INFECTION AND IMMUNITY, July 1997, p. 2965–2969 Vol. 65, No. 7
0019-9567/97/$04.00+0
Copyright © 1997, American Society for Microbiology

NOTES

Characterization of A New Isolate of *Chlamydia trachomatis* Which Lacks the Common Plasmid and Has Properties of Biovar trachoma

ALDO FARENCENA,[1] MAURIZIO COMANDUCCI,[2] MANUELA DONATI,[1] GIULIO RATTI,[2]* AND ROBERTO CEVENINI[1]

Sezione di Microbiologia, DMCSS, Policlinico S. Orsola, Università di Bologna, Bologna,[1] and IRIS Research Centre, Chiron Vaccines, Siena,[2] Italy

Received 23 December 1996/Returned for modification 5 February 1997/Accepted 27 March 1997

A *Chlamydia trachomatis* urethral isolate, α/95, yielding pgp3-negative but otherwise normal inclusions by immunofluorescence also gave negative results when pCT-homologous DNA was searched by PCR and Southern blotting. *omp-1* sequence analysis identified α/95 as a new genotype B variant. These findings confirm that pCT is not required for chlamydial growth in vitro.

The obligate intracellular bacterium *Chlamydia trachomatis* (12) has a genome comprising ca. 1,000 kb of chromosomal DNA (3) and 5 to 10 copies of a well conserved plasmid, pCT, of approximately 7.5 kb (6). This plasmid appears to be under positive selective pressure in the natural habitat of *C. trachomatis* (essentially, epithelial cells of human mucosal tissues). Although there are reports of clinical specimens yielding negative results when *C. trachomatis* is diagnosed by a plasmid-based PCR (1), only one plasmid-free strain, belonging to biovar lymphogranuloma venereum, has been fully described and characterized (13). We now report on the isolation and characterization of a second strain which appears to lack the common plasmid pCT, grows well in vitro, and belongs to the less virulent biovar trachoma. The interest in finding pCT$^-$ isolates which can grow well in vitro stems essentially from two considerations. First, the fact that *C. trachomatis* so efficiently retains an extrachromosomal element suggests that pCT may provide some important advantage either for the chlamydial life cycle (e.g., eukaryotic cell infection, intracellular replication, or release) or for the modulation of host immune responses, which for obligate pathogens like chlamydiae is also a process essential for survival. So, the availability of plasmid-free strains would favor studies on the possible function of pCT in *C. trachomatis* pathogenicity. Second, although the feasibility of genetic transformation of *C. trachomatis* by electroporation has been described (18), better tools are still needed for routine work on chlamydial molecular genetics. One of these would be a pCT-based vector-host system capable of shuttling DNA fragments between *Escherichia coli* and *C. trachomatis*, and for this purpose a plasmid-free strain with good in vitro growth properties would be an important starting point. For these reasons, we searched for possible pCT$^-$ strains in a collection of *C. trachomatis* clinical isolates by immunofluorescence screening with antibodies to the protein pgp3, the presence of which is a pCT phenotype (7). The isolates were collected in various years from patients with urogenital infection attending the outpatient clinic for sexually transmitted diseases at the St. Orsola Polyclinic of Bologna, Italy. Monolayers of rhesus monkey kidney (LLCMK2) cells were grown on glass coverslips in Eagle minimal essential medium (with Earle salts, 5% fetal bovine serum, gentamicin at 50 μg/ml, and cycloeximide at 1.5 μg/ml) and infected with the elementary bodies (EBs) from the clinical isolates as previously described (5). Thirty hours after infection, cells were washed with sterile phosphate-buffered saline and fixed with methanol at −20°C. Monolayers were incubated with anti-pgp3 rabbit serum (diluted 1:100 in phosphate-buffered saline) at room temperature for 30 min and then with fluorescein-labelled mouse anti-rabbit immunoglobulin serum as previously described (5). Cells were counterstained with Evans blue before microscopic examination under UV light. For a control, a duplicate of each infected cell culture was also stained with antibodies to the chlamydial common lipopolysaccharide (LPS) antigen by using the Merifluor Chlamydia kit (Meridian Diagnostics Inc.). Of 30 isolates selected for screening, 29 gave positive results with both tests and only 1 urethral isolate from a male patient with urethritis-conjunctivitis (identified as α/95) yielded inclusions which had a normal microscopic appearance and could be immunostained with antibodies to chlamydial LPS but gave no response when tested with anti-pgp3 serum. Typical immunofluorescence results for α/95 and controls are shown in Fig. 1, where the normal appearance of α/95 cytoplasmic inclusions can also be seen. When this strain was used to infect monolayers of McCoy (mouse fibroblast) or Vero (green monkey kidney) cells, the same type of pgp3-negative inclusions were observed.

DNA was extracted with phenol-chloroform (16) from gradient-purified α/95 EBs grown in McCoy or LLCMK2 cells (11) and used as a template for PCR amplification (15) of the pCT plasmid segment comprising *orf-3* and the chromosomal gene *omp-1*, which encodes the major outer membrane protein (MOMP). DNA amplifications were performed by using the GeneAmp kit (Perkin Elmer) in accordance with manufacturer protocols and a Perkin Elmer 9600 thermocycler. The PCR primers used for *orf-3* were ORF3SN (AAAGTTACTTTTTC CTTG) and ORF3DX (GGAAATTCTGGTTTTT). The

* Corresponding author. Mailing address: IRIS Research Centre, Chiron Vaccines, 1 Via Fiorentina, 53110 Siena, Italy. Phone: 39-577-243 239. Fax: 39-577-243 564. E-mail: ratti@iris02.biocine.it.

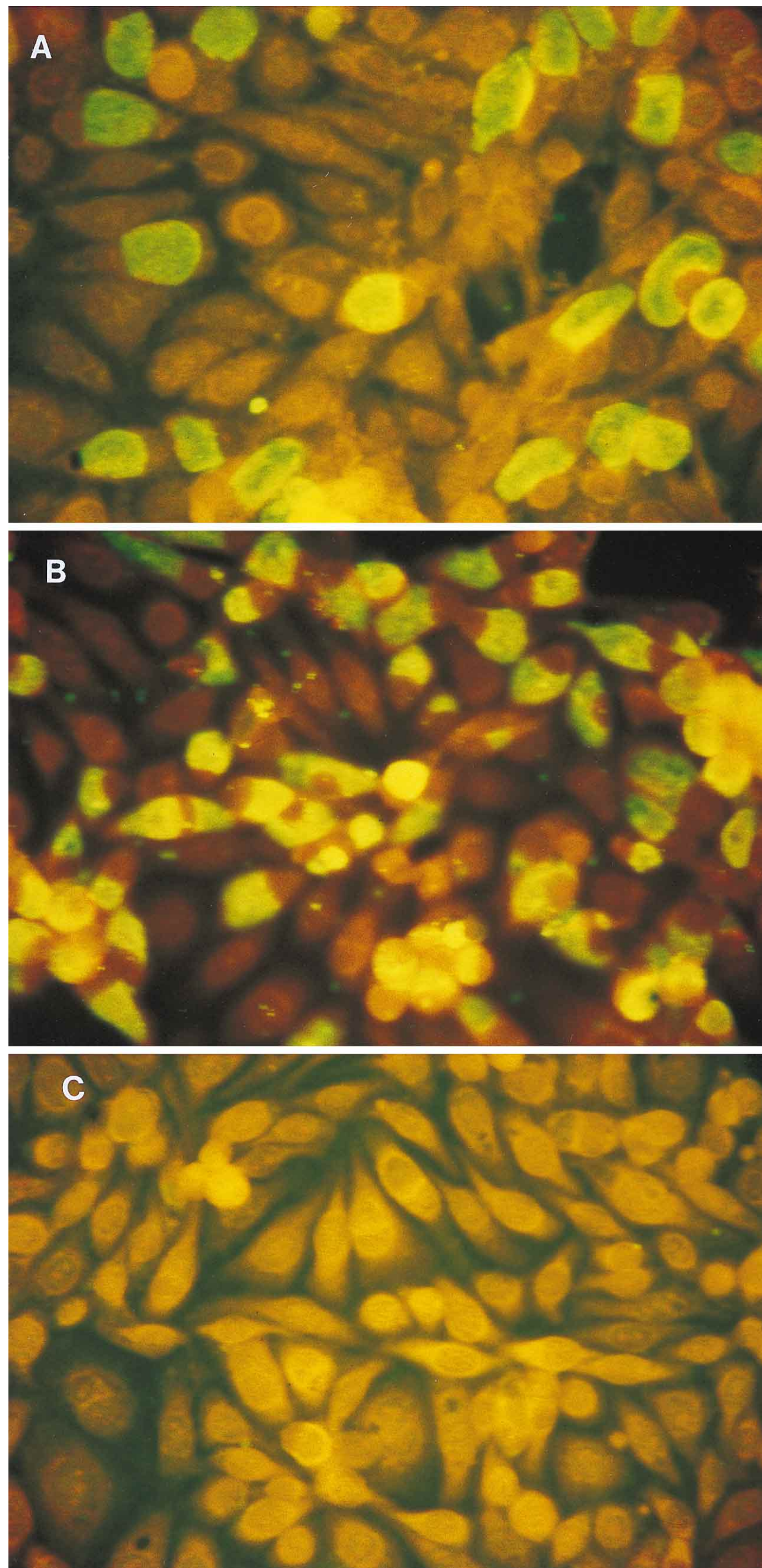

FIG. 1. Immunofluorescence results obtained by staining of *C. trachomatis*-infected LLCMK2 cell cultures with fluorescein-labelled antibodies to pgp3 and LPS. Cells were fixed with methanol 30 h after infection with the strain L2/434/Bu (A) or the α/95 isolate (B and C). Control pCT^{+} strain L2 inclusions show bright signals (A) when stained with antibodies to pgp3 (7). LPS staining of α/95-infected cells shows the normal appearance of the inclusions (B), whereas staining of the same culture with antibodies to pgp3 is negative (C).

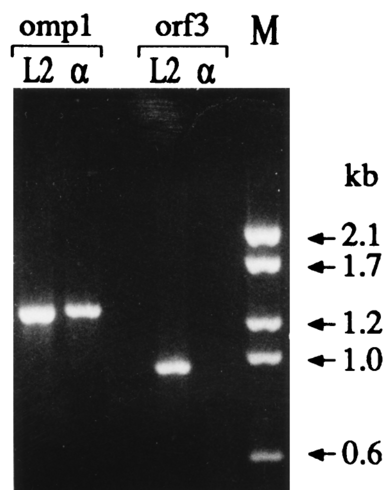

FIG. 2. Electrophoretic analysis of PCR results obtained with *orf-3*-specific and *omp-1*-specific primers. Control DNA from pCT$^+$ strain L2 was amplified and analyzed in parallel with DNA from strain α/95. The omp1 lanes (L2 and α) show that DNA segments of the correct size are produced when PCR is targeted to the single-copy *omp-1* gene. On the contrary, PCR performed in parallel with *orf-3*-specific primers (orf3 lanes) gives a DNA segment of the expected size with L2 DNA (lane L2) but a negative result with α/95 DNA (lane α). Lane M contained the molecular size markers.

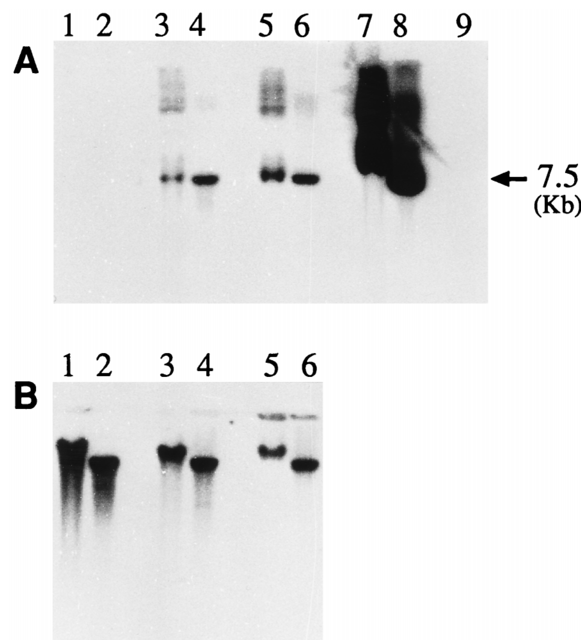

FIG. 3. (A) Southern blot probed with ^{32}P-labelled pCT-D DNA (7). Lanes: 1 and 2, 5 μg of total DNA extracted from α/95 EBs before (lane 1) and after (lane 2) digestion with *Bam*HI; 3 to 6, total DNA from purified EBs of pCT$^+$ control strains, i.e., 3 μg of L2/434/Bu DNA before (lane 3) and after (lane 4) *Bam*HI digestion and 3 μg of D/GO/86 DNA (7) before (lane 5) and after (lane 6) digestion with *Bam*HI; 7 and 8, a positive control and a size marker provided by pCT-D (7) cut at its unique *Bam*HI site, cloned in vector pUC8, and purified from *E. coli* before (lane 7) and after (lane 8) digestion with *Bam*HI; 9, negative control, i.e., 3 μg of total DNA from pCT$^-$ *C. pneumoniae* IOL 207. The arrow shows the position of full-length linearized pCT-D DNA. (B) Southern blot probed with a ^{32}P-labelled DNA probe specific for the single-copy chromosomal gene *omp-1* (α/95 variant). Lanes 1 to 6 were loaded the same way as the corresponding lanes in panel A.

primers used for *omp-1* amplification were OMPSN (CCCCg gatccATCTTGTCTCGCTTTAACT) and OMPDX (CCCCgg atccTTAACAAAGATAAACTTGCC). Lowercase letters indicate the *Bam*HI endonuclease restriction site added for cloning (see below). PCR conditions were 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C plus a final 10-min extension at 72°C. Thirty cycles were normally performed on 50 ng of purified DNA. An amplified DNA segment of the expected size for the *omp-1* gene was obtained (Fig. 2). The same DNA preparation, however, did not support PCR amplifications targeted either to *orf-3* (Fig. 2) or to a conserved segment (not shown) comprising the origin of replication of pCT as previously described (14). Both PCR assays targeted to pCT DNA are normally used in our laboratory and can detect approximately 10^2 pCT molecules per sample (14).

The possible presence in the α/95 genome of DNA segments broadly homologous to pCT DNA was tested by Southern blotting. Three- to 5-mg aliquots of DNA extracted from gradient-purified α/95 EBs were digested with *Bam*HI endonuclease (Boehringer) and analyzed on Southern blots by standard procedures (16) with ^{32}P-labelled whole-pCT DNA, the *orf-3* DNA segment, and the *omp-1* DNA segment as a control. Probes were labelled with ^{32}P by random priming with the Prime-a-gene kit (Promega). Blots were pretreated for 30 min at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS)–5× Denhardt's solution (16). After denaturation of the probe (95°C for 3 min), hybridization was performed overnight at 65°C in the same solution. The membranes were washed twice for 10 min each time in 0.2% SDS–2× SSC at room temperature, once in 1× SSC–0.1% SDS for 15 min at 65°C, and once in 0.1% SSC–0.1% SDS for 10 min at 65°C. Blots were air dried and exposed to Phosphor Screens (Molecular Dynamics). The resulting images were scanned with a PhosphorImager apparatus (Molecular Dynamics). In parallel, the same amount of DNA extracted from either pCT$^+$ *C. trachomatis* L2/434/Bu or D/GO/86 (6) or pCT$^-$ *C. pneumoniae* IOL-207 (4) was also analyzed on the same blot and with the same probes (Fig. 3). The results show that the amount of chlamydial DNA present on the blots was adequate for clear detection of the presence of the single-copy chromosomal gene *omp-1* and that a whole-pCT DNA probe (Fig. 3) or an *orf-3*-specific probe (not shown) could detect pCT-homologous DNA in the serotype L2 and D strains but not in the α/95 isolate or in the pCT-negative *C. pneumoniae* control.

All of the assays done (immunofluorescence screening of several thousand inclusions and Southern blot and PCR analyses of purified DNA) indicate that the α/95 isolate is essentially deprived of pCT (either plasmid free or unusually plasmid poor). Of course, all of the above techniques have detection limits and the possibility of the presence of a few pCT$^+$ chlamydiae in the population examined cannot be excluded. However, the negative PCR results, obtained with a 50-ng sample of purified DNA, which in a pCT$^+$ isolate should normally contain some 5×10^8 pCT molecules, against an expected PCR sensitivity on the order of 10^2 molecules per sample (14), indicate that most α/95 chlamydial cells must be plasmid free and the expected frequency of a pCT$^+$ chlamydial cell in the isolate could be on the order of 1 in every 5×10^6 cells if a pCT copy number of 10 is assumed or 1 in every 5×10^5 cells if one assumes a mutation reducing the plasmid to a single copy per cell. We submit that most chlamydiae of the α/95 isolate, which has been repeatedly cultured in our laboratory for over 2 years, are able to replicate and grow well in cell culture in the absence of pCT. Whether repeated passages in vivo, e.g., in an animal model of infection and disease, would

```
                                                                                          VS1
                                                                             *******  **********
L2          ..........  ..........  ..........  ..C.......  ..........  ..Q.......  ...-.....A  ....A....C
95          ..........  ..........  ..........  ..C.......  ..........  ..........  ...-.....A  ....T.....
B/Jali      ..........  ..........  ..........  ..C......I  ..........  ..........  ...-......  ....A.....
Ba                                                                           ......  ...-....A.  ....T.....
B      1    LPVGNPAEPS  LMIDGILWEG  FGGDPCDPCT  TWVDAISMRM  GYYGDFVFDR  VLKTDVNKEF  QMG-AKPTTT  TGNAVAPSTL
                                                C
                                                                                          VS2
            *****                                                                **********  **********
L2          .-........  ..........  ...Y......  ..........  .T........  ..........  D...HAT..D  SKL.......
95          .-........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Jali        .-........  ..........  ..........  ..........  ..........  ..........  ..........  ST........
Ba          .-........  ....                                                 .......  ..........  ST........
B     81    T-ARENPAYG  RHMQDAEMFT  NAACMALNIW  DRFDVFCTLG  ASSGYLKGNS  ASFNLVGLFG  NNENQTKVSN  GAFVPNMSLD

                                                                                          VS3
            **                                                                     *****  *********
L2          ..........  T....A....  ..........  ..........  ..........  ..........  .....Q.F..  ..K....GV.
95          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Jali        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
Ba          ........                                                                 .........  ...S......
B    161    QSVVELYTDT  AFAWSVGARA  ALWECGCATL  GASFQYAQSK  PKVEELNVLC  NAAEFTINKP  KGYVGKELPL  DLTAGTDAAT

                                                                                    VS4
                                                                     *  **********  **********  ****_*****
L2          ..........  ..........  ..........  ..........  ..........  .T.V......  ..........  .A..-.....
95          ..........  ..........  ..........  ..........  ..........  ..........  ..........  ....-.....
Jali        ..........  ..........  ..........  ..........  ..........  ..........  ..........  ....-.....
Ba          ...                                                    .....  ..........  ..........  ....-.....
B    241    GTKDASIDYH  EWQASLALSY  RLNMFTPYIG  VKWSRASFDA  DTIRIAQPKS  AETIFDVTTL  NPTIAGAGDV  KTSA-EGQLG


L2          ..........  ..........  ..........  ..........  ..........  .....
95          ..........  ..........  ......V...  ..........  ..........  .....
Jali        ..........  ..........  ..........  ....I.....  ..........  .....
Ba          ....
B    321    DTMQIVSLQL  NKMKSRKSCG  IAVGTTIVDA  DKYAVTVETR  LIDERAAHVN  AQFRF
```

FIG. 4. Alignment with reference MOMP sequences showing that the protein encoded by α/95 *omp-1* is a serotype B MOMP variant. The amino acid sequence of the reference serotype B MOMP on the bottom line is that reported by Stephens et al. (17) for strain B/T-W5/OT. Gaps (dashes) normally introduced for overall MOMP alignments (2, 8, 20) were also added here to maintain residue numbering in accordance with that in the chlamydial MOMP literature. The four hypervariable segments (VS1 to VS4) are marked by asterisks. For the α/95 MOMP, the L2/434/Bu MOMP, and two other serotype B variant MOMPs shown in this alignment (the serotype Ba prototype Ba/AP-2 and B/Jali-20/OT), only differences are shown; dots indicate identical amino acid residues. Two residues are shown at position 33 in the reference MOMP sequence because there is a discrepancy with respect to the sequence reported by Baher et al. (2), which has a cysteine at position 33.

cause selection in favor of pCT^+ chlamydiae is a very interesting point which remains to be demonstrated and is, in fact, one of the possible research applications of this strain which we envisage.

We further characterized the α/95 isolate by cloning and determining the nucleotide sequence of its *omp-1* gene. The α/95 *omp-1* gene was obtained by PCR amplification with the OMPSN and OMPDX primers described above, and the sequence of the amplified DNA segment was initially determined directly by using the Sequenase PCR Product Sequencing Kit (Amersham) and a set of 10 synthetic primers deduced from other *omp-1* sequences. Subsequently, the same amplified *omp-1* DNA was cloned into the *Bam*HI site of the vector BlueScript KS. The nucleotide sequence of the cloned *omp-1* insert was again determined, with identical results, with an automated DNA sequencer (Perkin Elmer). Nucleotide sequence analysis performed with the GCG software (9) showed that the protein encoded by the α/95 *omp-1* gene is most homologous to the MOMP variants reported in the literature for *C. trachomatis* serovar B isolates (Fig. 4). Compared with the sequence of the reference serovar B isolate B/TW-5/OT reported by Stephens et al. (17), the MOMP amino acid sequence deduced from α/95 *omp-1* shows four amino acid changes (Fig. 4): one in N-terminal conserved segment C1, restoring the generally conserved cysteine-33 residue; two in variable segment VS1; and one in conserved C-terminal segment C5, where α/95 has a valine-for-isoleucine substitution at position 347 as in the serotype A MOMP. In particular, the VS1 region of the α/95 MOMP has an alanine-for-threonine substitution at position 70 of the MOMP multiple alignments (8, 20), which is unique among all of the serotype B variants yet described (2, 8, 10, 17) but is present in the LGV-2 serotype (also belonging to serogroup B; see reference 20). A mutation at position 70 of the aligned MOMPs is also present in isolate Ba/J104 (8), where threonine is replaced with proline. The second VS1 mutation is a threonine-for-valine substitution at position 75 which has also been described for most serotype Ba variants (8, 20). Both VS1 mutations occur within a peptide that has been shown to be a B-cell epitope for several serovars, including B (19, 2). This combination of mutations would generate a new VS1 peptide variant (TTATGNATAPS) which closely resembles the VS1 putative epitope of serotype Ba;

however, since α/95 differs from the serotype Ba strains in both the VS2 and VS3 segments, it is possible that the observed *omp-1* genotype variations of α/95 also result in a serotype B variant of the expressed MOMP.

Interestingly, α/95 has normal growth properties and belongs to biovar trachoma and so can be expected to have pathogenicity properties different from those of the L2 (biovar lymphogranuloma venereum) plasmid-free strain previously described (13). Together with this strain, α/95 could therefore represent a useful additional tool for studies on the role of the pCT plasmid in vivo and for the development of chlamydial molecular genetics, e.g., as a recipient strain for pCT-based shuttle vectors. Our preliminary attempts to introduce native pCT extracted from *C. trachomatis* L2/434/Bu into α/95 by electroporation as described by Tam et al. (18) have given occasionally positive but not reproducible results. Further work along this line is in progress; however, the possibility that, at least under certain conditions, the genome of α/95 may not sustain pCT replication, for instance, because of DNA modification-restriction mechanisms, cannot be excluded.

These findings confirm the previous report by Peterson et al. that pCT genes are not essential for growth and replication (13). Consequently, in our view, since pCT is clearly maintained in multiple copies in most clinical isolates, these observations support the hypothesis that plasmid genes may, in fact, have a role in chlamydial pathogenicity.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank database under accession no. U80075.

This project was supported by the program Human Capital and Mobility of the European Union (contract ERBCHRXCT920040).

REFERENCES

1. **An, Q., G. Radcliffe, R. Vassallo, D. Buxton, W. J. O'Brien, D. A. Pelletier, W. G. Weisburg, J. D. Klinger, and D. M. Olive.** 1992. Infection with a plasmid-free variant chlamydia related to *Chlamydia trachomatis* identified by using multiple assays for nucleic acid detection. J. Clin. Microbiol. **30:** 2814–2821.
2. **Baher, W., Y.-X. Zhang, T. Joseph, H. Su, F. E. Nano, K. D. Everett, and H. D. Caldwell.** 1988. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc. Natl. Acad. Sci. USA **85:**4000–4004.
3. **Birkelund, S., and R. S. Stephens.** 1992. Construction of physical and genetic maps of *Chlamydia trachomatis* serovar L2 by pulsed-field gel electrophoresis. J. Bacteriol. **174:**2742–2747.
4. **Carter, M. W., S. A. al-Mahdawi, I. G. Giles, J. D. Treharne, M. E. Ward, and I. N. Clarke.** 1991. Nucleotide sequence and taxonomic value of the major outer membrane protein gene of *Chlamydia pneumoniae* IOL-207. J. Gen. Microbiol. **137:**465–475.
5. **Cevenini, R., M. Donati, V. Sambri, F. Rumpianesi, and M. La Placa.** 1987. Reactivity of elementary and reticulate bodies of *C. trachomatis* LGV2 with monoclonal antibodies specific for the major outer membrane protein. FEMS Microbiol. Lett. **42:**47–51.
6. **Comanducci, M., S. Ricci, R. Cevenini, and G. Ratti.** 1990. Diversity of the chlamydial common plasmid in biovars with different pathogenicity. Plasmid **23:**149–154.
7. **Comanducci, M., R. Cevenini, A. Moroni, M. M. Giuliani, S. Ricci, V. Scarlato, and G. Ratti.** 1993. Expression of a plasmid gene of *Chlamydia trachomatis* encoding a novel 28 kDa antigen. J. Gen. Microbiol. **139:**1083–1092.
8. **Dean, D., J. Schachter, C. R. Dawson, and R. S. Stephens.** 1992. Comparison of the major outer membrane protein variant sequence regions of B/Ba isolates: a molecular epidemiologic approach to *Chlamydia trachomatis* infections. J. Infect. Dis. **166:**383–392.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
10. **Hayes, L. J., M. A. Pickett, J. W. Conlan, S. Ferris, J. S. Everson, M. E. Ward, and I. N. Clarke.** 1990. The major outer-membrane proteins of Chlamydia trachomatis serovars A and B: intra-serovar amino acid changes do not alter specificities of serovar- and C subspecies-reactive antibody-binding domains. J. Gen. Microbiol. **136:**1559–1566.
11. **Moroni, A., M. Pavan, M. Donati, and R. Cevenini.** 1996. Differences in the envelope proteins of *C. trachomatis* and *C. psittaci* shown by two dimensional electrophoresis. Arch. Microbiol. **165:**164–168.
12. **Moulder, J. W., T. P. Hatch, C. C. Kuo, J. Schachter, and J. Storz.** 1984. *Chlamydia*, vol. 1, p. 729–739. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore, Md.
13. **Peterson, E. M., B. A. Markoff, J. Schachter, and L. M. de la Maza.** 1990. The 7.5-kb plasmid present in *Chlamydia trachomatis* is not essential for the growth of this microorganism. Plasmid **23:**144–148.
14. **Ratti, G., A. Moroni, and R. Cevenini.** 1991. Detection of *Chlamydia trachomatis* DNA in patients with non-gonococcal urethritis using the polymerase chain reaction. J. Clin. Pathol. **44:**564–568. (Erratum, **45**, 1992.)
15. **Saiki, A. K., D. H. Gelfand, S. Stoffell, S. J. Scharf, R. Higuchi, G. T. Horne, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
16. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Stephens, R. S., R. Sanchez-Pescador, E. A. Wagar, C. Inouye, and M. S. Urdea.** 1987. Diversity of *Chlamydia trachomatis* major outer membrane protein genes. J. Bacteriol. **169:**3879–3885.
18. **Tam, J. E., C. H. Davis, and P. B. Wyrick.** 1994. Expression of recombinant DNA introduced into *Chlamydia trachomatis* by electroporation. Can. J. Microbiol. **40:**583.
19. **Villeneuve, A., L. Brossay, G. Paradis, and J. Hébert.** 1994. Characterization of the humoral response induced by a synthetic peptide of the major outer membrane protein of *Chlamydia trachomatis* serovar B. Infect. Immun. **62:** 3547–3549.
20. **Yuan, Y., Y.-X. Zhang, N. G. Watkins, and H. D. Caldwell.** 1989. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. Infect. Immun. **57:**1040–1049.

Editor: P. J. Sansonetti