Deoxyribonucleic Acid Relatedness of *Chlamydia* sp. Strain TWAR to *Chlamydia trachomatis* and *Chlamydia psittaci*

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*Chlamydia* sp. strain TWAR is an important cause of acute respiratory disease in humans. To determine the genetic relatedness of the TWAR organism to *Chlamydia trachomatis* and *Chlamydia psittaci*, solution hybridization followed by S1 nuclease assays were performed, and guanine-plus-cytosine (G+C) contents were determined. Deoxyribonucleic acid (DNA) preparations from three TWAR isolates were radioactively labeled and hybridized against DNAs from three serovars of *C. trachomatis* and five strains of *C. psittaci*. Reciprocal experiments were also done by using radioactively labeled *C. trachomatis* and *C. psittaci* DNAs against TWAR DNA. In all cases, the degree of DNA relatedness between TWAR and the two *Chlamydia* species was 10% or less. Within the group of TWAR isolates, there was 94% or greater sequence similarity. The G+C values for TWAR were intermediate between the G+C values for *C. trachomatis* and *C. psittaci*. The degrees of DNA relatedness among the strains within the species *C. psittaci* ranged from 20 to 100%.

A newly recognized strain of *Chlamydia*, designated TWAR, has been shown to be an important human respiratory pathogen (9, 19, 25). Two species of *Chlamydia* are currently recognized, *Chlamydia psittaci* and *Chlamydia trachomatis* (21, 23). Like *C. psittaci*, TWAR inclusions are dense and oval shaped and contain no glycogen. These phenotypic characteristics clearly distinguish it from *C. trachomatis* (8, 15). Recent ultrastructural analysis has shown that the TWAR elementary body is unique compared with the elementary bodies of the other *Chlamydia* spp. (3). TWAR shares the genus-specific lipopolysaccharide antigen with the other *Chlamydia* spp. (15). Otherwise, the TWAR organism is serologically unique, with all TWAR isolates belonging to one serovar (9, 15). TWAR strains can be readily distinguished from the other *Chlamydia* spp. by restriction endonuclease analysis and Southern hybridization, using cloned TWAR deoxyribonucleic acid (DNA) fragments as probes (2). Qualitative DNA homology studies by dot blot analysis have shown that TWAR does not share extensive homology with the human biovars of *C. trachomatis* or with five mammalian and avian strains of *C. psittaci* (2).

DNA-DNA liquid hybridizations were performed in order to quantitate the degree of genetic relatedness that TWAR strains have to each other and to the other *Chlamydia* spp. The level of DNA sequence similarity among the *C. psittaci* strains used in this study was also determined. In addition, the guanine-plus-cytosine (G+C) contents were determined for *C. trachomatis*, *C. psittaci*, and TWAR.

**MATERIALS AND METHODS**

*Chlamydia* strains. The TWAR isolates used in this study included strains TW-183, AR-39, AR-388, AR-458, and LR-65 (9, 15). The *C. trachomatis* strains used were B/TW-5/OT, D/UW-3/Cx (27), and L2/434/Bu (26), which is the reference strain for *C. trachomatis* biovar LGV (ATCC VR-902) (21). The *C. psittaci* strains used included avian isolate 6BC (8) and the mammalian strains meningopneumonitis (Mn) (5), feline pneumonitis (FP) (1), guinea pig inclusion conjunctivitis (GPIC) (13), and ovine abortion (OA), a local isolate obtained from P. Dulbeck, Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman.

All isolates were grown and serially passed in HeLa 229 cell cultures (17). Elementary bodies were purified with a linear gradient of meglumine diatrizoate (Hypaque-76; Winthrop-Breon Laboratories, New York, N.Y.). Strains Mn and B/TW-5/OT were also propagated in embryonated chicken eggs, either in allantoic cavities (strain Mn) or in yolk sacs (strain B/TW-5/OT) as described previously (16). Purification of organisms from allantoic fluids was done by three cycles of differential centrifugation. Purification of organisms from yolk sacs was done by three cycles of differential centrifugation followed by sucrose gradient centrifugation (16).

**DNA isolation.** DNAs were isolated from purified organisms as described previously (2). Elementary bodies were suspended in 10 mM tris(hydroxymethyl)aminomethane–methylamine–1 mM ethylenediaminetetraacetate–50 mM NaCl and were treated with deoxyribonuclease (4 U/ml) for 30 min at 37°C to reduce contaminating HeLa cell DNA (done only on those strains grown in HeLa cells). After heat inactivation of the deoxyribonuclease (10 min at 70°C), the elementary bodies were solubilized by treatment with proteinase K (100 µg/ml) and 1% sodium dodecyl sulfate at 37°C for 1 h. DNA was extracted with tris(hydroxymethyl)aminomethyl-saturated phenol and was treated with ribonuclease (50 µg/ml). After phenol–chloroform extraction, the DNA was ethanol precipitated. By using this procedure, 50 to 150 µg of DNA per strain was obtained from eight 105-cm² culture bottles.

**Preparation of probe DNA.** DNA was labeled in vitro by nick translation with either [32P]deoxyadenosine triphosphate or [32P]deoxyguanosine triphosphate, using a standard protocol (18), to an average specific activity of 3 × 10⁶ cpm/µg of DNA. The appropriate deoxyribonuclease concentration for nick translation was calibrated so that the probe fragment size was 300 to 600 base pairs. Each probe was stored at 4°C and was used within 5 days.

**DNA-DNA liquid hybridization and S1 nuclease assay.** Determinations of DNA homology were done by using the method of Crosa et al. (4), with minor modifications. The unlabeled DNA (test DNA) to be used for hybridization with the labeled probe DNA was sonicated to a fragment size of approximately 500 base pairs. Probe DNA (5,000 cpm) was mixed with 3 to 15 µg of test DNA. The final ratio of probe
DNA to test DNA ranged from 1:1,000 to 1:5,000. After normalization, both ratios gave identical results. The final volume was adjusted to 200 μl in 0.42 M NaCl. For each set of experiments, all samples being processed had the same DNA ratio, DNA concentration, and mixture volume. The samples were denatured by boiling for 11 min and were hybridized overnight at 65°C.

After 18 to 48 h, ice-cold 0.42 M NaCl was added to a final volume of 1 ml. To determine the percentage of reassociation between probe DNA and test DNA, 200-μl portions were removed and incubated with S1 nuclease (Pharmacia, Inc., Piscataway, N.J.), an enzyme that preferentially digests single-stranded DNA (4). The optimal concentration of the enzyme necessary for complete hydrolysis of single-stranded DNA without any hydrolysis of double-stranded DNA was determined by testing serial dilutions of both double- and single-stranded salmon sperm DNA (4). A homologous control (the probe DNA hybridized against unlabeled DNA identical to the DNA from which the probe was made) and a heterologous control (the probe DNA hybridized against HeLa or salmon sperm DNA) were run in each assay.

Standardization of assay. The degree of DNA relatedness was determined as described by Crosa et al., with minor modifications (4). When chlamydial DNA was prepared from egg-grown organisms, the probe was always less than 7% homologous to HeLa DNA. For the chlamydial DNA probes prepared from HeLa cell-grown organisms, hybridization of the probe against HeLa DNA resulted in values ranging from 8 to 12%. This number was subtracted from the value for homologous DNA reassociation before normalizing it to 100%. The levels of homology of each probe to the various chlamydial DNAs were determined relative to the normalized homologous control. Duplicate tests were performed for each assay. The values reported below are averages of at least two assays.

Tₚ profile and G+C content. The G+C contents of whole-cell DNAs were determined for TWAR organisms (strains TW-183 and AR-388), C. trachomatis B/TW-5/OT and L2/434/Bu, and C. psittaci 6BC and Mn by using the thermal melting (Tₚ) method (11). DNA preparations were dialyzed overnight against 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Escherichia coli HB101 was used as the standard, and the G+C contents were calculated by using the following equation: G+C content = 51 + (Tₚ - Tₚ of E. coli) 1.99 (11). The G+C values reported below were calculated from the Tₚ profiles of three separate runs determined with a Response Series UV-VIS spectrophotometer equipped with a Response II thermal programmer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). All Tₚ determinations were repeated by using different DNA samples and a Gilford model 250 spectrophotometer equipped with a model 2526 thermostprogrammer. Tₚ values varied less than 3°C. This variability, due to the use of two different spectrophotometers, was consistent (i.e., the Tₚ values varied by the same number of degrees between tests for each species).

RESULTS

HeLa DNA calibration. Regardless of the purification method used, removal of HeLa DNA contamination from chlamydial DNA preparations was not absolute. To determine how varying amounts of HeLa DNA contamination in the test DNA would affect the results, we attempted to quantitate the amount of HeLa DNA present in a given sample. DNA reassociation was determined by using a HeLa DNA probe against samples containing graduated amounts of HeLa DNA (range, 0.05 to 15.0 μg of HeLa DNA). E. coli DNA was added in the appropriate amounts to maintain a constant concentration of test DNA (15 μg). Because this protocol was designed for analysis of procaryotic DNA (4), only 36% reassociation was observed when HeLa DNA made up the entire sample. A concentration difference of 100-fold (0.05 to 5.0 μg of HeLa DNA per 15 μg of DNA sample) resulted in small increases in the average percentage of homology (11 to 28%). This precluded quantitation of HeLa contamination in the chlamydia DNA preparations.

When probe chlamydial DNA was hybridized against HeLa DNA, the degree of reassociation ranged from 8 to 12%, which was routinely 3 to 5% above the value for the salmon sperm DNA heterologous control. From these results, we concluded that small amounts of HeLa contamination in the test DNAs resulted in negligible hybridization differences. Chlamydioid probes were tested by dot blot hybridization against homologous chlamydial DNA and against HeLa DNA. Probes showing little or no hybridization to HeLa DNA in this assay resulted in less than 12% reassociation with HeLa DNA. Any probe resulting in more than 12% reassociation with HeLa DNA was not used in the study.

Quantitative whole-cell DNA homology. As shown in Table 1, when TWAR DNA (from isolate TW-183, AR-39, or LR-65) was used as a probe, TWAR showed less than 10% DNA relatedness with the strains of C. trachomatis and C. psittaci tested. Using DNAs from C. psittaci and C. trachomatis as probes against TWAR DNA gave consistent results. Within the group of TWAR isolates, the level of reassociation was 94 to 100%. The level of relatedness among the strains of C. psittaci was variable, ranging from 20 to 100%. Strains 6BC, OA, and Mn shared between 63 and 100% sequence similarity, while strains FP and GPIC were only 24 to 37% related to the other C. psittaci strains.

G+C content. The G+C ratios were 40 mol% for TWAR, 42 mol% for C. trachomatis, and 39 mol% for C. psittaci (Table 2). The values for TWAR consistently fell between the values for C. trachomatis and C. psittaci.

DISCUSSION

In our initial studies of the TWAR organism, we tentatively classified TWAR as a C. psittaci strain because of similarity in inclusion morphology (9, 15). Recent evidence showing that the TWAR elementary body is unique among Chlamydia spp. (3), coupled with the results of serological (9, 15) and nucleic acid studies (2), has prompted a more precise investigation into the taxonomy of TWAR. Under the conditions used in this study, TWAR shows less than 10% DNA relatedness with either C. trachomatis or C. psittaci. Our findings, showing less than 10% DNA sequence similarity between C. trachomatis and C. psittaci and a high degree of genetic relatedness (≥94%) among serovars comprising the human biovar of C. trachomatis, are in agreement with the results of previous DNA reassociation studies (14, 29).

The G+C values reported in this study fall within the range of those previously reported (7, 14, 21) and follow a continuous spectrum which has been observed for Chlamydia spp. (21), with C. psittaci at the lower end and C. trachomatis at the higher end. The G+C content of TWAR is intermediate between the G+C values for C. trachomatis and C. psittaci. This finding is compatible with other char-
The genetic relatedness of the C. psittaci strains was also determined. This is the first report that has compared, by DNA liquid hybridization, more than two C. psittaci strains in the same study. Gerloff et al. (6), using DNA agar techniques, found relatively high levels of homology among members of C. psittaci. Kingsbury and Wiess (14), also performing immobilized DNA reassociation experiments, found that strain Mn DNA shared 96% DNA sequence similarity with strain 6BC, but no other C. psittaci strains were tested. As shown in Table 1, the species C. psittaci is a more genetically heterogeneous group than either C. trachomatis or TWAR. This is to be expected from the diversity of the group in terms of disease manifestations (21), natural hosts (21), antigenicity (24), and restriction fragment patterns (10, 20). Our results show that strains 6BC, OA, and Mn share a relatively high level of DNA sequence similarity. On the other hand, strains FP and GPIC showed ≦37% and ≧35%, respectively, DNA relatedness with all of the C. psittaci strains tested.

DNA hybridization studies have proven to be a useful and accurate means to determine taxonomic classification of microorganisms (12, 28). Bacterial strains that show approximately 70% or more DNA-DNA relatedness are considered to comprise a single species (28). All four TWAR isolates tested share ≧90% DNA sequence similarity and thus belong to the same species. The species that currently belong to the genus Chlamydia share limited genetic similarity. Thus, taxonomic classification depends not only on genetic relatedness; morphological, biological, and antigenic characteristics are important criteria as well. Based on these parameters, TWAR is a member of the genus Chlamydia. Previous studies have demonstrated phenotypic traits of the TWAR organism that differentiate it from both C. psittaci and C. trachomatis (3, 15). The results presented in this study show that TWAR shares less than 10% genetic relatedness with either Chlamydia species. This cumulative information will be presented in a formal proposal recommending separate species status for the TWAR organism (J. T. Grayston, C.-C. Kuo, L. A. Campbell, and S.-P. Wang, submitted for publication).

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