Molecular Biology of Spiroplasma Plasmids

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With one exception, all spiroplasma strains examined contained extrachromosomal DNA, most of which was in the form of covalently closed circular plasmids. One plasmid, pIJ2000, carried by Spiroplasma citri strain ASP-1, was purified and characterized and used to probe for related plasmids in other strains. Unsuccessful attempts were made to clone pIJ2000 into Escherichia coli using the vectors pAT153 and pBR322. However, spiroplasma chromosomal DNA fragments could be cloned without difficulty.

INTRODUCTION

Plasmids carried by spiroplasmas were first described by Ranhand et al. [1], who showed that ten of 12 spiroplasma strains examined possessed indigenous plasmids. Electron microscopy and agarose gel electrophoresis were used to characterize covalently closed circular DNA molecules purified by CsCl-ethidium bromide buoyant density centrifugation and it was found that each strain carried multiple plasmids, from two to eight in number.

Archer [2] obtained similar results in a survey of over 30 strains. With one possible exception, all the strains gave from one to ten bands on electrophoresis. The non-helical Spiroplasma citri strain ASP-1 [3] gave only one band, and the plasmid, designated pIJ2000, was purified and a restriction map produced [4]. No functions encoded by spiroplasma plasmids have yet been identified.

In this paper we present some data on the relationships between the plasmids of certain spiroplasmas, and we report attempts to clone the plasmid pIJ2000 into Escherichia coli so that the powerful methods of recombinant DNA technology can be harnessed to analyzing the functions of the plasmid.

MATERIALS AND METHODS

**Spiroplasmas**

Strains and growth conditions described by Daniels et al. [5] were used, with the following additional strains: S. citri ASP-1, ASP-2, SP2, SP4-6aS, SP-V3 (all isolated in this laboratory from Israeli citrus material), BR6 (from R.M. Goodman); flower spiroplasma SR9 (R.E. Davis), and coconut palm spiroplasma N525 (from S.J. Eden-Green).

**Plasmid Detection and Isolation**

Spiroplasmas were harvested and cleared lysates were prepared as described previously [4]. The lysates were either examined directly by agarose gel electrophoresis or plasmids were purified by CsCl-ethidium bromide isopycnic density

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gradient centrifugation [4]. Purified plasmid DNA (pAT153, pBR322, and pBR328) was donated by A.G. Hepburn and C. Waldron.

Hybridization

Ethidium bromide-stained gels were photographed and then immersed successively in 0.25 M HCl (20 minutes), water, 0.2 M NaOH–0.6 M NaCl (one hour), and 1 M tris-HCl (pH 7.4)–1.5 M NaCl (30 minutes), and denatured DNA fragments were transferred to Gene Screen membranes (NEN, Boston, MA) using a procedure similar to that of Southern [6]. The membranes were placed in polyethylene bags containing polyvinylpyrrolidone (0.02 percent), bovine serum albumin (0.02 percent), Ficoll (0.02 percent) [7], sodium dodecyl sulphate (SDS) (0.1 percent), and formamide (40 percent) dissolved in 4 x SSC [8]. Probe DNA was labeled by “nick translation” with [32P] d CTP [9], and was denatured by heating at 100°C for five minutes, followed by rapid cooling, before addition to the hybridization bags. Hybridization was carried out for 16 hours at 37°C, after which the membranes were washed in 2 x SSC (five times at 20°C) and 0.1 x SSC–0.5 percent SDS (one hour at 40°C). Radioactive DNA bands were located by autoradiography.

DNA Cloning

0.2–1 µg portions of spiroplasma DNA were cleaved with the restriction endonucleases HindIII, BglII, MboI, or EcoRI and ligated with appropriately cleaved pAT153, pBR322, or pBR328 DNA. The products were used to transform E. coli 803 [10]. Colonies transformed with recombinant plasmids were tentatively identified by their resistance to carbenicillin and sensitivity to tetracycline (for pAT153 and pBR322) or chloramphenicol (pBR328), and were definitively characterized by cleaving the partially purified plasmids [11] with the appropriate enzyme to demonstrate the production of linear vector molecules plus single fragments of spiroplasma DNA whose origin was verified by hybridization.

RESULTS

Electrophoretic examination of cleared lysates of spiroplasmas showed that, with one possible exception (S. floricola BNR-1), they possessed up to ten extrachromosomal DNA species (Fig. 1). Because of unavoidable preparative manipulations, nicks may be introduced into DNA molecules so a plasmid may exist in three forms: covalently closed supercoiled circular (ccc), linear, and open circular (in order of decreasing electrophoretic mobility). Thus a strain containing several plasmids may give complex band patterns, and moreover the extent of nicking may vary from one preparation to another. Two techniques were useful in resolving this difficulty. First, plasmid DNA recovered from CsCl-ethidium bromide gradients is almost exclusively ccc, so that comparison of electrophoretic patterns of cleared lysates with purified plasmids is revealing. Second, two-dimensional electrophoresis, with exposure to ultraviolet light to introduce single-strand nicks between the successive runs, permits unambiguous assignment of the forms [12]. In addition, the “stepladder” patterns sometimes seen [4] were shown by this procedure to represent single plasmids with a variable number of superhelical turns.

Nothing is yet known about functions encoded by spiroplasma plasmids. One attractive possibility is that pathogenicity determinants may be plasmid-borne, and if so one would expect to find some sequence homology between plasmids of different isolates of spiroplasmas from similar ecological niches. In our initial experiments, plasmid pIJ2000 was used to probe Southern “blots” from gels containing plasmids
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from a number of other strains, and homology was found with a small number of S. citri strains only (strain Iran and the Israeli isolates SP-A, ASP-2, and SP4-6aS—the initial isolates from which ASP-1 was subsequently cloned, Table 1). In all cases the single radioactive band had the same electrophoretic mobility as pIJ2000, and the remaining bands were not labeled.

**TABLE 1**

Sequence Homology Between Plasmids from Different Spiroplasma Strains

| [³²P]-probe DNA:                  | Homology with:                                                                 |
|----------------------------------|-------------------------------------------------------------------------------|
| pIJ2000 (from *S. citri* ASP-1)  | *S. citri* SP-A, SP4-6aS, ASP-2, Iran (homologous plasmids have same mobility as pIJ2000) |
|                                  | No homology with *S. citri* R8A2, C189, SP-2, SP-V3, 608, 750, Scaph, L163, OTM, BR6, and other spiroplasmas 277F, BC3, G1, SR9, and N525 |
| Mixed plasmids from *S. citri* Iran | (i) *S. citri* ASP-1, SP4-6aS (same mobility as pIJ2000)                           |
|                                  | (ii) *S. citri* 608, Scaph, and honeybee spiroplasma BC3 (lower mobility plasmids) |
|                                  | Other strains not tested                                                       |
| Mixed plasmids from honeybee spiroplasma BC3 | *S. citri* SP4-6aS, Iran, Scaph, 608 (lower mobility than pIJ2000)              |
|                                  | Other strains not tested                                                       |
In order to search for further relationships we also labeled the complex of plasmids from *S. citri* Iran and the honeybee spiroplasma strain BC3, and probed further gel "blots." The homology found is described in Table 1.

We attempted to clone linearized plasmid pIJ2000 and *MboI* fragments thereof into *E. coli* in order to facilitate the preparation of large quantities of the plasmid DNA and also to enable us to exploit techniques available for studying transcription and translation of cloned DNA in *E. coli*, in the hope that we might identify plasmid gene products. In the event, despite many attempts using two vectors (pBR322 and pAT153) we were unable to obtain any clones. In contrast, spiroplasma chromosomal DNA fragments could be "shotgun-cloned" into *E. coli* using pBR328 without difficulty. Six *E. coli* transformants resistant to carbenicillin but sensitive to chloramphenicol were chosen for further study. All possessed plasmids larger than the vector. When cleaved with *EcoRI* two DNA fragments were produced from each plasmid: one had the electrophoretic mobility of linear pBR328 and the other (insert) ranged in size from about 3 to 10 kb. Three of the recombinant plasmids were labeled and shown to hybridize with spiroplasma BC3 DNA. Conversely, when BC3 DNA was used as the radioactive probe, only the insert fragment of the cleaved plasmids hybridized.

**DISCUSSION**

We have confirmed that almost all spiroplasmas harbor plasmids, and using the purified plasmid pIJ2000 as a probe we have shown that some *S. citri* strains contain a related plasmid of the same size, although we are unable to say whether the whole plasmid or parts of it are conserved. Preliminary experiments using mixed plasmids from strains Iran and BC3 as probes pointed to a complex network of relationships. Definitive experiments would require the use of single purified plasmids from the mixtures, and it is possible that one plasmid (or sequences present in one plasmid) is widely distributed among spiroplasmas and is responsible for the homology; alternatively there may be a number of plasmid "families" showing some degree of base sequence conservation. It is believed that DNA homology often underestimates protein homology [13], so some plasmid-encoded proteins may be highly conserved between spiroplasmas.

Recombinant DNA techniques provide a promising approach to the molecular genetics of spiroplasma plasmids. It is unfortunate that the plasmid chosen for these experiments could not be cloned, for reasons which are not clear. It is possible that spiroplasma plasmid DNA of low G + C content (22 percent, [4]) cannot be tolerated in *E. coli* replicons. However chromosomal DNA (26 percent G + C) could be readily cloned. Other possibilities are that the plasmid DNA contains "poison" sequences inhibiting replication in *E. coli* (cf. [14]), or palindromic sequences may facilitate precise excision of inserted DNA at a high frequency. Alternatively, a product toxic to *E. coli* may be produced. However, the fact that fragments of the plasmid could also not be cloned argues against the last three possibilities.

Perhaps other plasmids will be more amenable so that the nature of plasmid gene products can be investigated. In addition, it should be possible to insert foreign DNA fragments containing genes giving a selectable phenotype (e.g., antibiotic resistance) into spiroplasma plasmids, so as to improve the chances of detecting transformation and gene exchange in spiroplasmas.

The possibility remains that some of the extrachromosomal DNA species may be forms of viral DNA. Spiroplasmas are known to harbor at least four types of viruses
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[15,16] but only the short-tailed, polyhedral SP-V3-type viruses have had their nucleic acid characterized; all isolates of this type have possessed double-stranded DNA of about 20 kb [17]. Until examples of the other virus groups are fully characterized, it is not possible to address this question further. However, both the size and restriction enzyme sensitivity of plJ2000 argue against its being related to type 3 virus DNA.

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