Diversity within inc genes of clinical Chlamydia trachomatis variant isolates that occupy non-fusogenic inclusions

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The obligately intracellular chlamydiae are bacterial pathogens that occupy intracellular vacuoles, termed inclusions, as they develop and multiply. Typical Chlamydia trachomatis isolates occupy inclusions that fuse with other C. trachomatis inclusions within cells infected with multiple elementary bodies (wild-type phenotype). The authors of this study have recently described C. trachomatis isolates that form multiply-lobed, non-fusogenic inclusions within single cells infected with multiple elementary bodies (variant phenotype). Inclusions formed by these isolates uniformly lacked the protein IncA on the inclusion membrane (IM). In the present work, the study of the C. trachomatis inclusion phenotype has been expanded to include 27 variant and 13 wild-type isolates. Twenty-four of the 27 variant isolates were IncA-negative, as detected by fluorescence microscopy and immunoblotting, but three variants localized IncA to the IM. The IncA-positive variants formed inclusions that fused, at a reduced rate, with those occupied by wild-type isolates and with inclusions formed by other IncA-positive variants. Nucleotide-sequence analysis of the incA sequences from the variant isolates identified a variety of distinct sequence polymorphisms relative to incA from wild-type strains. The authors also demonstrate that a second Inc protein, CT223p, is not found in the IM in selected C. trachomatis isolates. No change in the structure or the fusogenicity of the inclusions was associated with the presence or absence of CT223p.

Keywords: inclusion membrane, intracellular pathogen, fluorescence microscopy

INTRODUCTION

The chlamydiae are obligate intracellular pathogens that cause a variety of diseases in humans and other animal species. There are at least 15 serovars of Chlamydia trachomatis and naturally occurring variants or subserovars of this species are relatively common (Dean et al., 1991; Hayes et al., 1992; Lampe et al., 1997; Yang et al., 1993). All chlamydiae develop within a membrane-bound, non-acidified vacuole termed the inclusion. The transfer of a fluorescent sphingomyelin derivative from the Golgi apparatus to the inclusion supports a model that places the developing inclusion in the host-cell exocytic vesicular trafficking pathway (Hackstadt et al., 1995, 1996). The list of chlamydial proteins that are associated with the inclusion membrane (IM) is growing and now includes a candidate protective antigen (Fling et al., 2001), a component of the type III secretory apparatus (Fields & Hackstadt, 2000) and a collection of chlamydial proteins known as Inc proteins (Bannantine et al., 1998a, 2000; Rockey et al., 1995; Scidmore-Carlson et al., 1999). Recently, a host protein (14-3-3β) was shown to localize at the IM through specific interactions with a single Inc protein, IncG (Scidmore & Hackstadt, 2001). It is likely that similar interactions between chlamydial IM proteins and unidentified host-cell proteins are critical for proper inclusion maturation and productive chlamydial development.

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; HSP60, 60 kDa heat-shock protein; IM, inclusion membrane; MOMP, major outer-membrane protein; (s), isolates that formed variant inclusions.

The GenBank accession numbers for the sequences reported in this paper can be found in Fig. 1.
We have recently identified and described a collection of *C. trachomatis* isolates that form unusual, non-fusogenic inclusions within infected cells (variant phenotype; Suchland et al., 2000). This is in contrast to typical *C. trachomatis*, which occupies inclusions that fuse with other wild-type *C. trachomatis* inclusions (wild-type phenotype; Ridderhof & Barnes, 1989). In this study, we have used immunofluorescence microscopy, immunoblotting and sequence analysis to expand on the characterization of the *C. trachomatis* isolates occupying the unusual inclusions. In results similar to those that we have reported previously, 24 of the 27 variants lacked IncA on the IM. However, the three remaining variants produced and correctly localized IncA to the IM. The IncA-positive variants formed multiply-lobed, but partially fusogenic, inclusions within cells. A variety of different mutations were found in incA within the variants, which led to truncated predicted proteins in most of the IncA-negative isolates. We also show that an additional Inc protein, *C. trachomatis* CT223p (encoded by ORF CT223; Bannantine et al., 2000), was variably present in the different isolates but that the presence or absence of CT223p did not correlate with the inclusion structure or its fusogenicity.

**METHODS**

**Chlamydiae.** Wild-type chlamydial reference strains of serovar G (G/UW-57) and serovar I (I/UW-12) were described by Wang et al. (1985). All other isolates were collected and serotyped during the routine evaluation of patients visiting the Seattle and King County Public Health Clinics (Suchland et al., 2000; Suchland & Stamm, 1991). The serovar and laboratory reference number are given for each clinical isolate. All isolates that formed variant inclusions are represented by (s). As reported previously (Suchland et al., 2000), 1:5–2:2% of approximately 11400 *C. trachomatis* clinical isolates collected over the course of 12 years showed the variant inclusion phenotype. Twenty-seven variant and 13 wild-type isolates were taken from this collection and used for these analyses (Fig. 1).

**Antibodies and reagents for microscopy.** mAb B9 is directed at a genus-common determinant of the 60 kDa heat-shock protein (HSP60; Yuan et al., 1992). The mAbs GG-11, CC-1 and AC-11 are specific for the *C. trachomatis* major outer-membrane proteins (MOMPs) of serovars G and I, respectively (Wang et al., 1985). The mAb 20F12, produced by the University of Oregon Monoclonal Antibody Facility, is specific for the *C. trachomatis* Inc protein CT223p (Bannantine et al., 2000). This antibody was shown, by immunoblotting, to react with both the wild-type and variant CT223p when either protein was produced in *E. coli* (not shown). Rabbit anti-IncA antisera were produced using described methods (Bannantine et al., 1998b). These antisera were pre-adsorbed with lysates of uninfected HeLa cells to remove any non-specific background. Mouse mAb 3H7 (IgG3), directed at *C. trachomatis* IncA, was produced using a *C. trachomatis* IncA-maltose-binding protein fusion as the immunogen. The specificity of the antisera was determined by immunoblotting and fluorescence microscopy. Secondary antibodies were purchased from the Pierce Chemical Co. DNA was labelled with 4',6-diamidino-2-phenylindole dihydrolorhodol (DAPI), which was added at 2 μg ml⁻¹ to the mounting medium.

**Chlamydial culture and immunofluorescence.** The non-fusogenic chlamydial phenotype was identified visually, by observing the inclusion morphology during large-scale serotyping studies in a low-passage microtitre-plate format (Suchland & Stamm, 1991). *Chlamydia*-infected cells were detected by fluorescence microscopy using mAb CF-2, which was directed at the genus-common lipopolysaccharide determinant of *Chlamydia* spp. (Washington Research Foundation, Seattle, WA).

Examination of the fusogenicity of inclusions was conducted via the analysis of mixed infections in HeLa-cell monolayers, using the strains indicated in the legends to Figs 2, 3 and 5. For the mixed infections, one isolate was inoculated onto cells in SPG (0·25 M sucrose, 10 mM sodium phosphate, 5 mM t-glutamic acid, pH 7·2) and incubated for 1 h at room temperature on a rocking platform. The inocula were removed and the cells were washed twice with Hank’s Balanced Salts Solution (Life Technologies). The cells were then incubated for 1 h at 37°C in Minimal Essential Medium plus 10% fetal calf serum (MEM-10; Life Technologies), prior to their inoculation with a second chlamydial isolate. Cells were again incubated for 1 h at room temperature prior to the removal of the inocula, the addition of the medium and incubation at 37°C in 5% CO₂. These sequential infections were used to reduce the likelihood of two different chlamydial elementary bodies entering the same vacuole. Cells were then cultured for 30 h prior to being fixed in methanol and prepared for fluorescence microscopy. Monolayers were stained as described previously (Rockey & Rosquist, 1994). Fluorescence images were visualized using a Leica DMLS microscope by using the 100× objective and collected digitally with a SPOT camera (Diagnostic Instruments). Images were processed by using Photoshop 6.0 (Adobe Software) and Canvas 5.03 (Deneba Software).

**Electrophoresis and immunoblotting.** Protein electrophoresis was conducted as described previously (Rockey & Rosquist, 1994). Briefly, *C.-trachomatis*-infected HeLa cells that had been cultured in 6-well trays were lysed with ice-cold electrophoresis sample buffer (Rockey & Rosquist, 1994) and boiled for 2 min. Samples were then aliquoted and stored at −20°C. Chlamydial lysates were standardized for electrophoresis using the relative level of chlamydial HSP60. Rabbit antibodies were detected with 35S-labelled staphylococcal protein A [37 kBq ml⁻¹ (1 μCi ml⁻¹); Amersham] followed by autoradiography. Mouse antibodies were detected using peroxidase-conjugated chicken-anti-mouse IgG (ECL reagents), followed by chemiluminescent detection.

**DNA amplification and sequence analysis of incA and CT223.** Analysis of the *incA* sequences from the 39 clinical isolates discussed in this work was performed (Fig. 1). Sequence analysis was conducted on the CT223 sequences from a total of 12 isolates; this included several serovars and isolates with both the variant and wild-type inclusion morphologies. Amplification products for sequencing were prepared and processed using the following techniques. McCoy cells cultured in 6-well tissue-culture trays were infected at an m.o.i. of approximately 3. Infected cells that had been cultured for 30 h were washed with Hank’s Balanced Salts Solution and lysed by a brief sonication. Cells and debris were pelleted in a microcentrifuge. The pellet was then resuspended in a commercial ligase chain reaction sample collection buffer (Urine Specimen Resuspension Buffer; Abbott Laboratories). The lysate was heated to 98°C for 15 min and then briefly...
**Fig. 1.** Sequence analysis of the chlamydial isolates examined in this study. For each isolate, the strain designation is indicated in the left column and the accession number for the *incA* sequence is indicated in the right column. The complete *incA* sequence was determined for the isolates shown in bold, whereas the first 450–500 nt were analysed for all other isolates. The sequences of *incA* from five additional wild-type isolates (E9332, J2364, J3464, J9311 and J9325) were identical to that of the serovar D sequence in the *Chlamydia* genome-project database (Stephens et al., 1998), and these sequences are not represented in the figure. One recent *incA*-positive variant, J(s)9045, was not subjected to sequence analysis. The results of the fluorescence-microscopy analysis are listed under *IncA IF*, with a † indicating labelling of the IM in cells infected with the isolate. The length of the *incA* sequence in the ORF predicted from the nucleotide-sequence analysis is indicated by the open bar for each isolate. □, Silent base changes in *incA*; †, a base change that leads to an amino-acid substitution; ▽, single base deletions or insertions that lead to premature truncation of the protein; ●, single base changes that lead directly to a stop codon. Solid bars indicate larger deletions and are drawn to scale.
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Fig. 2. Fluorescence-microscopy analysis comparing wild-type and IncA-positive variant isolates. Wild-type isolate G/UW-57 (A and D) and IncA-positive variant G(s)459 (B, C, E and F) were inoculated onto cells that were incubated for 30 h prior to methanol fixation. Each vertical pair of images represents identical cells. The cells shown in (A) and (D), and (B) and (E) were inoculated at an m.o.i. of 5. The cells shown in (C) and (F) were inoculated at an m.o.i. of 0.01. Fixed cells were labelled with an mAb specific for IncA (D–F) and counter-stained with DAPI to show the cell nuclei (brightly labelled in A, B and C) and chlamydial developmental forms (grey labelling in A, B and C). Bar in (F) represents 10 µm and applies to all panels.

RESULTS

In Fig. 1, wild-type and variant isolates are separated on the basis of their inclusion phenotype in HeLa cells infected at an m.o.i. of 5. Variant clinical isolates of serovars B, D, D-, E, F, G, H, Ia, J and K were identified and characterized in this work. The anti-IncA immunofluorescence data are included for each isolate. Sequence data are presented graphically and a GenBank accession...
number is provided for each isolate (Fig. 1). The following sections provide details about the isolates examined in this study.

**Fluorescence microscopy and immunoblotting of cells infected with different variants**

Wild-type isolates of *C. trachomatis* form single inclusions following the infection of HeLa cells at high multiplicities (Fig. 2A, D). The localization of IncA to the IM has been demonstrated in over 50 fusogenic wild-type clinical isolates (Fig. 1, and data not shown). In contrast, multiple, or lobed, inclusions form in HeLa cells infected under similar conditions by variant *C. trachomatis* isolates (Fig. 2B, E). Two distinct groups of the variant isolates were identified with respect to the presence or absence of IncA in the IM. Twenty-four of the 27 variant isolates lacked IncA on the IM [isolates J(s)893 (Fig. 3C, D) and J(s)1980 (Fig. 3E, F)]. Trace-labelling of the developmental forms of *C. trachomatis* with the anti-IncA antisera can occasionally be observed in some images (Fig. 3C). This background labelling was never seen at the IM. The remaining three variants occupied multiply-lobed inclusions that were IncA-positive [for example, G(s)459 (Fig. 2B, E, C, F) and J(s)5942 (Fig. 3A, B)]. The IncA-negative variant isolates were found in each oculogenital serovar, whereas the three IncA-positive variants were of serovars J(s) and G(s) (Fig. 1).

The examination of the Inc protein CT223p was initially included as a positive control for IM staining of the IncA-deficient strains. In the course of these studies, however, several isolates were identified that did not accumulate CT223p in the IM (represented in Fig. 3F). One of the 13 wild-type isolates (J9325) and three of the 27 variants [J(s)6276, J(s)6686 and J(s)1980] were shown to lack CT223p, as measured by immunofluorescence. All of the identified CT223p-negative isolates belonged to serovar J or J(s). There was no correlation between fusogenicity and non-fusogenicity, or any other observable aspect of inclusion development, and the presence or absence of CT223p.

Immunoblotting of infected-cell lysates was used to confirm the antigenic profiles identified by immunofluorescence, and to demonstrate that the IncA-negative or CT223p-negative chlamydiae not only failed to localize IncA and CT223p to the IM, but that they also failed to accumulate detectable levels of either protein within the infected cells (Fig. 4). Chlamydial HSP60 was included as a control, to demonstrate that similar amounts of total chlamydial antigen were loaded for each sample. Also included in these immunoblots were lysates of J(s)893 and D(s)2923, isolates shown in this
Vacuoles with any ability to fuse with other
whether the IncA-positive variant isolates formed
Mixed-infection experiments were conducted to exam-
2000), the number of vacuoles per cell varied directly
those occupied by wild-type strains. To determine if the
inclusions. Studies were undertaken to
determine if the inclusions fused with those occupied by
either IncA-positive or IncA-negative variants, or with
those occupied by wild-type strains. To determine if the
IncA-positive variants occupied inclusions that fused
with wild-type inclusions, monolayers of HeLa cells
were serially infected with C. trachomatis I/UV-12, a
wild-type strain, followed by infection with an IncA-
positive serovar J variant [isolate J(s)9045]. Chlamydial
growth and inclusion development were then monitored
with mAbs specific for the MOMP of each serovar.
Fluorescence-microscopy analysis of cells infected with
I/UV-12 and J(s)9043 demonstrated that both serovars
were commonly found within the same vacuole (Fig.
5A). This is in contrast to the results from mixed
infections with two IncA-negative non-fusogenic strains,
which were never found in the same vacuoles as the
wild-type in similar experiments (Suchland et al., 2000).
These data indicate that, in contrast to the IncA-negative
non-fusogenic isolates, the IncA-positive variants occu-
py vacuoles that can fuse with inclusions formed by
wild-type C. trachomatis.

Next, we examined whether IncA-positive variants
occupied inclusions that could fuse with one another or
with inclusions formed by IncA-negative isolates. For
these analyses, an IncA-positive G(s) variant was used in
serial infections with either an IncA-negative or an IncA-
positive J(s) variant. These experiments were com-
plicated by the unavailability of subisotypically distinct
serovar-specific anti-MOMP mAbs for the double-label
fluorescence microscopy of chlamydiae belonging to
serovars J and G. Hence, infected cells were labelled
with mAb 20F12, which labels CT223p in the IM of
inclusions formed by each isolate (shown red in Fig. 5B,
C), and with mAb GG-11, which is specific for the
MOMP of serovar G (shown green in Fig. 5B, C). The
images in Fig. 5(B, C) represent cells that were co-
infected with G(s)459 and either IncA-negative J(s)893
(Fig. 5B) or IncA-positive J(s)9045 (Fig. 5C). As shown
in Fig. 5(A), each of the multiple inclusions in these cells
was fully laden with chlamydia developmental forms.

Because no antibody specific for the MOMP of serovar
J was used, inclusions containing only serovar J(s)
developmental forms appeared as empty vacuoles,
inclusions containing only serovar G(s) developmental
forms appeared as dense green vacuoles and fused
inclusions, containing both isolates, appeared as spotted
green vacuoles. No fused vacuoles were evident in cells
infected with both an IncA-negative variant and an
IncA-positive variant (Fig. 5B). However, examples of
both fused and non-fused inclusions were common in
monolayers infected with two IncA-positive variants
(Fig. 5C). Collectively, these experiments demonstrated
that the IncA-positive variants formed inclusions that
retained some fusogenicity and that these vacuoles can
fuse with inclusions formed by wild-type isolates and
those formed by other IncA-positive variants.

Analysis of the incA sequences from the wild-type
and variant clinical isolates

The nature of the lesion leading to the lack of IncA seen
in the non-fusogenic variants was addressed by using
nucleotide sequencing. Amplification products of incA

(Fig. 1) and in previous work (Suchland et al., 2000) to
encode an apparently intact peptidpeptide. In these and all
other tested isolates, the results from the immunoblotting
confirmed the results from the immunofluorescence. In all cases, isolates that lacked detectable
IncA or CT223p in the IM were also negative for these
proteins upon immunoblotting.

Characterization of the inclusion occupied by the
IncA-positive variants

The three IncA-positive isolates (Figs 2B, C, E, F
and 3A, B) that form multiply-lobed, apparently non-
fusogenic inclusions at a high m.o.i. represent a new
class of variant isolate that is distinctly different from
the IncA-negative, non-fusogenic variant class. The
IncA-positive variants were contrasted with the IncA-
negative group to examine why these isolates formed
multiply-lobed inclusions. First, limiting-dilution exper-
iments were performed to determine if the number of
inclusions per cell was a function of the initial number
of elementary bodies that contacted the cell. In cells
infected at an m.o.i. much less than 1, cells infected
with the IncA-positive variants nearly uniformly con-
tained single inclusions (Fig. 2C, F). Therefore, as is
seen with the IncA-negative variants (Suchland et al.,
2000), the number of vacuoles per cell varied directly
with the m.o.i.

Mixed-infection experiments were conducted to exam-
in whether the IncA-positive variant isolates formed
vacuoles with any ability to fuse with other C. trachomatis
inclusions. Studies were undertaken to
determine if the inclusions fused with those occupied by
either IncA-positive or IncA-negative variants, or with
those occupied by wild-type strains. To determine if the

Fig. 4. Immunoblot analysis of wild-type and variant C. trachomatis isolates. Infected HeLa cells were lysed at 30 h post-
infection. The lysates then underwent electrophoresis, and the proteins were transferred to nitrocellulose membranes.
Immunoblots were probed with ant-IncA, anti-CT223p or anti-
HSP60. The immunoblots probed with HSP60 were included to
demonstrate that similar amounts of chlamydial antigen were
loaded into each well. Lanes: 1, mock-infected lysate; 2, wild-
type serovar D; 3, D(s)2923; 4, D(s)8039; 5, wild-type serovar J;
6, J(s)893; 7, J(s)1980.
from a total of 26 independent variant isolates and 13 wild-type strains were subjected to sequence analysis (Fig. 1). A wide variety of distinct sequences were identified in the variant isolates that lacked IncA. Single-nucleotide frameshifts were found within incA of 12 variants, which led to prematurely terminated protein sequences. In seven of the variants, single base changes led directly to translational stops. Larger deletions were identified in three of the variants. A single isolate, J(s)6686, had a 14 nt deletion that altered the reading frame and led to a very short truncated predicted amino-acid sequence. Two J(s) isolates, J(s)6462 and J(s)893, had identical 51 nt in-frame deletions that encoded an internally deleted polypeptide sequence. A 671 nt deletion was identified in isolate H(s)5642 that included the 3’-terminal 457 nt of incA and extended into the 3’-untranslated region. The complete incA sequence was also determined for two of the three IncA-positive variants, G(s)459 and J(s)5942 (Fig. 1). The incA gene of both of these variants encoded intact, full-length proteins that had no unique changes relative to either the IncA-negative variants or the wild-type isolates.

In a previous report (Suchland et al., 2000) we identified three nucleotide changes that led to two amino-acid changes in a single non-fusogenic isolate, D(s)2923. The analysis presented in Fig. 1 demonstrates that the changes found in the incA sequence of this isolate are also found in the incA sequences of several additional variants and in the incA sequences from three of the 13 wild-type strains. Therefore, these changes are not unique to the non-fusogenic isolates. These conclusions are consistent with the results of Pannekoek et al. (2001), who also demonstrated that these mutations are not unique to the incA sequences of the non-fusers.

An analysis was also done on the 400 nt sequence upstream of the start codon of the incA gene of isolate D(s)2923. This was conducted to determine whether changes in the promoter sequence might be responsible for the lack of IncA seen in this strain – D(s)2923 has an intact coding sequence. Surprisingly, the sequence of the region upstream of incA in this isolate was identical to that found in the genomic sequence of serovar D (Stephens et al., 1998, and GenBank accession no. AF163773).

The complete gene sequence was determined for eight independent CT223 ORFs, including three serovar J or J(s) isolates that were CT223p-negative, as determined by fluorescence microscopy and immunoblotting, and five isolates that localized CT223p to the IM. The predicted CT223p amino-acid sequences for serovars L2 and D are 91% identical. Each of the CT223p-positive isolates encoded a protein that was identical either to the serovar D sequence or to a protein that was a mosaic of the CT223p sequences of serovars L2 and D. Each of the three CT223p-negative isolates encoded an identical predicted protein sequence, but these sequences had two

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Fig. 5. Fluorescence microscopy of HeLa cells infected with different pairs of C. trachomatis isolates. In each case, the HeLa cells were infected sequentially with each C. trachomatis strain and incubated for 30 h prior to their fixation with methanol and labelling with specific antibodies. (A) Wild-type isolate I/UW-12 (green) and IncA-positive variant J(s)9045 (red) were labelled with antibodies specific for the MOMP of each isolate. (B) Cells infected with IncA-negative variant J(s)893 and IncA-positive variant G(s)459. (C) Cells infected with IncA-positive variants J(s)9045 and G(s)459. The antibodies used in (B) and (C) were anti-CT223p (red) and anti-serovar J MOMP (green). In each panel, cells were counter-stained with DAPI to label host nuclei blue. Examples of inclusions containing mixtures of different strains (spotted green), or inclusions containing single strains (dense green or empty), are shown in (B) and (C). Bar in (C) represents 10 μm and applies to each panel.
DISCUSSION

In a previous study we identified clinical isolates that formed multiple inclusions in cells infected at a high m.o.i. (Suchland et al., 2000). This is in contrast to wild-type *Chlamydia trachomatis* isolates that form inclusions that fuse in cells infected at an m.o.i. >1. In that study, three non-fusogenic *C. trachomatis* isolates were examined; each lacked detectable IncA on the IM. This led us to propose that IncA was associated with the fusogenic nature of the chlamydial inclusion, a concept that was supported by the micro-injection and two-hybrid studies conducted by Hackstadt et al. (1999). We continued to examine the culture collection used in our previous study (Suchland et al., 2000) for variants, and in doing so have identified a phenotypically distinct collection of variant isolates. A detailed examination of a total of 27 variant isolates led to the identification of three independent IncA-positive isolates that occupy multiply-lobed, apparently non-fusogenic inclusions following infection at high multiplicities. The IncA-positive variant inclusions were similar to the inclusions formed by the IncA-negative variants, but the size and shape of the former were more irregular (Fig. 2B, E). Mixed-infection studies subsequently demonstrated that the inclusions formed by the IncA-positive variant isolates can fuse with wild-type inclusions and with inclusions formed by other IncA-positive strains (Fig. 5). Similar mixed-infection studies did not, however, identify any examples where inclusions formed by IncA-positive chlamydiae fused with IncA-negative inclusions. These data support the conclusion that IncA is important in the homotypic fusion of *C. trachomatis* inclusions. The data expand on this concept to demonstrate that in some isolates additional, yet unidentified, factors also play a role in the efficiency or rate of fusion of *C. trachomatis* inclusions.

Initially, CT223p was included in this study as a positive control for the localization of the IM in the fluorescence-microscopy detection of the IncA-negative inclusions. Screening of our isolate collection, however, identified four isolates that did not localize detectable amounts of CT223p to the IM. Three CT223p-negative isolates were non-fusogenic and one was wild-type with respect to fusogenicity. Therefore, the absence of CT223p was not correlated with the non-fusogenic phenotype. Our ability to randomly identify the isolates that lacked CT223p suggests that these variants may be common. As several different Inc proteins are produced by different chlamydiae (Bannantine et al., 1998a, b, 2000; Rockey et al., 1995; Scidmore-Carlson et al., 1999), it is possible that variation similar to that seen for IncA and CT223p is common among other Inc proteins and that this may be a means of modulating other, unknown, phenotypes.

The sequence analyses performed in this study demonstrated considerable diversity within the *incA* sequences from the non-fusogenic clinical *C. trachomatis* isolates. Several different types of sequence changes were identified, including base exchanges that directly introduced stop codons, single base frameshifts and larger deletions. In most cases (i.e. for 21 of the 24 IncA-negative isolates), the absence of IncA was associated with the interruption of the proper reading frame. In the remaining three isolates, however, the reasons for the absence of detectable IncA remain unclear. The *incA* sequence of the original IncA-deficient isolate, D(s)2923, has 3 nt changes that were also found in three of 13 wild-type strains, and in wild-type sequences identified by Pannekoek et al. (2001). Two IncA-negative J(s) isolates were analysed whose *incA* sequences each had a 51 nt, in-frame deletion that encoded a predicted protein that lacked 17 aa. One possibility for the lack of IncA in the isolates that encode apparently intact polypeptides is that differences in the promoter sequence lead to the production of truncated IncA. This idea was examined by performing an analysis on the *incA* sequence of isolate D(s)2923. Analysis of the 400 nt upstream of *incA* in this isolate showed that this region was identical to that in the serovar D genomic sequence. Therefore, the mechanism used by these strains to block the synthesis or the accumulation of IncA remains an open question and is a major focus of research in our laboratory. We are currently examining the transcription and possible translation of *incA* in these unusual isolates.

The identification of the variant chlamydial inclusions has allowed us to perform a focused examination of genetic variation within the chlamydiae and to associate this variation with a phenotype. While much of the biology associated with variations in IncA or CT223p remains to be elucidated, there is evidence that differences in clinical presentation following infection by *C. trachomatis* may be associated with differences in the inclusion phenotype of the infecting chlamydiae. Geisler et al. (2001) used a retrospective case-control analysis to demonstrate that infection with non-fusogenic variants is associated with less severe clinical signs of infection, and lower numbers of recoverable chlamydiae, than infection with wild-type strains. The relatively high percentage of non-fusogenic isolates (1.5% of approximately 11400 isolates; Suchland et al., 2000) supports the possibility that the non-fusogenic phenotype offers a selective advantage to the variant strains, perhaps by increasing their opportunity for cryptic infection. We anticipate that a continued analysis of these variants will, in many ways, add to our basic understanding of the mechanisms used by chlamydiae for their growth and development within infected host cells and to our understanding of the mechanisms used by chlamydiae to cause disease.

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