Antigenic and Structural Analysis of Treponema denticola

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Polypeptide and Western immunoblot profiles of subcellular fractions of Treponema denticola ATCC 33520 have been determined by SDS-PAGE of Triton X-100-soluble and -insoluble fractions, a lipopolysaccharide-enriched fraction and purified flagella. Major Triton X-100-soluble polypeptides of 72, 68, 54 and 52 kDa were detected. The 54 kDa polypeptide appeared to be a breakdown product of a larger, heat-modifiable polypeptide. Based on the results of SDS-PAGE analysis and immunoblotting of proteinase K digests of T. denticola, a ‘rough’ lipopolysaccharide appeared to be present. Electron microscopy has been used to monitor the effect of detergent treatment on the morphology of the organism and to examine the detailed structure of the flagella. Treatment with Triton removed the T. denticola outer membrane, resulting in exposure of the flagella. The flagella were shown to have a complex sheath and core structure and polypeptide composition characteristic of that observed for other treponemes. Polypeptides of 38, 35, 32 and 28 kDa were present in purified flagella preparations. Immunoelectron microscopy, iodine-labelling and Western blotting were used to demonstrate the exposure of antigens on the T. denticola surface. Surface iodination located polypeptides of 72, 68 and 54 kDa. Antiserum raised against whole cells of T. denticola recognized these polypeptides and an additional polypeptide of 52 kDa. These data provide a basis for future detailed molecular analysis of the ultrastructure and antigenicity of T. denticola.

INTRODUCTION

The microflora associated with destructive periodontal disease is highly complex (Moore et al., 1982) but amongst the bacteria consistently present are spirochaetes, readily visible by dark-field microscopy, the numbers of which are associated with the severity of disease (Armitage et al., 1982; Listgarten & Levin, 1981; Loesche et al., 1985). In advanced disease, spirochaetes can comprise up to 50% of the observable bacteria (Loesche & Laughon, 1982) but the difficulties of culturing these organisms means that there is as yet little detailed information on the number of different species present, their relationships, or their contribution to the disease process.

Treponema denticola is the best-characterized of the oral species, as it is regularly isolated from periodontal disease sites (Moore et al., 1982; Fukomoto et al., 1987) and is relatively easily grown in vitro. Evidence for an association between periodontal disease and T. denticola includes the observed correlations between total number of spirochaetes present and levels of the marker ‘trypsin-like enzyme’ (Bretz & Loesche, 1987) and increases at diseased sites of T. denticola detected by a method using monoclonal antibodies (Simonson et al., 1988a). Although these studies suggest that T. denticola may be the most prevalent treponemal species, there is as yet no direct evidence for its role as an aetiological agent in periodontal disease.

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There has been limited analysis of *T. denticola* at the molecular level. PAGE analysis has revealed a large number of polypeptides in whole organisms and some evidence for variation among isolates has been presented (Cheng et al., 1985; Tall & Nauman, 1986; Sela et al., 1987; Fukumoto et al., 1987). There is also evidence of antigenic heterogeneity, shown by a variety of techniques (Cheng et al., 1985; Sela et al., 1987; Tall & Nauman, 1986). Fractionation of *T. denticola* by detergent-extraction was investigated by Jacob & Taumann (1982) but in contrast to other pathogenic treponemes such as *Treponema pallidum*, detailed information on the subcellular location of antigens and other components in *T. denticola* is not presently available. Such information may allow identification of additional components of use in the differentiation of *T. denticola* from other treponemes present in periodontal lesions, and of potential virulence determinants such as outer-membrane components which may be involved in pathogenesis.

We have applied subcellular fractionation techniques, SDS-PAGE and immunoblotting to define the ultrastructural location and antigenicity of polypeptides in *T. denticola*. These data suggest both similarities and fundamental differences in structure between *T. denticola* and other pathogenic and non-pathogenic treponemes.

**METHODS**

Maintenance and growth of T. denticola. *Treponema denticola* ATCC 33520 was grown anaerobically in static culture for 3 d at 37°C. The basal growth medium consisted of (g l\(^{-1}\)): tryptone (Difco), 20 g; yeast extract (Difco), 10 g; brain heart infusion (Difco), 5 g; K\(_2\)HPO\(_4\) (Sigma), 2 g; and cysteine hydrochloride (Sigma), 0-68 g. This was supplemented with 10% (v/v) newborn calf serum (Gibco) and 5 μg cocarboxylase ml\(^{-1}\) (Sigma). The medium was pre-reduced prior to use. After 3 d growth, bacterial counts (determined by dark-field microscopy) reached 1 × 10\(^8\) ml\(^{-1}\). After growth, bacteria were pelleted by centrifugation at 11 600 g in a microcentrifuge and were washed three times in phosphate-buffered saline (PBS).

PAGE and immunoblotting. SDS-PAGE was performed in 12.5% (w/v) polyacrylamide gels essentially as described by Towbin et al. (1979), with minor modifications (Cockayne et al., 1987). In addition, 1 mm-phenylmethylsulphonyl fluoride (PMSF) was included in the running buffer. Polypeptides were detected by silver staining (Wray et al., 1981). Molecular masses of treponemal polypeptides were determined by comparison with known molecular mass standards (Bio-Rad).

For immunoblotting, polypeptides were transferred to nitrocellulose membrane as described by Towbin et al. (1979), with minor modifications (Cockayne et al., 1987). Immunoblots were reacted for 2 h with 1:200 dilution of anti-*T. denticola* polyclonal rabbit serum and bound antibody was detected as previously described (Cockayne et al., 1987). For the electrophoretic experiments illustrated in Fig. 2, methods described by Russell et al. (1985) were used. Radiolabelling of *T. denticola* with \(^{125}\)I catalysed by Iodo-beads (Pierce & Warriner Ltd) and autoradiography were done as described previously (Russell et al., 1983).

Fractionation of *T. denticola* cells. Triton X-100-soluble polypeptides were identified using the method previously described by Penn et al. (1985). Pelleted washed bacteria (5 × 10\(^8\)) were incubated with 200 μl 0-2% (v/v) Triton X-100 in 100 mM-Tris, pH 8, for 30 min at 37°C. Bacteria were then recentrifuged at 11 600 g for 10 min and the supernatant removed and retained. The remaining Triton-insoluble pellet was rewashed twice in 100 mM-Tris buffer.

A lipopolysaccharide (LPS)-enriched fraction of *T. denticola* was prepared using proteinase K treatment as described by Hitchcock & Brown (1983). Following solubilization by boiling in Laemmli sample buffer and proteinase digestion, PMSF was added to the sample to a final concentration of 1 mm. The final concentration of *T. denticola* in these preparations was 4 times that of the cell fractions described above.

Small quantities of *T. denticola* flagella were purified from treponemes previously treated with Triton X-100. Treponemes (6 × 10\(^8\)) were pelleted and treated with 2:4 ml 0-2% Triton for 30 min at 37°C. Bacteria were pelleted and the pellet washed twice in 100 mM-Tris. The pellet was resuspended in 400 μl PBS and approximately 1/3 vol. of 1 mm diameter glass beads was added. The bacterial pellet was vortexed vigorously for 45 s. Bacteria were recentrifuged at 11 600 g for 10 min and the supernatant containing sheared flagella was removed. Triton X-100 was added to the supernatant to a final concentration of 0-2% v/v and the flagella were pelleted by centrifugation at 100 000 g for 15 min in a Beckman Airfuge. The pellet was washed twice in 0-2% Triton.

Prior to application to gels, all cell fractions, and a whole cell control, were mixed with Laemmli sample buffer and were boiled for 5 min. In addition, a duplicate sample of whole cells was solubilized by incubation in sample buffer at 37°C for 30 min. Unless otherwise indicated, approximately 4 × 10\(^7\) treponemes or equivalent loading of individual cell fractions was applied per lane.

Production of antiserum. Polyclonal antiserum to disrupted *T. denticola* was produced by repeated injection of sonicated *T. denticola* cells into a New Zealand White rabbit over a 2 month period. Immunization was initially performed using subcutaneous administration of 1 × 10\(^9\) treponemes in Freund’s complete adjuvant and
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subsequently in Freund's incomplete adjuvant. The immune response was boosted by intravenous administration of \( 5 \times 10^8 \) bacteria in PBS at weekly intervals. Serum was obtained from the rabbit 10 d after the final administration. A rabbit antiserum to intact \( T. denticola \) (washed once in physiological saline) was raised by a similar injection schedule but using subcutaneous injections with incomplete adjuvant throughout.

Electron microscopy. Whole cells of \( T. denticola \), Triton X-100-treated bacteria and purified flagella were applied to Formvar-coated copper grids and negatively stained with ammonium molybdate as previously described (Cockayne et al., 1987). In addition, whole cells and Triton-treated bacteria were reacted with polyclonal anti-\( T. denticola \) rabbit serum and bound antibody was localized using an immunogold conjugate and previously described techniques (Cockayne et al., 1987).

RESULTS

Analysis by SDS-PAGE

Silver-stained SDS-PAGE polypeptide profiles of whole cells and subcellular fractions of \( T. denticola \) are shown in Fig. 1. Polypeptides ranging from 110 to 14 kDa were detected in boiled whole cells (lane 3) with major polypeptides of 68, 54, 42, 38 and 35 kDa particularly prominent. The polypeptide profile of whole cells solubilized at 37 °C (lane 1) differed markedly from this profile. Major differences included the presence of a high-molecular mass component (arrow) and the absence of a 54 kDa polypeptide in the profile of unboiled cells.

Identification of surface proteins

Proteins which were exposed at the surface of \( T. denticola \) were identified by labelling with \(^{125}\text{I}\) using Iodo-beads, which are unable to penetrate cell membranes. The only proteins which were accessible for iodination were of 72, 68, 54 and 52 kDa (Fig. 2a). When iodination was catalysed by lactoperoxidase, the same proteins were labelled (not shown).

Selective extraction of \( T. denticola \)

The relative ease with which different proteins can be extracted with detergents can give an indication of their location and organization in the organism; the polypeptide profiles of Triton X-100-insoluble and -soluble fractions are shown in Fig. 1 (a), lanes 4 and 5. Major polypeptides of 68, 54, 52 and 40 kDa were detected in the Triton X-100-soluble fraction although a large number of minor polypeptides were also solubilized by this treatment. Two polypeptides of 52 and 40 kDa were found almost exclusively in this fraction, suggesting complete extraction by the detergent. A number of other polypeptides, including one of 72 kDa, were significantly enriched in the Triton-soluble fraction. In addition, a number of polypeptides were detected in approximately equal quantities in both the Triton-soluble and -insoluble fractions, suggesting only partial extraction. Two major polypeptides of 38 and 35 kDa were present exclusively in the Triton-insoluble fraction.

Removal of proteins aids the identification of other cellular components, and treatment of \( T. denticola \) with proteinase K resulted in almost complete digestion of the organism (Fig. 1, lane 6). Two proteinase-K-resistant polypeptides of 110 and 90 kDa were detected. In addition, three other silver-staining bands were visible in the molecular mass range 28–18 kDa. A densely staining component, migrating as a broad band almost at the dye front, was also evident.

Analysis of \( T. denticola \) by Western immunoblotting

Western blotting was employed to determine which proteins of \( T. denticola \) were antigenic and to identify antigens which did not stain with the silver method. Western immunoblots of duplicates of the samples used for silver-staining, reacted with polyclonal rabbit serum raised against disrupted \( T. denticola \), are shown in Fig. 1 (b). The pattern closely resembled that of the stained gels, indicating that most proteins were antigenic, but in addition three immunodiffuse antigens of 33–31, 28–26 and 24–22 kDa were observed in whole-cell preparations. The distribution of antigenic polypeptides between Triton-soluble and -insoluble fractions closely
followed that seen on the silver-stained gel. A major antigenic component migrating as a smear in the range 28–10 kDa was seen in treponemes treated with proteinase K. The serum did not however recognize the densely staining component migrating close to the dye front which was seen on the silver-stained gel. The position of the dye front on Fig. 1 (b) is indicated by the large arrowhead.

Similar Western blots reacted with preimmune rabbit serum showed faint reactivity with a small number of polypeptides in the approximate range 60–40 kDa (data not shown).

Antiserum which was raised against intact T. denticola in contrast recognized only four bands, of 72, 68, 54 and 52 kDa (Fig. 2b), which corresponded to those labelled with $^{125}$I.
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Two polypeptides of 38 and 35 kDa were detected in preparations of purified flagella (Fig. 1, lane 7). The migration of these polypeptides was identical to those of two polypeptides found to be associated with the Triton-insoluble fraction of T. denticola (lane 4). The antiserum to disrupted T. denticola reacted with the major 38 and 35 kDa polypeptides and weakly with two other polypeptides of 32 and 28 kDa in preparations of purified flagella (Fig. 1b, lane 7). None of these flagellar components was recognized by the antiserum to intact cells.

Electron microscopy

Electron micrographs of negatively stained whole cells, Triton-treated T. denticola and purified flagella are shown in Fig. 3. Whole cells showed typical treponemal morphology, with two flagella inserted at each pole of the cell enclosed within the bacterial outer membrane (Fig. 3a). Membrane vesicles and tubular structures were also seen associated with the bacterial surface. These structures are shown at higher magnification in Fig. 3(b).

Treatment of T. denticola with Triton X-100 removed the outer membrane and released the flagella (Fig. 3c). Examination of the flagella at higher magnification showed the presence of a distinct sheath and core structure (Fig. 3d). Flagella were seen which had lost a part of the flagellar sheath, exposing the underlying core structure (arrow). Purified flagella are shown in Fig. 3(e). Again fragments of flagella lacking part or all of their sheaths were visible.

Electron micrographs of whole cells and Triton-treated T. denticola reacted with anti-T. denticola rabbit serum and anti-rabbit immunogold conjugate are shown in Figs. 3(f) and (g), respectively. Antibody was detected at the surface of whole bacteria, indicating exposure of antigen(s) at the bacterial surface. Both the flagella and protoplasmic cylinder reacted with antibody following treatment with Triton X-100.
Fig. 3. Electron micrographs of T. denticola. (a, b) Whole cells; (c) Triton-treated cells; (d) higher magnification of (c) showing flagellum structure; arrow indicates point at which the underlying core structure has been exposed due to loss of part of the flagellar sheath; (e) purified flagella; (f, g) immunogold-labelled whole cells; (f) and Triton-treated cells (g) reacted with rabbit serum to disrupted T. denticola. Bars, 0.1 µm.

DISCUSSION

The data presented here extend previously published reports on the ultrastructure, polypeptide composition and antigenicity of T. denticola. The results indicate both similarities and fundamental differences between T. denticola and the other pathogenic and non-pathogenic treponemes that have been studied. The Triton X-100 solubility of the outer membrane of T. denticola is similar to that previously reported for both Treponema pallidum and Treponema phagedenis (Penn et al., 1985b; Cockayne et al., 1987) suggesting the lipid-rich nature of the outer membranes of all three organisms. Exposure of antigens at the T. denticola surface is indicated by our immunogold labelling experiments, which support the earlier findings of other workers who used agglutination (Jacob & Nauman, 1982; Tall & Nauman, 1986) or immunogold-labelling with a monoclonal antibody (Simonson et al., 1988) to detect surface exposure of antigens. These findings are in contrast to those for T. pallidum where direct evidence for surface exposure of antigens is still lacking (Bailey et al., 1987). The electrophoretic behaviour of the proteinase K-resistant antigen suggests the presence of an LPS in the T. denticola outer membrane and this component may contribute to the antigenicity of the T. denticola surface. Unpublished data (J. H. McDougall) indicate that the monoclonal antibody used by Simonson et al. (1988) reacts with this LPS-like moiety. LPS has not previously been reported in T. pallidum but has been observed in T. phagedenis (Van Embden et al., 1983; Bailey et al., 1986). The LPS in the latter treponeme has a characteristic ‘smooth’ electrophoretic mobility compared to that seen in T. denticola in the present study which suggests a ‘rough’ structure.

Protein antigens may also contribute to the antigenic reactivity of the T. denticola surface. Of particular interest are the 72, 68, 54 and 52 kDa proteins which are both accessible for iodine-labelling and are also exposed as immunogens on intact cells. The Triton solubility of three of these polypeptides and the effect of the detergent on the morphology of T. denticola also supports their association with the bacterial outer membrane. We do not yet know why the 40 kDa
protein is readily extracted but was not detected by $^{125}$I or by the anti-intact-cell serum. The 52 kDa Triton X-100-soluble polypeptide detected in the present study may be identical to a 52 kDa polypeptide previously detected in a sodium desoxycholate extract of *T. denticola* (Jacob & Nauman, 1982). The association of this polypeptide with the bacterial surface was inferred from agglutination experiments using antisera raised against such extracts. However, detergent treatment may also release periplasmic components, and definitive evidence for the composition of the outer membrane in *T. denticola* requires its purification. The ease of cultivation of this organism *in vitro*, and the relative abundance of the outer membrane in the bacterium, should allow this to be achieved using methods based on standard procedures such as fractionation on density gradients. The isolation and detailed characterization of the outer membrane of *T. denticola* will be the subject of future research efforts.

Another group of components of interest are the heat-modifiable polypeptides associated with the Triton-soluble fraction. Their anomalous electrophoretic behaviour resembles that of known outer-membrane proteins involved in transport functions at the surface of other Gram-negative bacteria (Hancock, 1987). Heat-modifiable polypeptides have previously been detected in another spirochaete, *Spirochaeta aurantia* (Kropinski et al., 1987), and porin activity was detected in a lipid bilayer system using purified outer membranes from this organism. No such studies have yet been done with *T. denticola*. However, the apparent oligomeric structure of the high molecular mass *T. denticola* polypeptide, its heat-modifiable nature and apparent relationship to an abundant 54 kDa Triton-soluble, surface-exposed polypeptide, are similar to those described for the porin of *S. aurantia*. The relationship between the high molecular mass polypeptide, the 54 kDa Triton-soluble polypeptide and outer membrane structure and function in *T. denticola* is currently being examined further.

In addition, a component of the high-molecular mass heat-labile moiety seen in *T. denticola* cells solubilized at 37 °C may be analogous to a high-molecular mass disulphide-bonded oligomeric polypeptide, the 4D antigen, observed in *T. pallidum* (Fehniger et al., 1984). In contrast to the 4D antigen however, disulphide bonds do not appear to be involved in the maintenance of the high-molecular mass form of the *T. denticola* antigen. Similar quantities of the antigen are solubilized by detergent from *T. denticola* in both the presence and absence of reducing agents, and higher oligomeric forms of the antigen have not been detected to date (data not shown). Similarly, reducing agents do not cause dissociation of the high-molecular mass form of the *T. denticola* antigen into its constituent polypeptide(s).

An additional similarity between *T. denticola* and *T. pallidum* is suggested by the detection of several immunodiffuse antigens in *T. denticola*. Antigens of similar heterogeneous electrophoretic mobility have been detected in *T. pallidum* (Van Embden et al., 1983) and more recent data suggest that these antigens may be lipoprotein in nature (L. M. Schouls, personal communication).

In contrast to the outer membrane, the flagella of *T. denticola* appear to be very similar to those of other treponemes in their structure and complexity, and in their location beneath the bacterial outer membrane (Penn et al., 1985a; Radolf et al., 1986; Cockayne et al., 1987; Norris et al., 1988). The detection of a complex sheath and core structure in *T. denticola*, as previously observed in both *T. pallidum* and *T. phagedenis*, re-emphasizes the fundamental differences between the flagella of the treponemes and those of other bacteria. It should be noted that the integral flagellar shaft structure comprised of more than one polypeptide in this type of 'sheath and core' arrangement is quite distinct from that seen for other complex flagella. A loosely associated sheath, apparently membranous and probably consisting of an extension of the bacterial outer membrane, is present on the flagella of some vibrios and campylobacters (Yang et al., 1977; Geis et al., 1989). Morphologically complex flagella, consisting of a single flagellin subunit, are also seen in some *Rhizobium* spp. (Krupski et al., 1985). The detection of four polypeptides associated with purified *T. denticola* flagella supports their structural complexity. Based on analogy with flagellar polypeptides of *T. pallidum*, it would be expected that in *T. denticola* the 38 kDa polypeptide would be a sheath component while one or more of the lower molecular mass components would be associated with the core structure. Further ultrastructural and antigenic analysis is required to confirm this suggestion.
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Work is currently in progress to analyse further the location, exposure and species specificity of T. denticola polypeptides using monoclonal antibodies, with the aim of identifying components of use in the identification of T. denticola and those of potential significance in the pathogenesis of periodontal disease.

Addendum. During preparation of this paper Umemoto et al. (1989) published an analysis of the outer membrane of T. denticola ATCC 33520 using a monoclonal antibody to a 53 kDa surface-exposed antigen. This work extended their earlier observations on the ultrastructure and antigenicity of T. denticola (Umemoto et al., 1988). Determination of the exact relationship of the 54 and 52 kDa polypeptides described in the present study with the 53 kDa antigen described by Umemoto et al. (1989) requires further investigation.

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