Cloning and Expression of *Treponema pallidum* Common Antigen (Tp-4) in *Escherichia coli* K12

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A library of *Treponema pallidum* DNA was constructed using a cosmid cloning system. Sixteen hundred *Escherichia coli* recombinant clones were generated covering the *T. pallidum* genome with a probability of 99%. Three hundred of the clones were screened for expression of *T. pallidum* antigens by a modified rocket immunoelectrophoresis technique using a polyspecific antiserum to *T. pallidum*. One clone was identified which produced the 'common antigen' (CA) of *T. pallidum* (Tp-4). CA shares epitopes with antigens present in more than 50 different bacterial species, but nothing is known about its structure, function and localization. The recombinant *E. coli* clone will be of value for a structural analysis of the CA gene.

INTRODUCTION

Common antigen (CA) is an antigen of *Escherichia coli* first described by Kaijser (1975). As the name indicates, antigens cross-reacting with *E. coli* CA are present in a wide range of bacteria: CA has been demonstrated in more than 50 different bacterial species (Hindersson *et al.*, 1984). Recently it has been shown that *Treponema pallidum*, the micro-organism causing syphilis, expresses CA (Tp-4) (Hindersson *et al.*, 1984). Although CAs of *Pseudomonas aeruginosa* and *Treponema phagedenis* biotype Reiter have been purified and characterized (Sompolinsky *et al.*, 1980*a, b*; Pedersen *et al.*, 1981; Petersen *et al.*, 1982) nothing is known about the function and localization of CA, but the antigen is probably important for the bacterial cells since CA is phylogenetically very stable. Antibodies to CA are often present in human and animal sera due to bacterial infections and the normal bacterial flora, and antibodies to CA might play a role in a presumed 'normal immunity' to bacterial infections in general. Cross-reacting antibodies to CA could explain some false serological tests for syphilis and other bacterial infections.

In this paper we describe the construction of a *T. pallidum* DNA library using a cosmid cloning system. An immunoprecipitation technique, which can be used for identification of *E. coli* recombinants expressing foreign genes, was used for identification of an *E. coli* recombinant which expresses the CA of *T. pallidum*. This *E. coli* recombinant will be of value for a structural analysis of the *T. pallidum* CA gene, which may give insight into the function of CA.

METHODS

Bacterial strains: growth, harvesting and purification. *T. pallidum* (Nichol's pathogenic strain) was grown in rabbit testicles as described by Pedersen *et al.* (1981). The testicular material containing treponemes was sliced, shaken vigorously, and centrifuged twice at 1500 *g* for 5 min to remove testicular debris. The resulting supernatant was centrifuged for 30 min at 40000 *g*. The pellet of treponemes was resuspended in HA buffer (6.6 mM-phosphate

Abbreviations: CA, common antigen; DMSO, dimethyl sulphoxide; DOS, dioctyl sulphosuccinate, sodium salt; TMB, tetramethylbenzidine.

buffer containing 0.15 M-NaCl, pH 7.2) and recentrifuged at 40000 g. The pellet was then resuspended in a small volume of HA buffer and run on a 45% to 19% Urografin (Schering) gradient at 80000 g for 60 min in a Beckman SW40 rotor (Baseman *et al.*, 1974). Two bands containing treponemes were pooled, diluted in 0.15 M-NaCl and recentrifuged for 60 min at 40000 g. The *T. pallidum* pellets were frozen at -20°C until used.

Test material for crossed immunoelectrophoresis (Axelsen, 1983), Western blotting (Burnette, 1981) and immunizations was prepared by resuspending 10^{10} *T. pallidum* in 3 ml HA buffer containing 1% (v/v) Tween 20 and sonifying the suspension for 3×30 s in an MSE 150W ultrasonic disintegrator (output frequency 20 kHz, amplitude 20 μm , 9 mm cylindrical probe) on an icebath.

T. phagedenis (biotype Reiter) was grown, harvested and sonicated as described by Pedersen *et al.* (1981).

P. aeruginosa was prepared as described earlier (Højby, 1977). The antigenic material was kindly provided by N. Højby, Department of Clinical Microbiology, Statens Seruminstitut, Copenhagen.

E. coli was grown at 37°C on LB plates or in liquid LB medium (Maniatis *et al.*, 1982). Ampicillin (sodium salt) or tetracycline was added to liquid media and plates as described by Maniatis *et al.* (1982). The following *E. coli* K12 strains were used: C600 (= CR34) [F^{-} *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^{-}]; HB101 [F^{-} *hsdS20 (r_mm_b) recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Sm^r) xyl-5 mtl-1 supE44* λ^{-}] (Maniatis *et al.*, 1982). C600(pHC79) was obtained by transformation of C600 with cosmid pHC79 according to the standard protocol given by Hanahan (1983).

Preparation of antibodies. Polyspecific rabbit antisera to *T. pallidum* were raised by subcutaneous inoculation of three rabbits with 5×10^9 purified *T. pallidum* in Freund's incomplete adjuvant every third week for 2 years. The most polyspecific antibodies to *T. pallidum*, as judged by crossed immunoelectrophoresis, were obtained after 48–90 weeks of immunization. These sera were pooled and stored at -20°C until used.

Polyspecific rabbit antiserum to *T. phagedenis* (immunoglobulin fraction) was prepared as described by Pedersen *et al.* (1981).

To prepare polyspecific rabbit antiserum to *E. coli* K12 strain HB101, cells were harvested by centrifugation at 40000 g, resuspended in HA buffer containing 1% Tween 20, and used for subcutaneous inoculation (10^{10} bacteria) of rabbits every third week for 7 months. The sera from the 4th to 7th month of immunization were pooled and frozen at -20°C .

Monospecific antibodies to the flagella of *T. phagedenis* were produced as described by Pedersen *et al.* (1981).

Monospecific rabbit antibodies to CA of *T. phagedenis* (anti-TR-c) were produced by immunization of rabbits with purified *T. phagedenis* CA (TR-c) as described by Pedersen *et al.* (1982).

To prepare monospecific rabbit antibodies to CA of *T. pallidum* (anti-Tp-4), *T. pallidum* sonicate (20 μl) was run against monospecific anti-TR-c in crossed immunoelectrophoresis. The resulting Tp-4/anti-TR-c immunoprecipitate was cut out from the gel and mixed with Freund's incomplete adjuvant. Rabbits were immunized by subcutaneous inoculation with one immunoprecipitate every third week for six months. High-titre antibodies to Tp-4 were obtained after 9–18 weeks of immunization. These sera were pooled and stored at -20°C until used.

Anti-Tp-4 specific for *T. pallidum* CA and not reactive with *E. coli* CA was obtained by absorption of anti-Tp-4 with sonified C600(pHC79). The absorption material was prepared as follows. One litre of LB medium containing ampicillin was inoculated with C600(pHC79). After cultivation overnight cells were harvested by centrifugation at 2500 g, resuspended in 10 ml IS buffer [immunostaining buffer: 0.16 M-Tris/HCl (Trizma base, Sigma), 0.5 M-NaCl, and 0.5% Tween, pH 7.4] and sonified three times as described for *T. pallidum*. This material (1 ml) was used to absorb 2 μl of anti-Tp-4 in a total volume of 10 ml IS buffer. The antibody was absorbed for 1 h at 4°C on an 'end-over-end mixer' just before use.

Immunochemical techniques. Crossed immunoelectrophoresis with intermediate gel was done as described in detail by Axelsen (1983) except that 1% Tween 20 was included in the gels to facilitate solubilization of *T. pallidum* antigens.

A modification of conventional rocket electrophoresis (Axelsen & Bock, 1983; Axelsen, 1983), was used for screening the cosmid library. Wells (15 μl) for samples to be tested were punched in a bottom gel ($20 \times 5 \times 0.15$ cm) containing 250 μl anti-*E. coli* per ml gel. The upper gel ($20 \times 5 \times 0.15$ cm) contained 50 μl polyspecific anti-*T. pallidum* per ml gel. As in crossed immunoelectrophoresis, 1% Tween 20 was used to facilitate solubilization of antigens and improve the quality of immunoprecipitates. In some experiments (Figs 5 and 6) a $20 \times 1 \times 0.15$ cm line gel containing C600(pHI1001) was cast below the circular wells in the bottom gel containing anti-*E. coli*.

Antigens and antibodies were dissolved in agarose at 56°C .

Polyacrylamide gel electrophoresis. Before electrophoresis, antigens were heated to 100°C for 3 min in a buffer containing (final concentrations) 8% (v/v) glycerol, 0.1 M-dithiothreitol, 0.6% SDS and 0.02 M-Tris/HCl, pH 6.8. Antigens were separated in polyacrylamide gradient slab gels using a discontinuous buffer system (Laemmli, 1970). The separation gel was a gradient gel of 10%/0.25% to 20%/0.5% acrylamide/bisacrylamide, 0.1% SDS in 0.06 M-Tris/HCl, pH 8.8. The stacking gel contained 10%/0.25% acrylamide/bisacrylamide, 0.1% SDS, 0.12 M-Tris/HCl, pH 6.8.

Transfer of separated antigens to nitrocellulose (Western blotting) (Burnette, 1981). After polyacrylamide gel electrophoresis, antigens were transferred for 16 h at 25 V to 0.45 µm nitrocellulose (BA85, Schleicher & Schuell) using a Trans Blot Cell (Bio-Rad) in a transfer buffer containing 0.025 M-Tris/HCl, 0.2 M-glycine (Merck), 20% (v/v) ethanol, pH 8.5.

Immunostaining of transferred antigen. Non-specific protein binding to the nitrocellulose was blocked by incubation of the filter for 30 min in IS buffer. The filters were then incubated for 1 h with first antibody diluted 1 in 10000 and washed extensively three times for 30 min in IS buffer. Binding of first antibody to antigen was demonstrated by using a peroxidase-labelled swine anti-rabbit IgG (P161, Dako, Copenhagen, Denmark) diluted 1 in 2000 in IS buffer. After three 30 min washes in IS buffer, peroxidase activity was visualized with TMB (tetramethylbenzidine, Merck). This procedure is a modification of one described by Buckel & Zehelein (1981). TMB was dissolved in DMSO (dimethyl sulphoxide, Merck) to a concentration of 70 mg ml⁻¹. The TMB solution was stable for up to 1 month when stored in the dark at room temperature. DOS (dioctyl sulphosuccinate, sodium salt, Merck) was dissolved in 96% ethanol (8 mg ml⁻¹). The solution was stable for up to 2 months at room temperature. Peroxidase activity was visualized by using a staining solution containing 0.5 ml TMB/DMSO, 15 ml DOS/ethanol, 60 ml CP buffer (0.08 M-sodium hydrogen phosphate, Merck, 0.05 M-citric acid, Merck, pH 5.0) and 30 µl 30% stabilized hydrogen peroxide (Merck, prod. 8597). The staining solution was used immediately after preparation. Nitrocellulose filters were developed for 5–15 min. The blue colour indicating peroxidase activity was stabilized and intensified by washing the filters in a solution containing 13 ml DOS/ethanol in 37 ml H₂O.

Isolation of T. pallidum DNA. Purified *T. pallidum* (10¹⁰) isolated from 102 infected testicles were suspended in 10 ml SET buffer [20% (w/v) sucrose, 50 mM-EDTA (Titriplex III, Merck), 50 mM-Tris/HCl (pH 7.5)], and 0.5% (w/v) SDS, 0.1 mg DNase-free RNase ml⁻¹ (Boehringer Mannheim) and 0.3 mg proteinase K ml⁻¹ (Boehringer Mannheim) were added. The treponemes were incubated for 60 min at 37 °C, with gentle shaking every 15 min. DNA was extracted with three successive phenol extractions and one phenol/chloroform extraction, dialysed overnight against TE buffer (10 mM Tris/HCl, 1 mM-EDTA, pH 8.0) at 4 °C and re-extracted with phenol/chloroform. Phenol (BRL, Ultrapure) and chloroform were prepared as described by Maniatis *et al.* (1982). DNA was precipitated with 70% (v/v) ethanol and 0.1 M-NaCl (Maniatis *et al.*, 1982) and pelleted by centrifugation for 30 min at 30000 g. The DNA pellet was washed with cold 70% (v/v) ethanol and resuspended in 150 µl TE buffer to give a final DNA concentration of 0.3 µg µl⁻¹. DNA showing a molecular mass of more than 90 kb in agarose electrophoresis was used for construction of the cosmid library.

Partial digestion of T. pallidum DNA. *T. pallidum* DNA (45 µg) was digested for 120 min at 37 °C with 2.5 U *Sau*3AI (Boehringer Mannheim) in 100 µl MS buffer (medium salt buffer; Maniatis *et al.*, 1980), giving rise to partial digestion products of 25–50 kb. Partially digested DNA was extracted with phenol/chloroform, precipitated with ethanol as described above and resuspended in TE buffer to a final concentration of 0.1 µg µl⁻¹.

Vector DNA. The cosmid pHC79 (Hohns & Collins, 1980) is a derivative of the vector pBR322 (Bolivar *et al.*, 1977) containing the 'cos-sequence' of the λ phage Charon 4A (Blattner *et al.*, 1977). Cosmid pHC79 (100 µg) (Boehringer Mannheim) was incubated for 2 h with 100 U *Bam*HI (Boehringer Mannheim) in 2 ml MS buffer. DNA was extracted with phenol/chloroform and ethanol-precipitated. *Bam*HI-cut pHC79 (50 µg) was dephosphorylated by two successive incubations with 50 U calf intestinal phosphatase (Boehringer Mannheim); the dephosphorylation reaction was stopped by addition of 0.1% SDS. Dephosphorylated DNA was purified by three phenol extractions and one phenol/chloroform extraction, precipitated with ethanol and finally dissolved in TE buffer to a final concentration of 0.1 µg µl⁻¹.

Ligation and packaging. *Bam*HI-cut, dephosphorylated pHC79 (2.8 µg) was ligated to 0.7 µg *Sau*3AI partially digested *T. pallidum* DNA, using 2.5 U T4 ligase (Amersham) in 70 µl ligation buffer (Maniatis *et al.*, 1982) at 13 °C for 30 h. DNA was packaged using a λ phage DNA packaging kit (Boehringer Mannheim) according to the manufacturer's protocol.

Construction of T. pallidum DNA library. *E. coli* C600 was grown overnight in 0.4% maltose, 0.1% glucose, 0.1% MgCl₂ in LB medium and harvested by centrifugation at 1000 g for 10 min. The cells were then resuspended in 10 mM-MgSO₄ and transduced with packaged DNA according to the manufacturer's protocol. Ligated concatemeric pHC79/*T. pallidum* DNA (3.5 µg) was packaged using 14 packaging reactions. The cells were plated out on LB plates containing 50 µg ampicillin ml⁻¹, resulting in 1600 ampicillin-resistant colonies.

Screening of E. coli transductants for expression of T. pallidum antigens. Ampicillin-resistant *E. coli* colonies (300) were grown overnight at 37 °C in 25 ml liquid LB medium containing 50 µg ampicillin ml⁻¹. *E. coli* cells were harvested by centrifugation at 2500 g for 20 min and resuspended in 4 ml IS buffer, sonicated as *T. pallidum* and tested in rocket immunoelectrophoresis as described above.

RESULTS

Fig. 1 shows a crossed immunoelectrophoresis of sonified *T. pallidum* against a polyspecific antibody to the same organism. The CA of *T. pallidum* (Tp-4) was identified by using a

Fig. 1. Crossed immunoelectrophoresis of 20 μ l sonified *T. pallidum* (3×10^9 ml $^{-1}$) against anti-*T. pallidum* (100 μ l per ml gel). Tween 20 was included in the gel to give an improved electrophoretic pattern. The TpD antigen and the Tp-4 antigen were identified as described previously (Hindersson *et al.*, 1984, 1986).

monospecific antibody to *T. phagedenis* CA (TR-c) in the intermediate gel as described by Hindersson *et al.* (1984). Fig. 2(a) shows a crossed immunoelectrophoresis of *T. pallidum* against the monospecific antibody to CA of *T. phagedenis* (TR-c). Tp-4/anti-TR-c immunoprecipitates were cut out from crossed immunoelectrophoresis plates and used for immunization of rabbits to produce a monospecific antibody to Tp-4. The rabbits immunized with Tp-4 immunoprecipitates developed monospecific antibodies as illustrated in Fig. 2(b), where sonified *T. pallidum* is run against the antiserum resulting from the immunizations. Only one precipitate is visible, indicating that the antiserum is monospecific for Tp-4. Antibodies specific for Tp-4 and not recognizing *E. coli* CA determinants were produced by absorption of the anti-Tp-4 antiserum with *E. coli* C600(pHC79). The specificity of the absorbed antiserum was tested by Western blotting using *T. pallidum*, (Fig. 3 lane a), and *E. coli* C600(pHC79) (Fig. 3 lane c), as antigen. The antibody recognized a protein subunit in *T. pallidum* with a molecular mass of 60 kDa. No reaction was observed when C600(pHC79) was used as antigen, demonstrating that the absorbed antiserum did not recognize *E. coli* CA but only the *T. pallidum*-specific epitopes of Tp-4.

Partially *Sau3AI*-digested *T. pallidum* DNA of molecular mass 25–50 kb was ligated to *Bam*HI-cleaved dephosphorylated cosmid pHC79 at high DNA concentration. The resulting concatemeric DNA was packaged *in vitro* (see Methods) and used to transform the *E. coli* C600. Of the 1600 ampicillin-resistant colonies obtained, less than 0.1% were resistant to tetracycline, indicating that virtually all the clones contained cosmids with *T. pallidum* DNA inserted into the *Bam*HI site of pHC79. Assuming an average insert of 40 kb and a total *T. pallidum* genome size of 14000 kb it can be calculated (Maniatis *et al.*, 1982) that the *T. pallidum* genome has been covered with a probability of approximately 99%.

Three hundred randomly picked colonies were grown in liquid LB medium containing ampicillin. Solubilized antigens of these cultures were tested for expression of treponemal

Fig. 2. (a) Crossed immunoelectrophoresis of *T. pallidum*, preparations as in Fig. 1, against monospecific anti-TR-c (100 μ l per ml gel). Tp-4/anti-TR-c immunoprecipitates were cut out and used for immunizations. (b) The resulting monospecific antiserum 'anti-Tp-4' in crossed immunoelectrophoresis using sonified *T. pallidum* as antigen. Only one precipitate is observed, indicating that the antibody is monospecific for the Tp-4 antigen.

Fig. 3. Western blot analysis of (a) *T. pallidum*, (b) *E. coli* C600(pHI1001), and (c) *E. coli* C600(pHC79). The equivalent of 3×10^7 micro-organisms was applied in lanes (a) and (b), and of 2×10^9 C600(pHC79) in lane (c). The Western blot was developed with anti-Tp-4 absorbed with C600(pHC79) diluted 1 in 10000. The second antibody was peroxidase-labelled swine anti-rabbit immunoglobulin diluted 1 in 2000. Anti-Tp-4 absorbed with *E. coli* identifies an antigen subunit of molecular mass 60 kDa in *T. pallidum* and in C600(pHI1001). The CA of *E. coli* transformed with pHC79 does not react with absorbed anti-Tp-4.

antigens using a modified rocket immunoelectrophoresis. Fig. 4 shows rocket immunoelectrophoresis of 23 clones. One clone, C600(pHI1001) produced an antigen which was precipitated by antibodies to *T. pallidum* (arrowed in Fig. 4). The rocket was slightly retained into the bottom gel. The plasmid pHI1001 encoding the antigen had a size of 36 kb when analysed by agarose electrophoresis (Maniatis *et al.*, 1982).

Sonified antigens of clone C600(pHI1001) were included in a line gel cast below the circular wells (rocket line immunoelectrophoresis, Fig. 5). The antigenic material in the line gel resulted

Fig. 4. Rocket immunoelectrophoresis of 23 *E. coli* recombinants. *E. coli* antigens are retained by a high concentration of polyspecific antibodies to *E. coli* ($\bar{a}$ -*E. coli*) in the bottom gel. One clone, C600(pHI1001) (arrow), expressed an antigen precipitated by polyspecific antibodies to *T. pallidum* ($\bar{a}$ -*T. pallidum*) in the upper gel.

in a large number of immunoprecipitation lines in the lower, anti-*E. coli*-containing, gel and a distinct line precipitate corresponding to the expressed antigen in the upper, anti-*T. pallidum*-containing, gel. When antigen of C600(pHI1001) was applied (well A), a positive deflection of the line precipitate was observed. Purified sonified *T. pallidum* (well B) also contained an antigen able to deflect the line precipitate. No spurs were observed at the legs (arrows, Fig. 5) of the rocket deflecting the line precipitate, indicating complete identity of the authentic antigen expressed by *T. pallidum* and the antigen expressed by C600(pHI1001). Several other *T. pallidum* antigens precipitated by the polyspecific antibody to *T. pallidum* were observed. Human syphilitic serum (a pool of serum from 20 cases of secondary syphilis, well C), reacted with the cloned antigen resulting in a negative deflection of the line precipitate. A weaker deflection was observed using non-syphilitic serum (a pool of serum from 20 blood donors with no history of syphilis, well D). These results indicate that antibodies to the *T. pallidum* antigen are present in syphilitics and, although in a lower concentration, in individuals not exposed to *T. pallidum* antigen. A polyspecific antiserum to *T. phagedenis* gave a very strong negative deflection of the line precipitate, showing indirectly that *T. phagedenis* expresses an antigen cross-reacting with the cloned antigen.

To identify the expressed antigen, a monospecific antiserum to TR-c was used (Fig. 6). This antiserum deflected the line precipitate, showing that the cloned antigen was the Tp-4 antigen of *T. pallidum*. A monospecific antibody to the flagellum of *T. phagedenis* was used as control and did not affect the line precipitate.

The identity of the cloned antigen was further substantiated by examination of clone C600(pHI1001) by Western blotting using an absorbed monospecific antibody to Tp-4, which does not recognize *E. coli* CA epitopes. This technique showed (Fig. 3, lane *b*) that clone C600(pHI1001) expressed a protein subunit with a molecular mass of 60 kDa, corresponding to the 60 kDa CA subunit found in *T. pallidum*. The absorbed antiserum could discriminate between CA of *T. pallidum* and *E. coli*, since no reaction with *E. coli* C600(pHC79) could be demonstrated despite the fact that the concentration of *E. coli* cells in lane *c* was 64 times higher than that in lane *b*. *T. phagedenis*, *E. coli* and *P. aeruginosa* antigens were examined by Western blotting using the non-absorbed antiserum to Tp-4. The molecular mass of the CA subunit detected by the non-absorbed antiserum was 60 kDa in all three species (data not illustrated).

Fig. 5

Fig. 6

Fig. 5. Rocket line immunoelectrophoresis. The electrophoretic design was the same as in Fig. 4, except that a line gel containing C600(pHI1001) was cast in the lower anti-*E. coli* ($\bar{\alpha}$ -*E. coli*) gel. The antigen expressed by the recombinant C600(pHI1001) results in a line precipitate. Well A contained sonified C600(pHI1001). A rocket corresponding to the expressed antigen deflecting the line precipitate is observed. Well B contained sonified *T. pallidum*. Several antigens of *T. pallidum* were precipitated by the polyspecific anti-*T. pallidum* antiserum. One rocket fused completely with the line precipitate without spur formation (arrows), indicating complete identity. Well C contained secondary syphilitic serum and well D human donor serum. A weak deflection by donor serum and a stronger deflection by syphilitic serum indicates the presence of antibodies to the cloned antigen in these sera. Polyspecific rabbit antibodies to *T. phagedenis* (well E) gave a very strong deflection of the line precipitate, indicating that *T. phagedenis* contains an antigen cross-reacting with the *T. pallidum* antigen expressed by C600(pHI1001).

Fig. 6. Identification of the antigen expressed by C600(pHI1001). The electrophoretic design was the same as in Fig. 5. Well A was an 'empty' control well. Well B contained polyspecific antibodies to *T. phagedenis* (see Fig. 5). The deflection of the line precipitate by monospecific antibodies to TR-c (well C) indicates that the cloned antigen is the Tp-4 antigen of *T. pallidum*. No deflection of the line precipitate could be detected when antibodies to the flagellum of *T. phagedenis* were applied (well D).

Two other *E. coli* clones expressing *T. pallidum* antigen were identified among the 300 colonies examined by rocket electrophoresis using the polyspecific anti-*T. pallidum* antiserum. Using the same technique as above it was shown that the antigen produced by these two clones was identical to TpD, a *T. pallidum*-specific surface-associated, immunodominant antigen identified in a cosmid gene bank constructed by van Embden *et al.* (1983). This antigen has been characterized and purified (Hindersson *et al.*, 1986) and will not be considered further here.

DISCUSSION

The preparation of a good polyspecific antiserum to *T. pallidum* was very difficult, since rabbits responded poorly to treponemal antigens as judged by crossed immunoelectrophoresis. This may have been due to contaminating testicular material or components of *T. pallidum* which can suppress the humoral immune response. A long immunization period with highly purified treponemes resulted in a good polyspecific antiserum to *T. pallidum*, which was useful for screening *E. coli* recombinants for expression of *T. pallidum* antigens. The solubilization of antigens to be analysed by electroimmunoprecipitation methods is very important. Earlier it was shown that the non-ionic detergent polyoxyethylene alcohol (C₁₀E₇) improved the quality and

number of immunoprecipitates when *T. pallidum* was analysed by crossed immunoelectrophoresis (Hindersson *et al.*, 1986). $C_{10}E_7$ is very expensive and other cheaper non-ionic detergents were therefore tested: Tween 20 was found to be a very good substitute. The number of detectable immunoprecipitates (Fig. 1) was almost the same using Tween 20 and this detergent was therefore used for all immunoelectrophoretic procedures described in this paper. The position and morphology of the Tp-4 precipitate is not affected by the inclusion of detergents in the gel.

Antibodies to *E. coli* antigens are often present in non-immune and hyper-immune sera. The presence of such antibodies makes it difficult to apply conventional rocket immunoelectrophoresis for detection of *T. pallidum* antigens expressed by *E. coli* recombinants. The direct absorption of the antiserum for use in rocket electrophoresis is difficult because the absorption material will result in a high background staining in the gels. For this reason we used the modified rocket electrophoresis system where *E. coli* antigens are precipitated in a bottom gel by a polyspecific antiserum to *E. coli* and *T. pallidum* antigens are precipitated by antiserum to *T. pallidum* in a gel above the anti-*E. coli* gel. The antigen produced by *E. coli* C600(pHI1001) was precipitated by anti-*T. pallidum* antibody in the upper gel but the rocket was slightly retained in the lower gel. This phenomenon reflects the effect of electroendosmosis or the fact that the antiserum in the lower gel recognizes epitopes of Tp-4 identical to epitopes present on *E. coli* CA.

Several recent reports have described cloning and expression of *T. pallidum* antigens in *E. coli* (Walfield *et al.*, 1982; Stamm *et al.*, 1982*a, b*; van Embden *et al.*, 1983; Norgard *et al.*, 1983; Hansen *et al.*, 1985). None of the antigens cloned until now have characters in common with the Tp-4 antigen, except the 60 kDa protein produced by *E. coli* phenotype A described by van Embden *et al.* (1983), which, like Tp-4, has a subunit molecular mass of 60 kDa. The phenotype A (kindly provided by Dr J. D. A. van Embden, Rijksinstituut voor Volksgezondheid en Milieuhygiëne, Bilthoven, Holland) was examined in Western blotting using the monospecific anti-Tp-4 absorbed with *E. coli*. No reactivity with absorbed antiserum to a 60 kDa band could be demonstrated, indicating that the two cloned antigens are non-identical.

Pedersen *et al.* (1981) demonstrated that the Tp-4 antigen has specific determinants when compared with the TR-c antigen of *T. phagedenis*. It might be – although it seems unlikely – that the species-specific determinants of Tp-4 are able to induce protective immunity against syphilis.

The existence of CA determinants common to a wide range of bacteria makes CA an unsuitable antigen for serological diagnosis of syphilis, but if the species-specific determinants can be defined using recombinant DNA technology or monoclonal antibodies, then serological diagnosis of syphilis using this antigen is feasible. An earlier attempt to use purified TR-c antigen of *T. phagedenis* for serological diagnosis of syphilis was unsuccessful (Hindersson *et al.*, 1984) due to the high background activity present in non-syphilitic sera. Bishop & Miller (1983) demonstrated by an *in vitro/in vivo* neutralization test that normal human immunoglobulin is able to 'kill' *T. pallidum*. This treponemicidal activity could be removed by absorption with sonified *T. phagedenis* antigen. Antibodies to CA are often present in normal serum as a result of earlier infections or presence of the normal bacterial flora. It is tempting to speculate that the treponemicidal activity demonstrated by Bishop & Miller (1983) in normal human serum was due to presence of antibodies to CA.

The molecular mass of purified CA from *T. phagedenis* (TR-c) was estimated to be 48 kDa (Pedersen *et al.*, 1982). Purified CA from *P. aeruginosa* had a molecular mass of 60 kDa (Sompolinsky *et al.*, 1980*b*). Our Western blot analysis of *T. phagedenis* and *P. aeruginosa* antigen using non-absorbed anti Tp-4 showed a CA subunit molecular mass of 60 kDa for both species. The lower molecular mass estimate of purified TR-c was perhaps a result of damage during purification.

Nothing is known about the function and localization of CA in the bacterial cell. Immunoelectronmicroscopy using monospecific antiserum to CA will probably clarify the latter. A structural analysis of the gene encoding Tp-4 is now possible using plasmid pHI1001. Work is in progress to reduce the size of the Tp-4-encoding plasmid and to localize the CA

coding region precisely. An analysis of the amino acid sequence of CA derived from the CA DNA sequence may cast light on the function and localization of CA. The sequence information relating to CA of other species, when available, might be a useful tool for studying DNA sequence drift and for classification of different bacterial strains.

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