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Molecular Cloning and Expression of *Treponema pallidum* DNA in *Escherichia coli* K-12

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A gene bank of *Treponema pallidum* DNA in *Escherichia coli* K-12 was constructed by cloning *Sau*I-cleaved *T. pallidum* DNA into the cosmid pHC79. Sixteen of 800 clones investigated produced one or more antigens that reacted with antibodies from syphilitic patients. According to the separation pattern of the antigens produced on sodium dodecyl sulfate-polyacrylamide gels, six different phenotypes were distinguished among these 16 clones. These antigens reacted also with anti-*T. pallidum* rabbit serum. No antibodies against the cloned antigens were found in normal rabbit serum and in nonsyphilitic human serum. The antigens produced by the *E. coli* K-12 recombinant DNA clones comigrated in sodium dodecyl sulfate-polyacrylamide gels with antigens extracted from *T. pallidum* bacteria, suggesting that the treponemal DNA is well expressed in *E. coli* K-12. Several of the cosmid recombinant plasmids have been subcloned, resulting in smaller *T. pallidum* recombinant plasmids which are more stably maintained in the cell and produce more treponemal antigen. Monoclonal antibodies were raised against *T. pallidum*, and one hybridoma produced antibodies that reacted not only with an antigen from *T. pallidum* but also with the antigen produced by one of the *E. coli* clones.

The inability to culture *Treponema pallidum* bacteria in vitro has hampered a detailed analysis of the antigens that play a role in the pathogenesis of syphilis. Although considerable progress has been made in recent years toward growing *T. pallidum* in tissue cultures (10), no practical method is yet available for obtaining *T. pallidum* cells in sufficient quantities to enable such an analysis. Molecular cloning and expression of *Treponema* DNA in *Escherichia coli* K-12 are alternative possibilities for obtaining large amounts of treponemal antigens.

The detailed chemical structure of the treponemal antigens is not known, and therefore the selection of *E. coli* clones that express treponemal antigens necessarily relies on their capacity to combine with antibodies. The treponemal DNA to be expressed in *E. coli* clones selected by antitreponemal antibodies is likely to encode for protein antigens because, in contrast to antigens such as polysaccharides or lipopolysaccharides, one or only a few contiguous chromosomal genes are needed for expression of protein antigens.

Recent investigations of the antigenic makeup of *T. pallidum* revealed that sera from syphilitic patients and from immunized rabbits contain antibodies against a fair number of treponemal protein antigens (1–3, 17). We have found that the level of these antibodies varies strongly depending on the stage of syphilis (R. V. W. van Eijk et al., manuscript in preparation). However, the immune response is remarkably uniform with regard to the relative levels of antibodies that react with the various individual protein compounds. This suggests that particular protein antigens can be used for serological diagnosis of syphilis. Production of protein antigens by *E. coli* would abolish a further need of rabbits, which are currently used for the growth of *T. pallidum*, to obtain treponemal antigens for serodiagnostic purposes.

During the course of our investigations two studies were published on the cloning of *T. pallidum* DNA in *E. coli* (24, 29). In one study plasmid pBR322 was used as the cloning vehicle and in the other one the λ phage Charon 30 was used as a vector. By an in situ immunoassay of unfractionated *E. coli* lysates, it was shown that certain recombinant DNA clones expressed treponemal antigen. The antigen expression of one clone was analyzed by electrophoretic transfer of total lysate proteins to nitrocellulose filters after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29). This clone produced seven different-sized polypeptides that reacted with antibodies in syphilitic sera.

Because of the relatively large size (14,000 kilobases [kb]) of the *T. pallidum* genome (18) and the relatively limited quantity of DNA that can be obtained, a highly efficient cloning system is desirable. For this reason, we have chosen the so-called cosmid cloning system. Cosmids are cloning vehicles that carry a small segment of the bacteriophage lambda genome, which enables recombinant DNA to be packaged in vitro into lambda heads (8). Due to the DNA requirements during packaging, large foreign DNA fragments of 30 to 40 kb are selectively cloned with high efficiency. In this paper we describe the construction of a gene bank of *T. pallidum* by means of the cosmid vector pHC79 and the selection of *Treponema* antigen-expressing *E. coli* clones by an immunoassay of fractionated proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. *T. pallidum* (Nichols) was maintained by serial passage in rabbit testes and was used as a source of DNA and protein. *Treponema phagedenis* (biotype Reiter) was cultured as described previously (9). *E. coli* K-12 strain HB101 (*recA hsr hsm lac leu pro*) was used as a recipient for transduction of recombinant cosmid DNA (8). *E. coli* K-12 strain SC181 (*leu thi lac thr hsr hsm sup*E) was used as a host for the deleted plasmid derivatives of pRIT3200, pRIT4600, and pRIT9000. The plasmids used were pHC79 (15), pBR327 (23), and pBR328 (23). The axial filament of *T. phagedenis* was purified as described by Hardy et al. (13).

Media. Unless otherwise stated, nutrient broth and nutrient agar (27) were used for growth of *E. coli* K-12 cells. Certain recombinant cosmid DNA clones grew poorly on these media. In such cases they were propagated on L-broth or L-plates (19). Plasmid-containing strains were grown on media supplemented with ampicillin, 200 μg/ml; tetracycline, 15 μg/ml; or chloramphenicol, 50 μg/ml.

Extraction of DNA and recombinant DNA techniques. DNA from *T. pallidum* was obtained from five rabbits that had been inoculated intratesticularly as described before (14). Twelve days after inoculation the rabbits were sacrificed, and *T. pallidum* bacteria were purified from the testicular tissue components by urografin gradient centrifugation as described by Baseman et al. (4). DNA from about 10^{10} bacteria was obtained as follows. The bacterial cells were suspended in 10 ml of TE buffer (0.01 M Tris, 0.001 M EDTA, pH 8.0). RNase (pancreas; Worthington Diagnostics) was added to 50 μg/ml, and the cells were lysed by the addition of SDS to 0.5%. After 30 min of incubation at 37°C, proteinase K (E. Merck AG) was added to 100 μg/ml, and the mixture was incubated again for 30 min at 37°C. Proteins were further removed by three successive phenol extractions. The DNA was repeatedly precipitated by ethanol and dissolved in TE buffer to a final concentration of 1 mg/ml.

Plasmid DNA was extracted by the alkaline method as described by Birnboim and Doly (5). When the plasmid DNA was used for subcloning, such preparations were further purified by cesium chloride-ethidium bromide density gradient centrifugation as described previously (27).

Restriction endonucleases and T4 ligase were used as previously described (26). For the cloning of *T. pallidum* DNA, about 1 μg of DNA was digested with 0.15 U of *Sau*I in a volume of 100 μl for 60 min at 30°C followed by 10 min at 65°C. This partially cleaved DNA was precipitated by ethanol and dissolved in TE buffer. *Treponema* DNA and vector DNA were ligated at 22°C for 16 h at concentrations of 100 and 200 μg/ml, respectively.

In vitro packaging of cosmid hybrid DNA was done according to V. Pirotta (personal communication) as described in detail by Grosveld et al. (12).

Detection of treponemal antigens in *E. coli* K-12. The expression of treponemal antigens in *E. coli* K-12 was determined by the binding of antibodies to SDS-PAGE-fractionated proteins on nitrocellulose ("Western blotting" [7]). For this purpose lysates containing an equivalent of 10^6 to 10^7 *E. coli* cells or approximately 10^6 *T. pallidum* cells were applied onto SDS-polyacrylamide gels (16). The acrylamide concentration was 12% unless otherwise stated. After electrophoresis the separated proteins were transferred to nitrocellulose filters (BA85, 0.45-μm pore size; Schleicher & Schuell) as described before (7). The filters were soaked in Tween buffer (0.15 M NaCl, 0.01 M Tris, 0.05% Tween 20) for 30 min and incubated with a 1:100 or 1:200 dilution of antiserum in Tween buffer for 1 h. After extensive washings in Tween buffer (three times, 30 min), the blots were incubated either with anti-human immune globulin labeled with horseradish peroxidase or anti-rabbit immunoglobulin labeled with horseradish peroxidase diluted 1:2,000 in Tween buffer. These peroxidase-labeled conjugates were prepared according to the method of Nakane and Kawaoi (20) and kindly provided by A. M. Hagenaars (Bilthoven, The Netherlands). The blots were again washed in Tween buffer, and the peroxidase activity was visualized by a variation of the method described by Buckel and Zehelin (6). A 30-ml amount of buffer containing 0.005 M citrate–0.01 M phosphate, pH 5.0, was mixed with 10 ml of an ethanol solution containing 24 mg of tetramethylbenzidine (Merck) and 80 mg of dioctylsodiumsulfosuccinate (Merck). H_2O_2 was added to 0.015%, and this solution was poured onto the nitrocellulose filters. Green bands developed after a few minutes and the filters were photographed. The sensitivity of intracellular antigen detection in *E. coli* K-12 was tested by the use of an antiserum against the dnaB protein of *E. coli* K-12, which was kindly provided by E. Lanka (Berlin, West Germany). The dnaB protein is known to be present at the extent of about 50 copies per cell, and in Western blots, using the anti-dnaB antiserum, the protein was still just detectable when an equivalent of 10^6 *E. coli* K-12 cells were applied onto the polyacrylamide gel. As an antiserum probe for screening of the gene bank, we used a serum pool composed of a mixture of equal volumes of five sera, obtained from patients with syphilis in the secondary stage. These sera showed a strong reaction in the *T. pallidum* hemagglutination assay, the fluorescent treponemal antibody absorption test, and the *T. pallidum* immobilization test. Other human sera used were from a single patient in the secondary stage of syphilis and normal human serum, obtained from a healthy individual who was negative in the *T. pallidum*

hemagglutination test, the fluorescent treponemal antibody absorption test, and the *T. pallidum* immobilization test. Anti-*T. pallidum* rabbit sera were obtained by intravenous immunization of rabbits with density gradient-purified *T. pallidum* cells for 3 weeks with 5 × 10^7 bacteria at 3-day intervals. The rabbits were bled 1 week after the last vaccination. Anti-*T. phagedenis* rabbit serum used was obtained from N. H. Axelsen (Copenhagen, Denmark).

Hybridoma techniques. Hybridoma cell lines were produced according to Osterhaus et al. (21). Three fusions were carried out. BALB/c mice were primed intraperitoneally with 10^7 viable *T. pallidum* cells (not purified on density gradients [14]) in 0.5 ml of phosphate-buffered saline (PBS) 1 (fusion 81/47), 3 (fusion 81/41), or 5 (fusion 82/19) months before the fusion. In all three experiments the mice were boosted intravenously 4, 3, and 2 days before fusion with 2 × 10^7 urografin gradient-purified *T. pallidum* cells. Hybridoma cultures were screened for antibody production by an enzyme-linked immunosorbent assay (ELISA).

Screening of hybridoma cultures. The screening of hybridoma culture media was done by an ELISA, as described by Veltkamp and Visser (28) with some modifications. Purified *T. pallidum* antigen was prepared by sonication of freshly isolated, density gradient-purified *T. pallidum* cells (10^8 cells per ml). Polystyrene micro-ELISA titer plates (Dynatech Laboratories, Inc.) were coated overnight with purified *T. pallidum* antigen diluted to 12 μg/ml in PBS (0.15 M NaCl, 0.01 M phosphate, pH 8.0), rinsed thrice with PBS plus 0.01% Tween 20 (PBS-T), and incubated with 0.15 ml of Dulbecco minimal essential medium-20 for 1 h. Subsequently, the wells were rinsed thrice with PBS-T and filled with 0.1 ml of the samples under investigation and 0.1 ml of PBS (as control), respectively; the plate was then incubated for 1 h. After the plate was rinsed thrice with PBS-T it was incubated for 1 h with 0.1 ml of goat anti-mouse total immunoglobulin horseradish peroxidase [GAMIgG (H+L)/PO; Nordic] diluted 1:500 in PBS-T, rinsed thrice with PBS-T, and incubated for 10 min with 0.1 ml of a substrate solution containing tetramethylbenzidine (1.2 mg/ml), H_2O_2 (0.003%), sodium acetate (6.8%), and citric acid (1.4%). The reaction was stopped by adding 0.05 ml of 2 N sulfuric acid. The extinctions of the cup contents were measured with a Multiscan (Titertek) at 450 nm. The results were presented as relative extinctions by subtracting the extinction of the reference from the extinction of the samples and dividing these values by the extinction of the standard. As a standard we used the culture fluid of one particular antibody-producing clone, 81/41 3-22D6F5.

To avoid the selection of monoclonal antibodies with anti-rabbit testicle specificities, the positive clones were screened by an ELISA in which the *T. pallidum* antigen coat was replaced by rabbit testicle material which was isolated by a procedure identical to that used for the density gradient-purified *T. pallidum* cells. The cross-reactivity of monoclonal antibodies with *T. phagedenis* was determined by an ELISA as described above, except that sonicated cells of *T. phagedenis* instead of *T. pallidum* were used as the first coat.

Western blots with monoclonal antibodies were done as described above, except that hybridoma culture fluids (diluted 1:100 in Tween buffer) were used, and the above-mentioned goat anti-mouse conjugate was used in a 1:500 dilution in Tween buffer. An anti-poliovirus monoclonal antiserum served as a control.

RESULTS

Construction and screening of a gene bank of *T. pallidum*. The 6-kb cosmid pHC79 was used as a vector for cloning *T. pallidum* DNA in *E. coli* K-12. pHC79 was cleaved with the restriction endonuclease *Bam*HI and dephosphorylated with alkaline phosphatase to prevent self-ligation of the vector. This vector DNA was ligated to partially *Sau*3AI-digested *T. pallidum* DNA. The majority of the latter DNA migrated in an agarose gel with mobilities corresponding to DNA fragments with molecular weights of >30,000. After in vitro packaging and transduction to strain HB101, about 3,000 ampicillin-resistant colonies were obtained. None of 300 clones tested was tetracycline resistant, indicating that in virtually all clones treponemal DNA had been inserted into the *Bam*HI site of pHC79, resulting in the inactivation of the tetracycline resistance gene.

A total of 800 randomly picked colonies were purified by restreaking, and lysates were prepared from cells grown in L-broth cultures. All 800 lysates were applied onto SDS-polyacrylamide slab gels to separate the proteins, and the presence of treponemal antigens among these separated proteins was detected by Western blotting. The pooled patient sera, obtained from five syphilitic patients in the secondary stage of syphilis, were used as a probe to detect treponemal antigens.

Figure 1 is a representative example of such blots loaded with lysates of different recombinant cosmid clones. Two lanes of the gel shown in Fig. 1a (indicated with B and E) and three lanes in Fig. 1b (indicated with F, B, and F, respectively) differ from all the other ones in the presence of components that bind antibodies from the pooled patient sera. These bands comigrate with antibody-binding components isolated from *T. pallidum* bacteria. In 25 of the 800 lysates tested by Western blotting, one or more colored, apparently non-*E. coli* bands appeared, indicating the production of putative treponemal components that reacted specifically with the syphilitic pooled patient sera.

All suspected positive clones were purified by repeated restreaking on ampicillin-containing plates, and lysates of these pure cultures were again tested for the production of treponemal antigens. Only 16 were found to be positive, probably due to loss of treponemal DNA during growth.

Based on the different banding patterns, six different antigenic phenotypes could be distinguished. Representative clones having these

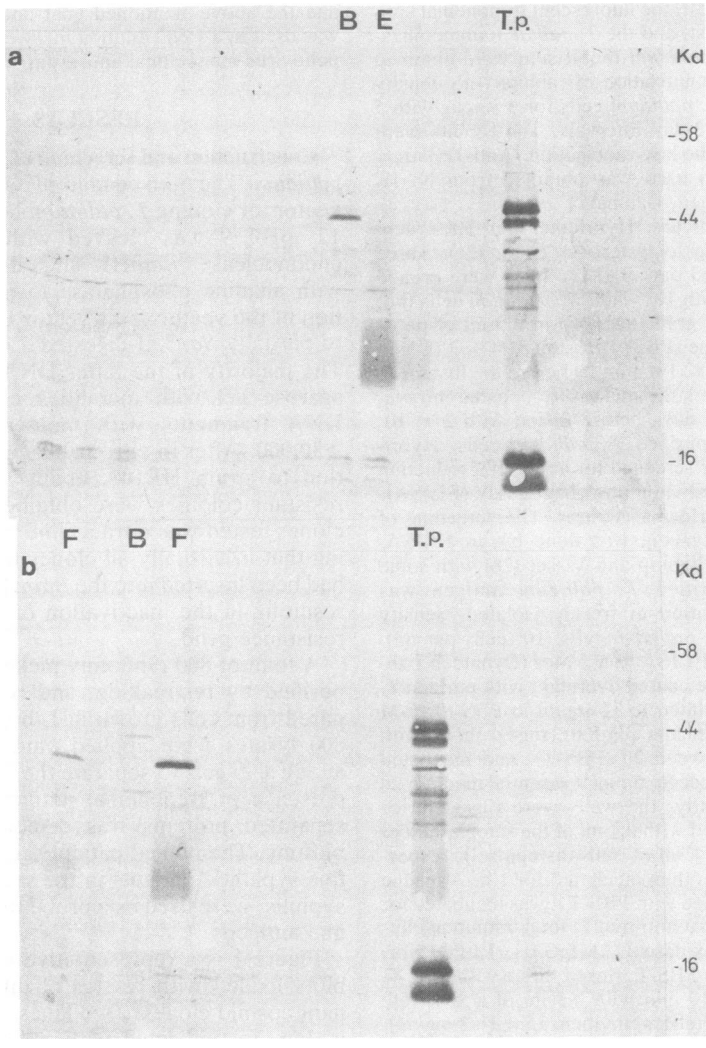

FIG. 1. Western blots of 34 *E. coli* clones obtained by cosmid cloning. Proteins in lysates of *E. coli* clones carrying recombinant cosmid DNA were separated by SDS-PAGE, and the separated proteins were transferred to nitrocellulose as described in the text. A lysate (containing about 0.4 μg of protein) of *T. pallidum* (T.p.) was included on each gel as a control. The blots were developed with the pooled sera of syphilitic patients. B, E, and F refer to the phenotypes in which these clones were grouped. An equivalent of about 3×10^7 *E. coli* cells containing about 5 μg of protein was applied to each slot of gel.

phenotypes are shown in Fig. 2a and b, designated A through F. Most bands observed in the blots of *E. coli* lysates corresponded in mobility to bands observed in lysates of *T. pallidum*. Clones with phenotypes D, E, and F produced antigens that did not manifest on the blots as discrete bands, but rather as smeared components, in the protein molecular weight range of 26,000 to 32,000 and 19,000 to 24,000, respectively. Smears with corresponding mobilities were also visible in the lanes loaded with *T. pallidum* lysates. The colored bands of the various recombinant DNA phenotypes were not seen when normal human serum was used to develop the Western blots. These data strongly suggest that the antigenic components produced by the *E. coli* K-12 clones react specifically with anti-treponemal antibodies.

Characterization and subcloning of recombinant DNA cosmids. Plasmid DNA was isolated from the positive clones, and the molecular weights of the circular DNAs were estimated by agarose electrophoresis, using appropriate covalently closed circular DNA standards. Molecular weights are given in Table 1. It should be noted that a number of the antigen-producing clones easily lost their plasmids. Particularly the plasmid DNAs in strains E5 and B71, both with the

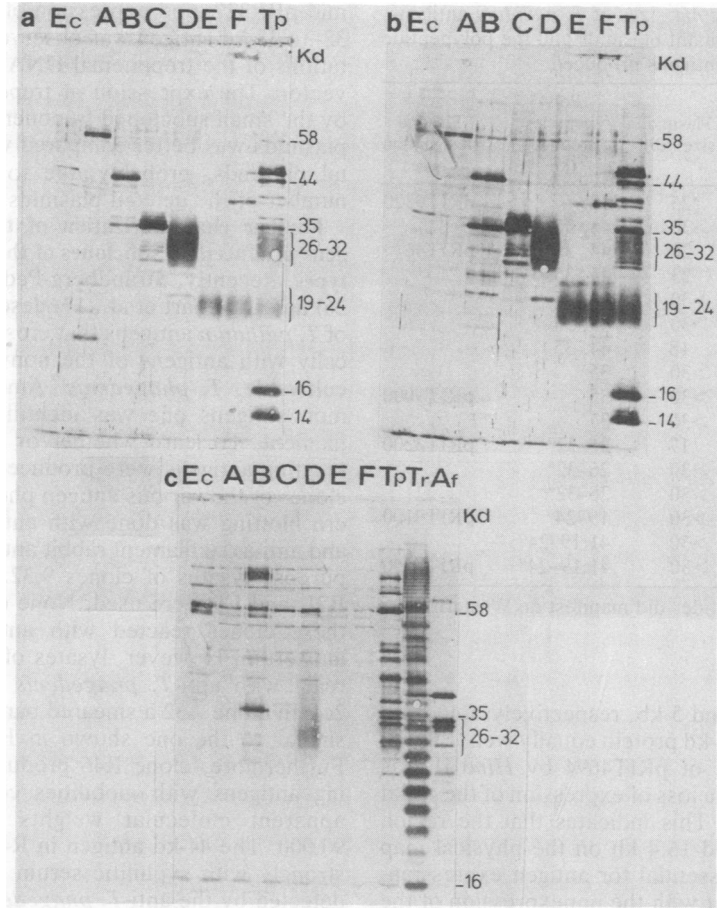

FIG. 2. Western blots of lysates of six representative *E. coli* clones showing the six different phenotypes, A through F. Lysates of *E. coli* (Ec) and *T. pallidum* (Tp) were included as a control. A, Clone 9.32; B, clone R46; C, clone P9; D, clone A32; E, clone D9; F, clone B71; Ec, strain HB101(pHC79). The latter lysate served as a control to show the nontreponemal bands reacting with the antiserum. The following sera were used: human serum from a patient in the secondary stage of syphilis (a), rabbit anti-*T. pallidum* serum (b), and rabbit anti-*T. phagedenis* serum (c). All sera were absorbed with an ultrasonicate of *E. coli* cells of strain M348 to reduce the binding of antibodies to *E. coli* proteins. In (c) 0.2 μg of purified axial filament (Af) and a lysate (containing about 3 μg of protein) of *T. phagedenis* (biotype Reiter; Tr) were also included on the gel as controls. In slots containing *E. coli* lysates about 1.5 μg of protein was loaded.

phenotype F, were found to be extremely unstable.

Many clones carried plasmid DNA smaller than 35 to 45 kb, as would be expected from the cosmid recombinant DNA molecules (8). Because of the high plasmid instability observed in some of the clones investigated, these smaller plasmids might have arisen by deletion after transduction of cosmid hybrid DNA of normal length. Clones 9.32, R46, B71, and A32 were chosen for further study.

To identify the regions of the cloned treponemal DNA, deletions were made either by subcloning or by endonuclease digestion of the recombinant plasmids, followed by self-ligation at low DNA concentration (1 to 5 μg/ml) and reintroduction into *E. coli* K-12. Clone 9.32 (phenotype A) carried a plasmid, designated pRIT9320, of 25 kb and it was reduced to 8 to 15 kb in size by treatment with *Eco*RI, *Bam*HI, *Bgl*II, *Cla*I, or *Hin*dIII. None of the deleted plasmids lost the capacity to produce the 59-kilodalton (kd) antigen. Although no physical map of pRIT9320 has yet been constructed, these data suggest that the antigen-encoding region of treponemal DNA is located close to the vector insertion site. Plasmid pRIT4600 from clone R46 with phenotype B was studied in more detail. The 20-kb treponemal DNA fragment in pRIT4600 was deleted by *Bam*HI and *Cla*I. The resulting derivatives, pRIT4693 and pRIT4694, carried treponemal DNA fragments that were

TABLE 1. Molecular sizes of *T. pallidum* antigen-expressing recombinant plasmids and the polypeptide antigens produced

Clone	Phenotype	Plasmid size (kb)	Polypeptides produced (kd)	Plasmid designation
9.32	A	25	58	pRIT9320
10.43	B	>30	44, 35	
R46	B	26	44, 35	pRIT46
E4	B	23	44, 35	
6.17	B	>30	44, 35	
A50	B	>30	44, 35	
D11	B	18	44, 35	
A13	C	>30	35	
P9	C	>30	35	pRIT9000
R59	C	>30	35	
A32	D	17	26–32[a]	pRIT3200
Z98	D	>30	26–32[a]	
P68	D	>30	26–32[a]	
D9	E	>30	19–24[a]	pRIT9100
E5	F	>30	41;19–24[a]	
B71	F	>30	41;19–24[a]	pRIT7100

[a] These polypeptides did manifest on Western blots as smears.

reduced to 9.5 and 5 kb, respectively, and both expressed the 44-kd protein equally well (Fig. 3). Further deletion of pRIT4694 by *Hin*dIII was accompanied by a loss of expression of the 44-kd protein antigen. This indicates that the region between 15.0 and 16.4 kb on the physical map (see Fig. 3) is essential for antigen expression. This is consistent with the nonexpression of the treponemal antigen by the *Xba*I and *Sal*I deletion derivatives of pRIT4693 which are deleted in the 15- to 19-kb and 11- to 20-kb regions of the treponemal fragment, respectively (see Fig. 3).

The phenotype D clone A32 contained the smallest recombinant plasmid, pRIT3200, with a treponemal DNA insert of only 10 kb. A physical map was constructed (Fig. 4). Plasmid pRIT3200 was subcloned by digestion with *Hin*dIII, and the fragments were inserted into the *Hin*dIII site of the vector pBR328. Plasmids of various sizes were obtained, and all seven strains carrying plasmids with an insert of the 4.5-kb *Hin*dIII fragment were positive by Western blot analysis. Six of these plasmids had a molecular size of 10.2 kb, and only one was 9.1 kb. A size of only 10.2 kb is expected from the 4.9-kb pBR328 plasmid plus the 5.2-kb treponemal *Hin*dIII fragment. Later analysis showed that the vector pBR328 and its recombinants were very unstable and frequently deleted a fragment of 1.1 kb in the tetracycline resistance determinant. A similar spontaneously occurring deletion in pBR322 was recently described by Garaev et al. (11). The 4.5-kb *Hin*dIII fragment was further recloned in the *Hin*dIII site of plasmid pBR327, and expression of the treponemal 32- to 38-kd antigen was observed in both orientations of the treponemal DNA fragment in the vector. The expression of treponemal antigens by the small subcloned treponemal recombinant plasmids was better compared with their parental plasmids, probably due to a higher copy number of the deleted plasmids.

Further characterization of treponemal antigens produced by subclones of the various phenotypes. Recently, Strandberg-Pedersen et al. (25, 26) and Lukehart et al. (17) described a number of *T. pallidum* antigens that cross-react serologically with antigens of the nonvirulent in vitro cultivable. *T. phagedenis*. Among these common antigens one was identified as the axial filament. To learn whether or not such cross-reacting antigens were produced by the *E. coli* clones of the various antigen phenotypes, Western blotting was done with anti-*T. phagedenis* and anti-axial filament rabbit antiserum. For this purpose lysates of clones 9.32, R46, P9, A32, B71, and D9 were used. None of the lysates of these clones reacted with anti-axial filament antiserum. However, lysates of two clones did react with anti-*T. phagedenis* antiserum (Fig. 2c). In clone A32 a smeared band was detected, similar to the one shown in Fig. 2a, lane D. Furthermore, clone R46 produced cross-reacting antigens with mobilities corresponding to apparent molecular weights of 35,000 and 90,000. The 44-kd antigen in R46 that did react strongly with syphilitic serum could barely be detected by the anti-*T. phagedenis* antibodies.

To obtain antisera that are monospecific for the cloned antigens, an attempt was made to raise monoclonal sera against *T. pallidum*. Three different fusions were done, and from each fusion we obtained only one hybridoma secreting anti-*T. pallidum* antibodies. The two hybridoma clones, 81/47 1-2C3E5 and 81/41 3-22D6F7, were positive in the *T. pallidum* ELISA and negative in the ELISA of rabbit testes. The antibodies produced by both clones were of the immunoglobulin G2b class. The binding of antibodies produced by both monoclonal antibodies was inhibited by syphilitic serum and not by normal human serum, indicating that these monoclonal antibodies react specifically with *T. pallidum* antigens (data not shown). The antibodies in the ascites of the hybridoma clones were tested by Western blotting, using lysates of *T. pallidum* and *E. coli* clones of each phenotype as antigen. Antibodies of hybridomas 81/47 2-1C3E5 and 81/41 3-22D6F7 reacted with a single *T. pallidum* protein of 46 kd (Fig. 5) and not with any of the *E. coli* clones. Accidentally we selected a hybridoma of which the antibodies were negative in the *T. pallidum* ELISA but positive by Western blotting. Monoclonal antibodies from this hybri-

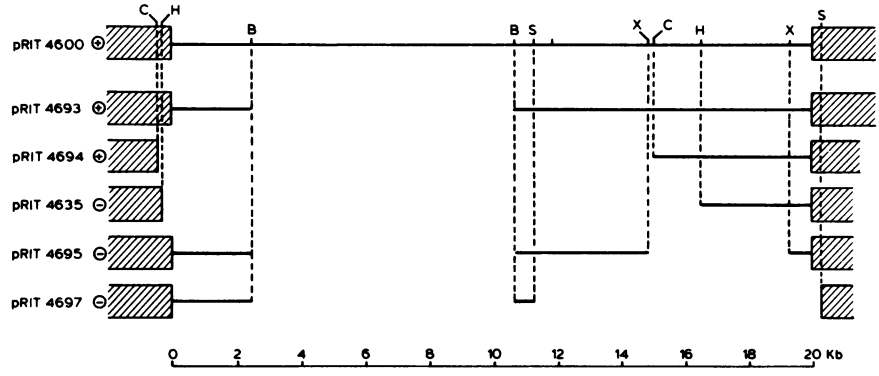

FIG. 3

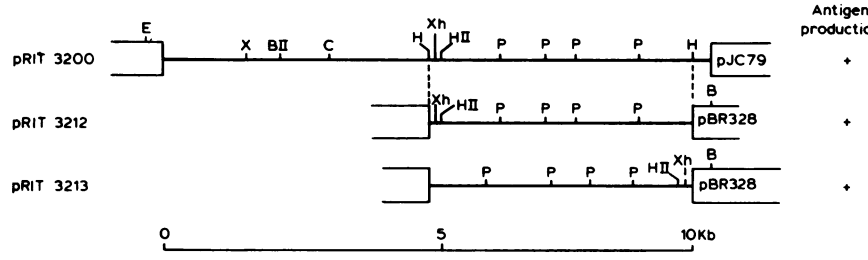

FIG. 4

FIG. 3 and 4. Physical map of plasmids pRIT4600 (Fig. 3) and pRIT3200 (Fig. 4) and their derivatives. The thick line denotes the *T. pallidum* DNA. Sites of the following restriction enzymes are depicted: *Eco*RI (E), *Xba*I (X), *Bgl*II (BII), *Cla*I (C), *Hin*dIII (H), *Xho*I (Xh), *Hin*dII (HII), *Pst*I (P), and *Sal*I (S). The expression and nonexpression of *T. pallidum* antigen are denoted with plus and minus signs, respectively.

doma cell line, 82/19 1-14M1, reacted with the *T. pallidum* polypeptide corresponding to a molecular weight of 44,000. These antibodies reacted also with the 44-kd polypeptide from *E. coli* clone R46 (Fig. 5). This indicates that the latter polypeptide carries the same antigenic determinant recognized by the monoclonal antibody. Deletion mutants pRIT4693 and pRIT4694 also expressed the 44-kd antigen. In contrast, no monoclonal antibodies were bound by proteins on blots of *E. coli* harboring the deletion mutant plasmid pRIT4635 (Fig. 5). This is consistent with the observations on the expression of these deletion derivatives as measured by the polyclonal sera from syphilitic patients. None of the three monoclonal antibodies was positive in the ELISA with *T. phagedenis* antigens, the fluorescent *T. pallidum* antibody absorption test, and the *T. pallidum* immobilization test.

To learn whether or not the antigens produced by the positive *E. coli* clones are proteinaceous in nature, lysates of clones of all phenotypes were digested with proteinase K (100 μg/ml) for 60 min at 37°C and were reexamined by Western blotting. After this treatment none of the clones showed any visible band with the human serum pool as a probe. The same result was obtained after proteinase K treatment of the *T. pallidum* lysate, indicating that the bands as observed by Western blotting are proteinaceous in nature. None of the *T. pallidum* antigen-producing *E. coli* clones was agglutinated by the syphilitic serum pool. Also, several other syphilitic sera and several anti-*T. pallidum* rabbit sera were tested by Western blotting with the clones of the various phenotypes. In all clones the specific banding patterns as shown in Fig. 2 were observed, in addition to a number of *E. coli* bands that reacted with antibodies in the serum. No *Treponema*-specific bands developed when normal human or rabbit serum was used as the probe.

DISCUSSION

In this study partially *Sau*3AI-cleaved *T. pallidum* DNA was cloned into the vector pHC79. One might expect that cloning with this cosmid vector results in recombinant DNA molecules that have inserts of treponemal DNA with an average size of 35 kb (8). If the *Treponema* DNA could have been cleaved at random, then each gene in a gene library of 800 clones would be

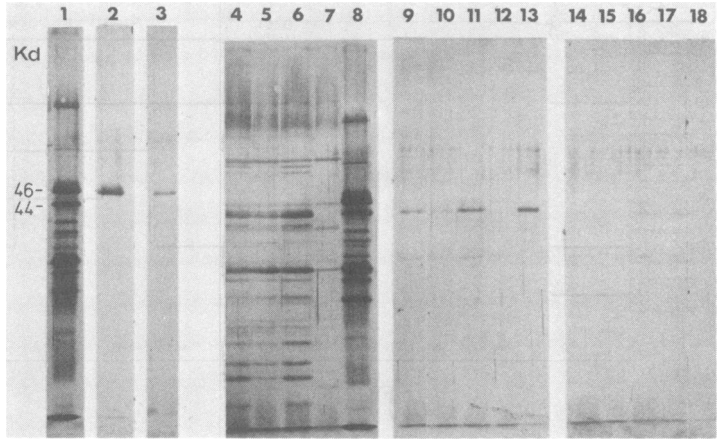

FIG. 5. Reaction of monoclonal antibodies with SDS-PAGE-separated proteins of *T. pallidum* and *E. coli*. Lanes 1 through 3 contained *T. pallidum* lysate, and the blots were incubated with antibodies from human syphilitic serum, hybridomas 81/47 2-1C3E5 and 81/41 3-226F7, respectively. Lanes 4, 9, and 14 contained lysates of *E. coli* carrying pRIT4600; lanes 5, 10, and 15, pRIT4693; lanes 6, 11, and 16, pRIT4694; lanes 7, 12, and 17, pRIT4635; and lanes 8, 13, and 18, *T. pallidum*. Lanes 4 through 8 were incubated with antibodies from human syphilitic serum; lanes 9 through 13, with hybridoma 82/19 1-14M1; and lanes 14 through 18, with antibodies from a hybridoma with anti-poliovirus specificity, which served as a control to check nonspecific binding.

expected to be present twice or thrice, since the genome size of *T. pallidum* is about 14,000 kb (18).

Among the 800 clones investigated, we found several types that were more frequently represented. For instance, we obtained six clones with phenotype B, but only one clone with phenotype A. The unexpected high frequency of phenotype B clones is probably a reflection of the nonrandom cleavage of the treponemal DNA by *Sau*3AI. This also might explain why the production of certain antigens has not been found among the 800 clones. Furthermore, *E. coli* K-12 might not be able to express particular treponemal antigens, such as the 46-, 16-, and 14-kd treponemal proteins.

Because we were primarily interested in obtaining clones producing treponemal antigens which can be used in the serodiagnosis of syphilis, we used an antibody probe obtained from a mixture of sera from syphilitic patients. According to the mobility and banding pattern in polyacrylamide gels, six phenotypically different antigen-producing *E. coli* clones were found among the positive reacting ones. Some clones produced a single antigenic component and others produced more components. In the case of clone R46 mainly two polypeptides of 44 and 35 kd reacted with anti-*T. pallidum* antibodies. Monoclonal antibodies of one of the hybridomas reacted with the 44-kd polypeptide, and anti-*T. phagedenis* antibodies did so with the 35-kd polypeptide. This indicates that clone R46 produces two antigens which have different antigenic determinants. Absorption of syphilitic serum with various ultrasonicates of *E. coli* carrying subcloned *T. pallidum* DNA results in removal of all antibodies that react with particular polypeptides isolated from *T. pallidum* cells (unpublished data). This indicates that *E. coli* can express all antigenic determinants of particular *T. pallidum* proteins as detected by Western blots.

Western blots of the clones of phenotypes B and C often show many more components, with mobilities higher than the main antibody-binding components in polyacrylamide gels. It seems unlikely that each antigenic component observed in these clones is encoded by a different structural gene, because then all of these genes would have been located in a single cluster on the *T. pallidum* genome. It seems more likely that these multiple bands are derived from a particular protein antigen which occurs in the cell in different molecular weights due to proteolytic cleavage or association to other cellular components or both. Interestingly, certain antigens produced by clones of phenotypes D, E, and F did not migrate in the polyacrylamide gel as a discrete band, but instead as a smear. Smears with corresponding mobilities are also found in extracts of *T. pallidum*. Therefore, the phenomenon is specific for these antigens and seems not to be caused by a difference in DNA expression between *T. pallidum* and *E. coli*. The nature of the apparent heterogeneity of these smeared antigens is not understood. Perhaps these proteins are only partially unfolded despite

the denaturing conditions during the SDS and heat treatments of the samples and the presence of SDS in the gel. Alternatively, these proteins might be heterogenous due to post-translational modifications such as glycosylation.

In one of two recent papers on the cloning of *T. pallidum* DNA in *E. coli* K-12, the gene products of one particular lambda recombinant DNA clone, Tp3A, were characterized by SDS-PAGE (29). This clone was also obtained by cloning a *Sau*3AI fragment. Tp3A produced at least seven polypeptide antigens with molecular weights of 18,500, 20,000, 23,000, 24,000, 38,000, 43,000, and 46,000. The cosmid recombinant obtained in our study that resembles Tp3A is pRIT4600. It expresses a number of proteins in the 30- to 44-kd range, but it differs from Tp3A in the absence of protein antigens smaller than 30 kd. Furthermore, the DNAs of Tp3A and pRIT46 differ in their endonuclease cleavage patterns, so it seems unlikely that we obtained clones that produce the same antigens as Tp3A in our study. Our attempt to raise monoclonal antibodies against *T. pallidum* resulted in three hybridomas producing antibodies that reacted with the 46- and 44-kd proteins, respectively. During the course of our studies Robertson et al. (22) published a paper on the production of monoclonal antibodies against *T. pallidum*. They obtained 39 hybridomas and 31 of these were specific for *T. pallidum* and did not react with *T. phagedenis* or rabbit testicular tissue. Our failure to isolate more hybridomas with various specificities is probably due to the non-optimal immunization schedule that we used for the BALB/c mice (22). The hybridoma line 82/19-1-14M1 recognizes not only 44-kd protein in *T. pallidum* lysates, but also the similarly sized antigen expressed by the recombinant plasmid pRIT4600 and its derivatives pRIT4693 and pRIT4694 in *E. coli*. This hybridoma will probably be helpful in purifying the 44-kd *T. pallidum* antigen produced by *E. coli*. The monoclonal antibodies will also be used as a highly specific probe for the 44-kd antigen during the process of further genetic manipulations designed to increase the production of this particular antigen.

Alderete and Baseman (1) showed that many of the treponemal antigens that react with antibodies from syphilitic patients are localized on the surface of *T. pallidum*. Although none of the isolated cosmid clones that were positive by Western blotting were agglutinated by antitreponemal serum, incorporation of treponemal antigens into the outer membrane of *E. coli* K-12 cannot be ruled out. We are currently investigating cell fractions of *E. coli* K-12 in which treponemal antigens are present.

Many of the cosmid recombinant plasmids were found to be unstable, some even to an extreme extent. Several antigen-producing clones have been lost because of this instability. Various stable deletion mutants were constructed, and these plasmids appear to express higher levels of antigen as judged from the Western blots. This probably reflects a gene-dose effect due to the increased number of copies per cell. Most antigens produced in *E. coli* clones are also found in extracts of *T. pallidum*. However, certain of such antigens, e.g., the 58-kd antigen produced by clone 9.32 with phenotype A, are found in *T. pallidum* extracts only as weak bands in Western blots. This suggests that the 58-kd antigen is produced by *T. pallidum* only in small amounts, although it evokes a good antibody stimulus during a natural infection. Clones of phenotypes B and D produce components that also bind antibodies from anti-*T. phagedenis* serum, but no such reacting antibodies were found in anti-axial filament antiserum. This suggests that classes B and D produce a nonflagellar *T. pallidum* antigen that cross-reacts with *T. phagedenis*. As can be shown by Western blots of *T. pallidum* proteins, virtually all patients in the various stages of syphilis have antibodies against a great number of *T. pallidum* antigens (van Eijk et al., in preparation). Therefore, it seems likely that at least a number of the antigens produced by the *E. coli* recombinant DNA clones obtained in this investigation might be of value in the serodiagnosis of syphilis. If in syphilis patients the levels of antibodies against the various treponemal antigens vary depending on the progression of the disease, the treponemal antigens produced by *E. coli* might be used in the serodiagnosis of syphilis to differentiate various stages of the disease. To investigate this, we are presently in the process of purifying the treponemal antigens produced by *E. coli* K-12.

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