate representative ApVs harboring GFP-positive bacteria. Results are representative of two to six experiments with similar results. DIC, differential interference contrast.

Figure 3.

Transformed *A. phagocytophilum* organisms express genes encoding GFP and spectinomycin/streptomycin resistance. (A) RT-PCR and (B and C) RT-qPCR analyses of *A. phagocytophilum* infected RF/6A cells that had been mock transformed, transformed with dendrimers only (G4), or transformed with dendrimer-pCis GFPuv-SS Himar A7 plasmid complexes (G4-GFP) were performed using *gfp* and *aadA* (aminoglycoside adenyltransferase, which confers spectinomycin/streptomycin resistance) gene specific primers at 72, 144, and 240 h. (B and C) Relative transcript levels of each target were normalized to *A. phagocytophilum* 16S rRNA transcript levels. Each RT-qPCR reaction was performed in triplicate. Results are representative of two independent experiments.

Figure 4.

Dendrimer-enabled transformation of host cell-free *A. phagocytophilum* organisms. *A. phagocytophilum* DCs were isolated from infected HL-60 cultures and treated with dendrimerpCis GFPuv-SS Himar A7 plasmid complexes, followed by incubation of the bacteria with naïve HL-60 cells. ApVs harboring GFP-positive *A. phagocytophilum* organisms were detected by immunofluorescence microscopy starting at 72 h post infection. P44 antibody was used to detect *A. phagocytophilum* bacteria. DAPI was used to stain host and bacterial DNA. The representative image was acquired on day 7.

Table 1

Primers used in this study

Designation ^I	Sequence (5' to 3')
<i>aadA</i> -Fwd	GAGGCGCTAAATGAAACCTTAAC
<i>aadA</i> -Rev	GATAAGCCTGCCTAGCTTCAA
<i>gfpuv</i> -Fwd	ACATCACGGCAGACAAACA
<i>gfpuv</i> -Rev	GTAATCCCAGCAGTTACA
<i>Ap 16S</i> -527F	TGTAGGCGGTTCGGTAAGTTAAAG
<i>Ap 16S</i> -753R	GCACTCATCGTTTACAGCGTG

^I F and R refer to primers that bind to the sense and antisense strand, respectively.