Dendrimer-enabled transformation of *Chlamydia trachomatis*

Rangaramanujam M. Kannan a,1, Hérve C. Gérard b, Manoj K. Mishra a, Guangzhao Mao c, Sunxi Wang c, Mirabela Hali b, Judith A. Whittum-Hudson b, Alan P. Hudson b,*1

a Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD 21235, USA
b Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, USA
c Department of Chemical Engineering, Wayne State University, Detroit, MI 48202, USA

**A R T I C L E   I N F O**

Article history:
Received 10 January 2013
Received in revised form 23 August 2013
Accepted 30 August 2013
Available online 25 September 2013

**Keywords:** Pathogenesis, Genetics, Transformation, Chlamydia, Transcription, Plasmids

**A B S T R A C T**

Lack of a system for genetic manipulation of *Chlamydia trachomatis* has been a key challenge to advancing understanding the molecular genetic basis of virulence for this bacterial pathogen. We developed a non-viral, dendrimer-enabled system for transformation of this organism and used it to characterize the effects of inserting the common 7.5 kbp chlamydial plasmid into strain L2(25667R), a *C. trachomatis* isolate lacking it. The plasmid was cloned in pUC19 and the clone complexed to polyamidoamine dendrimers, producing ~83 nm spherical particles. Nearly confluent McCoy cell cultures were infected with L2(25667R) and reference strain L2(434). At 16 h post-infection, medium was replaced with dendrimer–plasmid complexes in medium lacking additives (L2(25667R)) or with additive-free medium alone (L2(434)). Three h later complexes/buffer were removed, and medium was replaced; cultures were harvested at various times post-transformation for analyses. Real time PCR and RT-PCR of nucleic acids from transformed cultures demonstrated plasmid replication and gene expression. A previous report indicated that one or more plasmid-encoded product govern(s) transcription of the glycogen synthase gene (glgA) in standard strains. In L2(25667R) the gene is not expressed, but transformants of that strain given the cloned chlamydial plasmid increase glgA expression, as does L2(434). The cloned plasmid is retained, replicated, and expressed in transformants over at least 5 passages, and GFP is expressed when transformed into growing L2(25667R). This transformation system will allow study of chlamydial gene function in pathogenesis.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

*Chlamydia trachomatis* is an obligate intracellular bacterial pathogen of humans. In developed nations, *C. trachomatis* is an important etiologic agent in genital infections [1]. In the USA this pathogen constitutes the most prevalent sexually-transmitted bacterial infection, and new genital chlamydial infections must be reported to the Centers for Disease Control and Prevention from all 50 states and the District of Columbia. Estimates of the number of people in the US with active urogenital chlamydial infections range from 4 to 6 million [2]. Importantly, genital chlamydial infections often engender severe sequelae, including reproductive problems in women, chronic inflammatory arthritis in both genders, and others [1,3–5].

*C. trachomatis* undergoes a biphasic developmental cycle [e.g., [6,7]]. This cycle is initiated when elementary bodies (EB), the extracellular form of the organism, attach to a target host epithelial or epithelia-like cell. Once bound EB are brought into a cytoplasmic inclusion, and during the first few hours in that inclusion EB undergo a developmental process resulting in production of reticulate bodies (RB). Each RB undergoes 7–8 cell divisions, and near the end of the cycle ~80% of RB dedifferentiate back to the EB form; these are released to the extracellular milieu by host cell lysis or exocytosis [16–9] for review]. In permissive cell lines ~48 h are required for cycle completion. This description was derived from study of *in vitro* culture systems which employed host cell lines that support passage of the organism through the entire cycle. Under some circumstances, including those relevant to disease sequelae, the cycle is arrested, obviating production and release of new EB. This state is designated “persistence”. During persistent infection, aberrant chlamydial RB form and accumulate replicated copies of the
bacterial genome in the absence of cell division [9,10]. Persistently-infecting chlamydiae display an unusual transcript profile [11–15 for review]. Much data indicate that disease sequelae involve persistently-infecting chlamydiae [e.g., [16]].

The C. trachomatis genome includes a chromosome of about 1 mbp specifying just over 900 open reading frames, more than 200 of which specify products of unknown function [17]. Importantly, studies from our group have indicated that many of these latter genes are involved in persistence in infected tissue [e.g., [18]; APH personal communication]. Virtually all chlamydial strains also harbor a plasmid of ~7.5 kbp [19–22], but studies have demonstrated that the functions encoded on it are not required for survival [e.g., [23–26]; see also [27]]. Interestingly, C. trachomatis produces iodine-stainable glycogen in its inclusions during the developmental cycle [e.g., [24]]. Glycogen production results from expression of several chromosomal genes including glgA [17,28]. A recent study showed that expression of glgA and several other chromosomal genes is governed by one or more functions on the plasmid [24]; see also [29]. Plasmid-lacking strains of C. trachomatis do not produce glycogen [e.g., [24]].

Many groups have attempted to produce a system for genetic manipulation of C. trachomatis, but to date success has been circumvented. An early study showed that DNA could be inserted into chlamydiae by electroporation, but stable transformants were not produced [30]. More recently, a system was described for transformation of Chlamydia psittaci, a bird pathogen, but that method is impractical to employ for routine purposes [31]. Most recently, a more workable system was published that uses CaCl2 in the transformation process, and this showed that insertion of the 7.5 kbp plasmid into an isolate of C. trachomatis that lacks it restores glycogen production [32]. This system requires several passages of transformed EB under antibiotic selection to produce a homogeneous population of transformants.

We developed a non-viral system for genetic manipulation of chlamydiae that uses synthetic non-cytotoxic nanostructured delivery vectors called dendrimers. These nanomaterials are emerging as viable vehicles for efficient intracellular delivery of nucleic acids [e.g., [33]]. Initial characterization of this system demonstrated that fluorescently-labeled dendrimers rapidly enter C. trachomatis-infected host cells in culture and the inclusions within them, that exogenous molecules can be reversibly attached to the dendrimers and that such complexes deliver those molecules to chlamydial inclusions, that entry of such complexes into infected cells does not elicit persistent chlamydial infection, and that the complexes enter inclusions containing persistently as well as actively infecting chlamydiae [34]. We further showed that dendrimers deliver antisense oligonucleotides to growing intracellular C. trachomatis, and that these oligos knockdown expression of a plasmid-encoded gene [35]. We have used the transformation system to insert exogenous genes into Chlamydia pneumoniae, a related respiratory pathogen [36]. In the present report, we describe development and use of this system for introduction of plasmids into growing C. trachomatis in cultured cells.

2. Materials and methods

2.1. Culture of C. trachomatis

Reference C. trachomatis strain L2(434) (L2/LGV-434/Bu) was originally obtained from the ATCC. Strain L2(25667R) was the generous gift of Dr. Julius Schachter; this strain lacks the 7.5 kbp plasmid [37] but its chromosome is essentially identical to that of reference strain L2/LGV-434/Bu [24]. Both strains were maintained in, and cultured for experiments in, the murine McCoy cell line as described [24]. Infection of nearly confluent monolayers of McCoy cells with either strain was done at a multiplicity of infection (MOI) 2:1 unless otherwise noted. Maintenance of host cell lines and preparation of chlamydiae were done as given [e.g., [11,12]].

2.2. Cloning of the C. trachomatis plasmid

The DNA sequence of the 7.5 kbp plasmid has been published, and many aspects of expression of plasmid genes have been described [e.g., [20,25,29,38]]. We derived a restriction map from that sequence information and determined that the plasmid included a single site for the restriction enzyme SacI. The plasmid was linearized at that site, amplified by PCR using the Platinum Taq® high-fidelity DNA polymerase (Invitrogen, Carlsbad CA USA) using primers given in Table 1, and cloned into the SacI site of pUC18; production of the cloned plasmid (pUC-pCL2) was by standard means in Escherichia coli [e.g., [39]].

2.3. Preparation of a GFP-encoding plasmid for transformation into C. trachomatis

The 5129 bp pMW82 plasmid was designed for use as a promoter-trap device; it includes the bla sequence to serve as the basis for selection upon transformation into bacterial systems [40]. We procured the pMW82 plasmid (generous gift of Dr. J. Withey) and cloned into it a 267 bp PCR product that included the origin of replication and several other plasmid sequences. That product was designed to have a promoter-trap device; it includes the lacZa promoter and several other plasmid sequences. This system requires several passages of transformed EB under antibiotic selection to produce a homogeneous population of transformants.

Table 1

| Primers used in this study for cloning, real time PCR and RT-PCR analyses. |
|-----------------------------|------------------------------|
| pCL2 plasmid construct      |                              |
| Sacl us                     | 5'-caagcagtgctcaatttaagaatcttc-3' |
| Sacl ds                     | 5'-tcatacatgacgcttgaattagct-3'  |
| Real-time PCR and RT-PCR analyses |              |
| 16S Cts                     | 5'-gggggtgcccttcg-3'           |
| 16S Ctds                    | 5'-ttttttctctcgacg-3'          |
| pl2-01us                    | 5'-ttttttctctcgacg-3'          |
| pl2-01ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-02us                    | 5'-ttttttctctcgacg-3'          |
| pl2-02ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-03us                    | 5'-ttttttctctcgacg-3'          |
| pl2-03ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-04us                    | 5'-ttttttctctcgacg-3'          |
| pl2-04ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-05us                    | 5'-ttttttctctcgacg-3'          |
| pl2-05ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-06us                    | 5'-ttttttctctcgacg-3'          |
| pl2-06ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-07us                    | 5'-ttttttctctcgacg-3'          |
| pl2-07ds                    | 5'-ttttttctctcgacg-3'          |
| pl2-08us                    | 5'-ttttttctctcgacg-3'          |
| pl2-08ds                    | 5'-ttttttctctcgacg-3'          |
| L2gGgAus*                   | 5'-catatgacgcttgaatttaagaatcttc-3' |
| L2gGgAdsf                   | 5'-tcatacatgacgcttgaattagct-3'  |
| 18S us                      | 5'-gggggtgcccttcg-3'           |
| 18S ds                      | 5'-tcatacatgacgcttgaattagct-3'  |

* Genome sequence designation for the C. trachomatis glgA gene is CILD67.
2.4. Preparation of dendrimer/plasmid complexes

Generation 4 polyamidoamine dendrimers (G4-NH₂ PAMAM) were purchased from Dendritech (Midland MI USA). Complexation of the cloned 7.5 kbp chlamydial plasmid DNA to G4-NH₂ dendrimers was achieved as described by us [36]. G4-NH₂ dendrimers have 64 primary amine groups (N), which form complexes with phosphate groups (P) of the DNA. As previously, we used ratios of 4 and 8 (i.e., N/P ratio = 4, 8). In control studies, we determined that dendrimer/plasmid complexes with N/P = 8 provided superior results over those with N/P = 4 (not shown). Thus all experiments described utilized complexes with N/P = 8. Complexes were characterized by dynamic light scattering (DLS) and atomic force microscopy (AFM), as described [36]. AFM images of plasmid—dendrimer complexes showed a stable, compact, globular confirmation; DLS determination of the zeta potential and particle size of complexes were +43.6 mV and 82.9 nm, respectively, consistent with data from our previous study [36].

2.5. Transformation of chlamydiae

 Cultures with nearly confluent monolayers of McCoy cells were treated by: mock infection, mock pulsing (dendrimers + cloned 7.5 kbp plasmid, not complexed), and complexed dendrimer/cloned 7.5 kbp plasmid at 0.2 μg and 1 μg input. Dendrimers and dendrimer/DNA complexes were suspended in medium (IMDM) without fetal bovine serum (FBS). In most experiments, input of dendrimer/plasmid complexes at 1 μg proved optimal, and data reported here primarily those from the 1 μg input experiments. Uncomplexed dendrimers/cloned plasmid in IMDM, complexed dendrimers/cloned plasmid in IMDM at the two concentrations, or IMDM alone were added to cell cultures at 16 h post-infection at 37 °C. At 19 h post-infection those were removed and fresh medium with glutamine and FBS and containing glucose (10 mg/ml) and cycloheximide (1 μg/ml) were added. Cultures were harvested at various times post-infection for analyses. In one set of experiments, McCoy cell cultures were infected at MOI 2:1 with strain L2(25667R), transformed as above with the dendrimer/pUC-pCL2 plasmid complexes, and EB from the transformed cultures were harvested at 48 h post-infection. These were used to infect another culture, with EB harvested again at 48 h post-infection, a procedure repeated 5 × to determine if the plasmid is stably maintained. All studies were approved by the Wayne State University Biosafety Committee, which NIH has ruled is all that is required for transformation of C. trachomatis L2 using a vector containing an ampicillin or related antibiotic resistance gene.

2.6. Preparation and analyses of nucleic acids; other methods

Total nucleic acids were prepared from infected and uninfected cultures of McCoy cells using the hot phenol method [e.g., [11—14,41]]. From those preparations DNA, or RNA/cDNA for transcript analyses, were prepared [e.g., [11,14,41]]. Real time PCR and RT-PCR analyses were done as described, using an Applied Biosystems model 7500 machine and the primer systems listed in Table 1. Microscopic analyses of infected and uninfected cultures were done using a Nikon E600 microscope with epifluorescence.

3. Results

3.1. Transformation of the chlamydial plasmid into growing chlamydiae

We cloned the C. trachomatis 7.5 kbp plasmid in pUC18 to produce pUC-pCL2, complexed the clone DNA to G4-PAMAM dendrimers, and gave the complexes to cultures of McCoy cells infected for 16 h with a plasmid-lacking isolate of C. trachomatis designated L2(25667R). Three hr later we removed the complexes and replaced complete growth medium, then harvested the cultures at 24, 36, and 48 h after transformation. Real time PCR analyses of DNA prepared from the cultures showed that accumulation of chromosomes in the untransformed and transformed strain L2(25667R), and in reference strain L2(434), was similar (Fig. 1A). Real time PCR analyses also showed that plasmid DNA accumulated in the transformed cultures with kinetics similar to those of the reference strain (Fig. 1B); control cultures given dendrimers alone, cloned plasmid DNA alone, or buffer alone showed that no plasmid was present in the cultures over time. Thus, dendrimer/plasmid complexes deliver the cloned DNA to growing chlamydiae, and those plasmids undergo reasonably normal replication within the metabolically-active organisms.

3.2. Genes on the 7.5 kbp plasmid are expressed in transformed C. trachomatis

The chlamydial 7.5 kbp plasmid includes genes specifying products of unknown function [17,20]. We next asked if the open

![Fig. 1. Replication of C. trachomatis chromosome and 7.5 kbp plasmid following transformation. Panel A, accumulation of chromosomal DNA in reference strain L2(434) and in untransformed and transformed strain L2(25667R) over 72 h post-infection of McCoy cells. Panel B, accumulation of cloned chlamydial plasmid (pUC-pCL2) DNA over 72 h post-infection of McCoy cells in transformed strain L2(25667R) and reference strain L2(434). Data represent two independent experiments, each analyzed in replicate twice. Variation between experiments and among triplicates <8%. L2, reference strain L2(434); L2p-, plasmid-less strain L2(25667R); Gh = pCL2, dendrimers (ghosts) and pUC-pCL2 DNA mixed together but uncomplexed for transformation; CD-pCL2, complexed dendrimer/pUC-pCL2 DNA for transformation; IMDM, medium alone for transformation. Normalization in real time assays was to host 18S rDNA; data indexed to value at t = 0 h in reference strain L2(434).]
reading frames on the plasmid were expressed in transformed strain L2(25667R), or whether the plasmid was present and undergoing replication but was transcriptionally inert. As shown in Fig. 2A, real time RT-PCR analyses targeting eight protein-coding genes on pUC-pCL2 demonstrated expression from each gene; no transcripts from plasmid-encoded genes were found in the various control cultures. Thus, the plasmid-encoded genes are transcribed. A recent study showed that expression of the glycogen synthase-encoding gene glgA from the chlamydial chromosome is attenuated in the plasmid-less chlamydial strain L2(25667R) compared to expression in reference strain L2(434) [24]. Given that coding sequences on the plasmid were expressed in transformants of strain L2(25667R), we reasoned that those transformants should display restored glgA expression, as in another recent study [32]. We assessed glgA expression in the transformed cultures, and real time RT-PCR analyses showed that the expected increase in transcripts from that gene indeed were present (Fig. 2B). Thus, the chlamydial plasmid is replicated apparently normally in growing C. trachomatis transformed with that DNA, the encoded genes are expressed after transformation, and those mRNA produce functional products.

3.3. Recovery and passage of plasmid-containing transformants

An important question concerns whether transformation of the cloned plasmid into strain L2(25667R) produces EB that stably retain and express it in subsequent passages of the organism, thus generating a strain functionally indistinguishable from reference strain L2(434). We recovered EB at 48 h post-infection from the initial culture of infected McCoy cells given the dendrimer/plasmid complex and passed those EB 5× to assess plasmid retention. The data presented in Fig. 3 indicate that EB given the 7.5 kbp plasmid retain it through at least that number of passages, as judged from PCR confirmation of plasmid in DNA prepared from each passage. Thus, transformed cells of strain L2(25667R) retain 7.5 kbp plasmid over multiple passages following its insertion in complexes with G4 dendrimers.

3.4. Insertion of expression of GFP in C. trachomatis

A recent study demonstrated that GFP is expressed following its insertion into C. trachomatis [32]. We produced pMW82: pL2-01-plL2-01P-GFP, a derivative of promoter-trap plasmid pMW82 containing a PCR product that included the origin of replication and the pL2-01 promoter sequence just 5′ to GFP, complexed it to dendrimers, and transformed it into strain L2(25667R) as above. As shown in Fig. 4A, this construct inserted into C. trachomatis engendered green fluorescence in the initial transformed culture. Counting green compared to total inclusions in 10 random fields indicated that ~80% were fluorescent in the transformed culture. We allowed the developmental cycle to complete in two transformed cultures, prepared EB from them, and used those EB to infect subsequent McCoy cell cultures. As demonstrated by the images provided in Fig. 4B, the GFP-containing plasmid was retained despite lack of any antibiotic selection, and it was expressed in that second culture. Counting again showed that ~80% of inclusions were green-fluorescent, suggesting that in the total absence of antibiotic selection the transformation efficiency was ~80% (see Discussion).

4. Discussion

The genome of C. trachomatis is small, but it includes more than 200 genes encoding products of unknown function [17]. Many of these latter are thought to be involved in pathogenic processes engendered by chlamydiae [e.g., [42]]; however, elucidation of their functions has been virtually impossible because no system for genetic manipulation of the organisms has been available. Development of a practical such system has been a goal of Chlamydia research for decades. One transformation system utilized electroporation of DNA constructs into tissue culture cells infected with C. psittaci [31]. The method requires large quantities of transforming DNA to produce low numbers of transformants and thus is cumbersome and impractical to use. The most recent system described improves ease and reliability for transformation over the previous system, but selection over several passages is required to produce a clonal population of transformants [32].

[Figures and diagrams are not transcribed here.]
We use dendrimers as vehicle to deliver modifying nucleic acids to *C. trachomatis* within inclusions in tissue culture cells. We inserted a wild-type plasmid into a plasmid-less strain of the organism in initial experiments, since we reasoned that retention of this plasmid in an organism that normally harbors it would require no external selection such as antibiotic resistance. This transformation system involves no electroporation or other manipulation(s) of infected cell cultures, uses small amounts of DNA complexed to dendrimers, produces largely uniform transformant populations, and in our hands is reliable and practical for regular use. Our impression has been that the system is efficient, and the assessment of GFP-plasmid retention following passaging supports that contention. Utilization of antibiotic selection in combination with this transformation procedure will give uniform transformant populations without multiple passages. Transformants of *C. trachomatis* given the 7.5 kbp plasmid retain and express encoded genes through multiple passages. Thus, this system will be useful for study of a many currently unaddressable questions regarding gene function in this pathogen.

As mentioned, initial characterization of this genetic manipulation system using various species of DNA or antibiotics complexed to dendrimers showed no significant effects on chlamydiae or on host cells containing them [34,35]. At the low MOI routinely used in our studies, ~30–50% of host cells are infected with chlamydiae, and labeled dendrimers were seen in uninfected as well as infected cells in those initial studies; interestingly, within infected cells the labeled plasmid appeared to be concentrated in the inclusions [34,35]. We do not understand why this is the case, but perhaps dendrimer-DNA complexes home to cellular regions with increased metabolic activity, such as inclusions, relative to neighboring regions. It would of course be advantageous to target dendrimer-complexes to infected cells, and we are investigating strategies for accomplishing that end.

Glycogen may be a virulence factor in some bacterial pathogens [e.g., [43]]. Interestingly, *C. pneumoniae* retains the genes required for glycogen synthesis in open form but does not express them at a significant level under standard *in vitro* culture conditions [44]. Expression of these genes in *C. trachomatis*, and retention of them with low expression in *C. pneumoniae*, suggests that their translation products are important to metabolic or other processes of these organisms. It is possible that the gene products involved in glycogen synthesis/catabolism, or perhaps the glycogen itself, is/are involved in the pathogenesis engendered by *C. trachomatis* but not (or less so) by *C. pneumoniae*. In another context we demonstrate that transformation of pUC-pCL2 elicits a large transcriptional up-regulation of *glgA* and consequent glycogen production in transformants of *C. pneumoniae* [36].

A recent report from another group demonstrated that the pl2-05 gene is responsible for transcriptional governance of chromosomal *glgA* and other genes in *C. trachomatis* [45]. That study used the same isolate employed in the present study, L2(25667R). While it is clear that *C. pneumoniae* can be transformed using the dendrimer-enable method [36], the question arises as to whether other *C. trachomatis* strains are amenable to genetic manipulation using this technique. An earlier report from this group showed that antisense oligonucleotides for targeted knockdown of gene expression are delivered efficiently to growing K serovar *C. trachomatis* when complexed to dendrimers [35]. We add that in experiments to be reported elsewhere, we show that the system can be used to transform the murine pathogen *Chlamydia muridarum* (in preparation). Thus, the system described here should be generally useful for transformation of multiple chlamydial species and strains/serovars.
The 7.5 kbp chlamydial plasmid may encode virulence functions other than that specified by the pl2-05 gene, possibly including products involved in the transition from active to persistent infection and/or the maintenance of the latter state. Indeed, a recent review reiterates the importance of virulence factors encoded by the chlamydial plasmid and argued that the functions therein specified must be elucidated [42]. That review stressed the need for a genetic system to study gene function in chlamydiae, as did the recent paper describing a method for making targeted mutations in the chlamydial genome in lieu of such a genetic system [46]. The system described here should contribute significantly to our understanding of the genetic underpinning of pathogenesis for chlamydia species.

5. Conclusions

- A transformation system for C. trachomatis is described that utilizes delivery of DNA by complexation with dendrimers
- The system is reliable, easy to use for routine purposes, and shows high transformation efficiency
- C. trachomatis cells lacking the normal 7.5 kbp plasmid stably retain and express it following its introduction by the transformation system

Acknowledgments

This work was supported by a grant from the President’s Research Enhancement Program at Wayne State University. We are grateful to Dr. Julius Schachter (University of California San Francisco) for the gift of the plasmid-lacking strain of C. trachomatis, (L2(25667R)) via Dr. Luis de la Maza (also University of California San Francisco). We are also grateful to Dr. Jeffrey Withey (Wayne State University School of Medicine) for his gift of the pMW82 plasmid. Support from the Wayne State University Nano- Incubator effort (RMK, APH, JWH) is also gratefully acknowledged.

References


