Survey of Plasmids in Various Mycoplasmas

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Thirty-three strains representing 15 distinct Mycoplasma, Acholeplasma, and Spiroplasma species were examined for the presence of plasmid DNA by agarose gel electrophoresis. The electrophoretic patterns of the DNAs of three strains, Mycoplasma sp. strain 747, Spiroplasma mirum strain SMCA, and M. hominis strain 1257, suggested the presence of a plasmid with molecular weights of approximately 70, 10, and 9 megadaltons, respectively. The functions of these plasmids are currently unknown.

INTRODUCTION

Although plasmids are commonly found in a variety of prokaryotes, there has been no systematic examination for plasmids in microorganisms belonging to the class Mollicutes except for the genus Spiroplasma [1,2]. Extrachromosomal DNA or plasmids have been detected in Mycoplasma arthritidis [3,4], M. hominis [5], Acholeplasma laidlawii [6,7], and from ten of twelve Spiroplasma strains tested from different sources [1,17].

Although the functions of these mycoplasma plasmids are unknown, their presence might provide a useful tool to study the genetic properties of this class of microorganisms.

MATERIALS AND METHODS

Cultivation of Mycoplasmas

The 33 strains examined are listed in Table 1 and represent 15 species (11 Mycoplasma, two Spiroplasma, and one Acholeplasma) and nine serotypes of Ureaplasma urealyticum. Mycoplasma sp. strain 747, isolated from the vagina of a baboon by M. Davidson, New York, was sent to H. Neimark, Brooklyn, who in turn sent it to us. Strain 747 has been found to be unrelated to the established species of Mycoplasma [Barile, Grabowski: unpublished observations]. Spiroplasma mirum strain SMCA was received from J.G. Tully, Bethesda, and M. hominis strain 1257 obtained from H. Morton, Philadelphia, PA. The Mycoplasma species were grown in mycoplasma broth (Baltimore Biological Laboratories, Cockeysville, MD) supplemented with 10 percent unheated horse serum (M.A. Bioproducts, Walkersville, MD), 5 percent yeast extract (Flow Laboratories, McLean, VA) and 1,000 units per ml of penicillin G potassium (Eli Lilly & Co., Indianapolis, IN), incubated at 35°C. Dextrose (Fisher Scientific Company, Fair Lawn, NJ) was added to broth for growth of the fermenters, and L-arginine monohydrochloride (Eastman Organic

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TABLE 1
Mycoplasma Strains Investigated for the Presence of Plasmids

| Species                      | Strains | Natural Habitat                          | Plasmid Presence |
|------------------------------|---------|------------------------------------------|------------------|
| *Mycoplasma mycoides* subsp. mycoides | PG1     | Cattle, contagious pleuropneumonia       | –                |
|                              | B3      | Swine, nasal cavity                      | –                |
|                              | UM30847 | Goat                                     | –                |
| *M. mycoides* subsp. capri   | PG3     | Goat, pleuropneumonia                    | –                |
| *M. agalactiae*              | PG2     | Sheep, contagious agalactia              | –                |
| *M. bovis*                   | Donetta | Cattle, exudate, mastitis                | –                |
| *M. hominis*                 | PG21    | Human, rectal swab                       | –                |
|                              | H34     | Human, abdominal wound                   | –                |
|                              | 1257    | Human, Reiters syndrome                  | +                |
| *M. pneumoniae*              | PI-1428 | Human, primary atypical pneumonia        | –                |
|                              | M129    | Human, primary atypical pneumonia        | –                |
|                              | FH      | Human, primary atypical pneumonia        | –                |
|                              | B176    | Human, primary atypical pneumonia        | –                |
|                              | Mac     | Human, primary atypical pneumonia        | –                |
| *M. fermentans*              | PG18    | Human, ulcerative balanitis              | –                |
| *M. capricolum*              | 14      | Goat                                     | –                |
| *M. gallisepticum*           | PG31    | Avian, trachea, chronic respiratory disease | –                |
| *M. arthritidis*             | PG6     | Rat, knee joint                          | –                |
| *Mycoplasma* sp.             | M14     | Irus macaque                             | –                |
|                              | 7457    | Baboon, vagina                           | +                |
| *Ureaplasma urealyticum*     | Serotype 1 | Human, nongonococcal urethritis      | –                |
|                              | Serotype 2 | Human, nongonococcal urethritis      | –                |
|                              | Serotype 3 | Human, nongonococcal urethritis      | –                |
|                              | Serotype 4 | Human, nongonococcal urethritis      | –                |
|                              | Serotype 5 | Human, nongonococcal urethritis      | –                |
|                              | Serotype 6 | Human, urethra, Reiters syndrome      | –                |
|                              | Serotype 7 | Human, urethra, Reiters syndrome      | –                |
|                              | Serotype 8 | Human, nongonococcal urethritis      | –                |
|                              | Serotype 9 | Human, nongonococcal urethritis      | –                |
| *Spiroplasma citri*          | Maroc (R8) | Citrus plant, stubborn disease       | –                |
| *S. mirum*                   | SMCA    | Rabbit tick pool, *Haemophysalis leporisalpaulstris* | +                |
| *Acholeplasma laidlawii*     | PG8     | Sewage                                  | –                |
|                              | PG9     | Sewage                                  | –                |

Chemicals, Rochester, NY) was added for growth of the non-fermenting, arginine hydrolyzing mycoplasmas at final concentration of 1.0 percent and 0.2 percent, respectively. Ureaplasmas were grown in the 10-B medium formulation described by Shepard and Lunceford [8] and the two *Spiroplasma* species (*S. citri* and *S. mirum*) in SP4 broth described by Tully et al. [9]. Each culture produced from 1 to $5 \times 10^6$ color-changing units (CCU) per ml.

**DNA Preparation**

All mycoplasma cultures were screened for plasmids by using our modification of two methods, one reported by Hansen and Olsen [10] and the other by Kado and Liu [11]. Three other procedures [6,12,13] were used as well to examine 13 of the 33 strains. In brief, the organisms were harvested from 50 ml broth cultures by centrifugation at 10,000 rpm at 4°C for 30 minutes in a Sorvall SS34 rotor. The
mycoplasma cell pellet was suspended in 50 µl of TE buffer (50 mM tris[hydroxymethyl]aminomethane [Tris], BRL, Rockville, MD), pH 8.0, containing 10 mM ethylenediaminetetraacetate [EDTA], (Fisher Scientific Company, Fair Lawn, NJ) at 22–24°C. The cells were lysed by adding 0.5 ml of 1 percent (w/v) sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, CA) in TE buffer, pH 12.4, followed by 8 cycles of heat pulse at 56°C for 30 seconds. The lysates were removed from the water bath and mixed gently by inverting the tubes quickly five times. The pH was reduced to 8.0 by adding 30 µl of 2M Tris, pH 7.0. The lysate was treated with ribonuclease A (Worthington Biochemical Corp., Freehold, NJ) at a final concentration of 50 µg per ml at 37°C for 30 minutes, and then added in equal volume to an unbuffered mixture of 1:1 parts distilled phenol (BRL, Rockville, MD) and chloroform (Fisher Scientific Company, Fair Lawn, NJ) in a 1.5-ml Eppendorf tube. The mixture was emulsified by shaking, and the aqueous phase was separated by centrifugation at 15,000 rpm for five minutes in an Eppendorf Microcentrifuge, model 5412. The upper aqueous phase was transferred to another microcentrifuge tube, constituting the DNA preparation.

Electrophoresis of DNA

Samples (35 µl) of the DNA preparation were mixed with a tracking dye solution (5 µl) consisting of 0.07 percent (w/v) bromophenol blue (Sigma Chemical Company, St. Louis, MO), 7.0 percent (w/v) SDS and 33 percent (v/v) glycerol (Fisher Scientific Company, Fair Lawn, NJ) in water. The resulting solution was applied to a gel slab containing 0.7 percent agarose (Type I, Low EEO, Sigma Chemical Company, St. Louis, MO) and Tris-borate buffer (89 mM Tris, 2.5 mM disodium EDTA, and 89 mM boric acid, pH 8.2) and electrophoresed in a BRL vertical gel electrophoresis apparatus model V16 at 110 volts for about three hours, or until the dye marker reached the bottom of the gel as described by Meyers et al. [14]. The gels were stained for 15 minutes with a 0.4 µg/ml aqueous solution of ethidium bromide (Sigma Chemical Company, St. Louis, MO), visualized with ultraviolet light, and photographed.

Escherichia coli strain V517 containing eight plasmids [15] and E. coli K-12, χ1849 bearing plasmid pBR322 (both obtained from B.M. Chassy, Bethesda, MD) were grown at 35°C in 10 ml of L-broth [16] supplemented with 0.01 percent DL-diaminopimelic acid (Sigma Chemical Company, St. Louis, MO). The E. coli cells were processed and subjected to gel electrophoresis as described above, and the bands produced were used as molecular weight reference markers for establishing the size of unknown mycoplasma plasmids.

RESULTS AND DISCUSSION

The results of the survey for the presence of plasmids in the 33 mycoplasma strains examined are given in Table 1. The electrophoretic patterns of the DNAs of three strains representing three species (Mycoplasma sp. strain 747, M. hominis strain 1257 (not shown), and S. mirum strain SMCA) [17] showed additional bands to those of chromosomal DNA, suggesting the presence of plasmids (Fig. 1). Extrachromosomal DNA bands were not detected in five strains of M. pneumoniae, including the two pathogenic strains PI-1428 and M129, nor in any of the nine human serotypes of Ureaplasma urealyticum. All other mycoplasma strains were also negative by the procedures used. Zouzias et al. [5] examined cultures of M. hominis, M. arthritidis, M. pneumoniae, and A. laidlawii (strain designations not given) for the presence of extrachromosomal DNA by electron microscopy and found a
plasmid only in *M. hominis*. In our study, only strain 1257 of *M. hominis* was positive; strains PG21 and H34 were negative for plasmids. Thus, there appears to be strain variation with regard to harboring plasmids.

It must be emphasized that the results obtained depend on the procedure used, e.g., plasmids were detected by using our modification of Hansen and Olsen [10] and Kado and Liu [11] procedures, but we failed to detect plasmids in two of the positive strains (747 and SMCA) using three other procedures [6,12,13]. Moreover, whereas Ranhand et al. [2] reported the presence of plasmids in the Maroc stain of *Spiroplasma citri*, we failed to detect the presence of plasmids in this strain under the conditions of our tests. Negative findings do not necessarily imply the absence of plasmids in the mycoplasmas tested, but rather that they were negative by the test procedures used.

Because viruses [18,19] and/or plasmids [2] have been commonly found in *Spiroplasma* and *Acholeplasma* species, it has been suggested that these extrachromosomal DNA components may pose difficult problems regarding the use of restriction enzymes for analysis of chromosomal DNA [20]. Conversely, since most *Ureaplasma* and *Mycoplasma* species tested appear to be free of viruses and plasmids, these species should lend themselves as excellent models for restriction enzyme studies and, in fact, experimental data support these conclusions [21,22].

The logarithmic plot of the relative migration of the reference *E. coli* plasmids and of the mycoplasma plasmids is shown in Fig. 2. Based on these values, the molecular weights of the *Mycoplasma* strain 747, *S. mirum* strain SMCA, and *M. hominis* strain 1257 plasmids were calculated to be approximately 70, 10, and 9 megadaltons (Mdal), respectively. The large size of the strain 747 plasmid was unexpected because the size of the mycoplasma genome itself is very small (5 × 10^6 Mdal), and because mycoplasma plasmids and viruses reported previously ranged from only 1.5 to 25.8 Mdal [2,19]. Thus, the strain 747 plasmid appears to be the largest extrachromosomal DNA particle as yet found in the class mollicutes. The molecular weight of the plasmids of *S. mirum* and *M. hominis* fall within the range of the cryptic plasmids isolated from other *Spiroplasma* and *Mycoplasma* species and strains [2,3,5]. Some of these plasmids were shown to be circular [2,4,5] and represent cryptic plasmids [2]. Some were detected by cesium chloride [3], or by

![Fig. 1. Agarose gel electrophoresis of plasmid DNA from cell lysates. Track (A), plasmid pBR322 of *E. coli* K-12 \( \times 18499\); (B), plasmids of *E. coli* V517; (C), plasmid of *Spiroplasma mirum* SMCA; (D), *Spiroplasma citri* Moroc; (E), same as (B); (F), plasmid of *Mycoplasma* sp. 747. The four smaller plasmids of *E. coli* V517 were not detected on these gels under the conditions of test.](image)
FIG. 2. Molecular weights of the plasmids of *Mycoplasma* sp. strain 747 and of *Spiroplasma mirum* (SMCA) were determined by using plasmids of *E. coli* V517 and χ1849 (pBR322) as references. Symbols: ○, four reference plasmids of *E. coli* V517: pVA517A (35.8 Mdal), pVA517B (4.8 Mdal), pV517C (3.7 Mdal), and pVA517D (3.4 Mdal); □, pBR322 (2.6 Mdal); ■, *Mycoplasma* sp. strain 747 plasmid; ■, *Spiroplasma mirum* strain SMCA plasmid; ▲, *Mycoplasma hominis* strain 1257.

ethidium bromide-cesium chloride or sucrose gradient centrifugation procedures [2]. The molecular masses of these plasmids were calculated to be 20 Mdal for *M. arthritidis* [4], 18 Mdal for *M. hominis* [5], and 16 and 30 Mdal [7] or 19 and 26 Mdal [23] for *A. laidlawii*. In addition, plasmids were detected in ten of twelve spiroplasma strains examined by Ranhand et al. [2]. Two to eight different size classes were present in each of the ten positive spiroplasma strains. The strains containing plasmids include *S. citri* strain C189; strains 4; 9; 608; 750; BC3 (honeybee), X (belonging to *S. citri* group), Cactus B (corn stunt), and Scaph. The molecular masses of the extrachromosomal bands from these ten spiroplasma strains ranged from 1 to 26 Mdal [2]. The genetic function of the plasmids isolated from mycoplasma remains to be determined.

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