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Identification of a Transferrin-Binding Protein from *Borrelia burgdorferi*

JAMES A. CARROLL,[1] DAVID W. DORWARD,[2] AND FRANK C. GHERARDINI[1]*

Department of Microbiology, University of Georgia, Athens, Georgia 30602,[1] and National Institutes of Health/Rocky Mountain Laboratories, Hamilton, Montana 59840[2]

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Bacterial pathogens have evolved various strategies to acquire iron from the iron-restricted environment found in mammalian hosts. *Borrelia burgdorferi* should be no different with regard to its requirement for ferric iron, and previous studies have suggested that transferrin (Tf) may be a source of iron in vivo. By probing blots with Tf conjugated to horseradish peroxidase, we have identified an outer membrane protein (28 kDa) from *B. burgdorferi* B31 that bound holo-Tf but not apo-Tf. The 28-kDa protein bound human, rat, or mouse Tf and was produced only by low-passage (less than passage 5), virulent isolates of strain B31. In addition, the Tf-binding protein (Tbp) from strain B31 retained the ability to bind Tf after treatment with 2% sodium dodecyl sulfate–1% β-mercaptoethanol and heating to 100°C for 5 min. These properties are remarkably similar to those of the Tbp of *Staphylococcus aureus* and Tbp2 from *Neisseria meningitidis*. *B. burgdorferi* Sh-2-82 produced an outer membrane protein different in size, i.e., 26 kDa, but with properties similar to those of to the protein from strain B31, suggesting variation in *B. burgdorferi* Tbps. The exact role of the 28-kDa protein in iron acquisition by *B. burgdorferi* remains to be determined.

Borrelia burgdorferi, the causative agent of Lyme disease, is an obligate parasite that relies on a host for a multitude of growth factors and nutrients. During the initial stages of the infection process, *B. burgdorferi* must overcome many environmental challenges, including the scarcity of free ferric iron. While there are significant levels of iron in mammalian tissue, virtually all of it is concentrated within cells. The iron that is found extracellularly is bound to carrier proteins called siderophilins (28). The principal siderophilin in blood is transferrin (Tf), while lactoferrin predominates in secretions at mucosal surfaces (20). The primary function of Tf or lactoferrin is to sequester iron, which is highly insoluble at physiological pH, and to transport that bound iron to tissues (20). As a result, free extracellular iron is found at levels (10^{-18} M) that will not support bacterial growth (5).

In order to overcome this limitation, bacterial pathogens have evolved different mechanisms to acquire iron needed for growth. Some bacteria, such as *Shigella* and *Streptococcus* spp., cause tissue damage and acquire iron by utilizing heme or hemin (9, 11, 24). Others, like *Escherichia coli*, produce low-molecular-weight, nonporphyrin, nonprotein, iron binding molecules (7). These compounds, called siderophores, have a higher affinity for iron than do host siderophilins. A third mechanism for iron acquisition, characterized for *Neisseria*, *Listeria*, *Haemophilus*, and *Staphylococcus* spp., involves the direct binding of host siderophilins, such as Tf, to inducible proteins that are located on the bacterial cell surface (12, 13, 22, 23). One well-characterized system is that of *Neisseria* species. Pathogenic strains produce two Tf-binding proteins (Tbps) (Tbp1 and Tbp2) that form a complex in the outer membrane and promote the effective binding and uptake of Tf. *Staphylococcus aureus*, on the other hand, has a receptor composed of a single surface protein of 42 kDa (22).

* Corresponding author. Mailing address: 546 Biological Sciences Building, Department of Microbiology, University of Georgia, Athens, GA 30602. Phone: (706) 542-4112. Fax: (706) 542-2674. Electronic mail address: FRANKG@BSCR.UGA.EDU.

Efforts to study iron acquisition by *B. burgdorferi* have been hindered by intrinsic components of the complex medium needed to culture the bacterium. Previous studies have demonstrated the ability of iron-starved *B. burgdorferi* cells to overcome bacteriostatic growth conditions by the addition of Tf or $Fe(NO_3)_3$ to the medium, but the addition of heme or lactoferrin had little or no effect (10). This suggests that one mechanism by which *B. burgdorferi* may acquire iron from the host is via the utilization of host Tf. In this article we report the surface labeling of *B. burgdorferi* cells with Tf conjugated to colloidal gold and the partial characterization of a 28-kDa outer membrane protein of *B. burgdorferi* with the ability to bind Tf. This outer membrane protein has properties that are similar to those of Tbp2 of *Neisseria* and *Haemophilus* spp., but it appears to have more characteristics in common with the Tf receptor recently detected in *S. aureus* (22).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Low-passage-number (LP; <5 passages) or high-passage-number (HP; >100 passages) *B. burgdorferi* B31 (6) or Sh-2-82 (32) was grown under an atmosphere of 3 to 5% O_2–5% CO_2–90% N_2 in modified Barbour-Stonner-Kelly (BSK II) medium (2) at 34°C. Virulent strains were tested in Syrian hamsters as previously described (16). Medium for chelation experiments was prepared by the addition of 150 μM 2,2′ dipyridyl or 5 mM ethylenediamine di(*o*-hydroxy phenylacetic acid) (EDDHA) (Sigma Chemical Co., St. Louis, Mo.) to BSK II medium. Five milliliters of the chelated BSK II was filter sterilized by being passed through a 0.45-μm-pore-size syringe filter (Corning, Corning, N.Y.) directly into sterile 15-ml culture tubes flushed with the above-described gas mixture. Once cultures reached the desired cell density of 5×10^7 cells per ml, cells were harvested by centrifugation (8,000 × *g*; 10 min; 4°C). Cell pellets were gently rinsed with 50 mM NaCl in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.6 (HEPES buffer); centrifuged a second time; and suspended in 10% sucrose in HEPES before the isolation of outer membranes (4).

Electron microscopy. Samples (1.5 ml) of normal or chelated cultures of *B. burgdorferi* were harvested by centrifugation (8,000 × *g*; 2 min; 24°C). Cell pellets were gently dislodged into 200 μl of 0.2 M sucrose in 10 mM Tris, pH 7.5 (TS); harvested; washed; and gently suspended in 100 μl of TS buffer. Parlodion-coated copper-palladium grids were floated, palladium side down, on 10-μl droplets of the bacterial cell suspensions (5 min). The grids were washed twice on droplets of TS buffer and were then incubated on 10-μl droplets containing a 1:24 dilution of human Tf (hTf) (CalBiochem, Inc., La Jolla, Calif.) conjugated to 5-nm-diameter colloidal gold particles (Polysciences, Inc., Warrington, Pa.) in

filter-sterilized stabilizing buffer composed of 1.0% (wt/vol) bovine serum albumin (BSA), 0.5 mg of polyethylene glycol 20,000 (Sigma Chemical Co.) per ml, 150 mM NaCl, and 50 mM Tris (pH 7.0) (20 min at 24°C) (26). For some experiments, grids were preincubated for 20 min in 1 mg of unconjugated Tf per ml in stabilizing buffer and washed twice in 10 μl of stabilizing buffer before labeling with colloidal gold conjugates. After labeling, the grids were washed with three changes of 0.2 M ammonium acetate and negatively stained with 0.5% ammonium molybdate in 0.2 M ammonium acetate. The grids were air dried and examined at 75 kV on a Hitachi model HU-11E transmission electron microscope.

Separation of *B. burgdorferi* inner and outer membranes. Inner and outer membranes from *B. burgdorferi* B31 and Sh-2-82 were isolated as described by Bledsoe et al. (4) with the following modification. One milligram of 4-(2-aminoethyl)-benzenesulfonyl-fluoride (Pefabloc SC; Boehringer Mannheim, Indianapolis, Ind.) per ml was added to the cell suspension 20 min before lysis. Membrane samples were suspended in 20% glycerol in HEPES buffer and stored in 50-μl aliquots at −80°C. The protein concentrations of membrane samples were estimated by a modified Lowry procedure (19) with BSA as the standard.

Electrophoresis and blotting. *B. burgdorferi* membrane samples were solubilized in Laemmli buffer (17) and separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE). Separated proteins were electrophoretically transferred to 0.45-μm-pore-size nitrocellulose (Schleicher & Schuell, Keene, N.H.) in a Trans blot apparatus (Bio-Rad Laboratories, Hercules, Calif.) using Towbin buffer with 20% methanol (14 h; 30 V; 4°C) (35). Blots were stained with 0.1% Ponceau red S (Sigma Chemical Co.) in 1.0% acetic acid and blocked for a minimum of 2.5 h in 5% Carnation dry milk (wt/vol) in 100 ml of 0.1% Tween 20 (Sigma Chemical Co.) in Tris-buffered saline (TTBS) at 24°C. In some cases, blots were probed with polyclonal antiserum to outer surface protein A (OspA) (10). Low-molecular-mass protein standards (97 to 14 kDa) were purchased from Bio-Rad Laboratories.

Partial purification of the 28-kDa Tf-binding protein. *B. burgdorferi* outer membrane proteins separated by SDS-PAGE were stained with 0.1% naphthol blue black (Sigma) in 5% acetic acid for 20 min. Stained acrylamide gels were destained with several changes of distilled water, and the 28-kDa region was excised. Protein from the excised gel slices was isolated with an Elutrap (4 h; 200 V; 4°C) (Schleicher & Schuell) and was recovered by addition of an equal volume of cold acetone to the eluted sample followed by centrifugation (16,000 × *g*; 25 min; 4°C). The protein pellet was resuspended in 50 μl of sterile distilled water and stored at −20°C.

Tf binding assay. Tf blotting was performed as described by Schryvers and Morris (31) with the following modifications. Purified membrane samples were solubilized in Laemmli buffer (17). For some experiments samples were heated to 100°C for 5, 15, or 30 min, and for others 9 M urea was added to sample buffer. Blots were probed with human, mouse, or rat Tf conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, West Grove, Pa.) diluted to a final concentration of 2.5 μg/ml of TTBS. HRP was used as a negative control in some blots. Blots were incubated at 24°C with gentle agitation for 1.5 h and washed four times in 200 ml of TTBS for 1 h per wash. Reactive bands were visualized with the Enhanced Chemiluminescence kit (Amersham, Arlington Heights, Ill.) according to the manufacturer's specifications.

All commercially available holo-Tf conjugates used in these experiments were 25 to 30% saturated with iron. Apo-Tf–HRP or "reloaded" diferric Tf-HRP was prepared in the following manner. Two milligrams of Tf-HRP was suspended 1 ml of 0.15 M acetate buffer (pH 5.5) and incubated at 4°C for 16 h with gentle agitation (23). The apo-Tf–HRP was precipitated by adding an equal volume of cold acetone, harvested by centrifugation (16,000 × *g*; 25 min; 4°C), washed, and suspended in 2 mM $NaHCO_3$ in 40 mM Tris buffer, pH 7.4. Reloaded diferric Tf-HRP was prepared from this apo-Tf–HRP by the method of Holbein (14).

RESULTS

Labeling of *B. burgdorferi* Sh-2-82 with hTf-colloidal gold. Previously we had reported that LP *B. burgdorferi* cells were able to grow in chelated media with the addition of hTf or ferric nitrate as the sole iron source (10). Also, we demonstrated that *B. burgdorferi* was unable to overcome iron starvation by the addition of sequestered (i.e., in a dialysis bag) holo-Tf to the medium, suggesting that no siderophores were being produced (10). These data indicate that *B. burgdorferi* was directly binding hTf. To investigate the potential mechanism(s) involved in iron uptake, we examined the interaction of *B. burgdorferi* cells with hTf.

LP and HP strain B31 cultures (passage 2 [p2] p5, and p100) were grown in BSK II or BSK II with 2,2′ dipyridyl or EDDHA added to chelate iron. The cells from these cultures were harvested, probed with hTf-colloidal gold, and examined by transmission electron microscopy (Fig. 1). p2 LP cells grown in nonchelated BSK II medium (Fig. 1a) bound the Tf-gold particles on the spirochetal cell surface, suggesting a receptor-mediated binding of hTf to the outer membrane of *B. burgdorferi*. However, p5 LP cells showed a decrease in binding of hTf-gold conjugate, suggesting that the ability to bind Tf was being lost (Fig. 1b) with in vitro passage. p5 LP cells from cultures containing 2,2′ dipyridyl maintained motility but grew more slowly (data not shown). When the cells (p5) from these chelated cultures were incubated with Tf-gold conjugates, the number of bound particles (Fig. 1c) was equal to or greater than those observed binding to p2 cells (Fig. 1a). In addition, LP (p2 or p5) cells that were preincubated with unlabeled hTf had no Tf-gold bound to them (data not shown). These same results were observed when EDDHA was used as the chelator. HP cells (>100 passages) that were grown in nonchelated BSK II medium completely lost the ability to bind the Tf-gold conjugates (Fig. 1d). We were unable to grow or restore Tf-gold binding to HP cells in the presence of 2,2′ dipyridyl or EDDHA.

Direct binding of hTf-HRP to *B. burgdorferi* membrane proteins. To determine the outer membrane component(s) that was interacting with hTf, we used the blotting technique described by Schryvers and Morris (31). This method has been used to identify Tbps from several pathogenic bacteria, including *Neisseria*, *Listeria*, *Haemophilus*, and *Staphylococcus* spp. Total membrane proteins were purified from LP B31 or Sh-2-82 by centrifugation, separated by SDS-PAGE, transferred to nitrocellulose, and probed with hTf-HRP (Fig. 2). Reactive bands were observed in membrane preparations from strains B31 and Sh-2-82, and the profiles showed differences. Membranes isolated from strain B31 (Fig. 2, lane a) contained two predominant low-molecular-mass proteins (31 and 28 kDa) that bound hTf-HRP, while membrane samples isolated from strain Sh-2-82 (Fig. 2, lane b) contained two slightly smaller proteins (30 and 26 kDa) that bound hTf-HRP. This suggested variation between strains in the proteins binding Tf.

The subcellar locations of the 31- and 28-kDa proteins were established by isolating membrane fractions from strain B31 and evaluating inner and outer membrane preparations for binding of hTf-HRP. The results indicated that the 31-kDa protein localized primarily to the inner membrane fraction (Fig. 3, lane IM [indicated by the asterisk]), while the 28-kDa protein was found in the outer membrane fraction (Fig. 3, lane OM [indicated by the arrow]). This suggested that the 31- and 28-kDa proteins were not breakdown products but, more interestingly, that they localized to different cellular fractions. It is possible that they represent precursor (31-kDa inner membrane protein) and mature (28-kDa outer membrane protein) forms of the Tf receptor.

Comparison of virulent and avirulent strain B31 outer membranes probed with hTf-HRP. In order to determine if the 28-kDa protein was potentially associated with the loss of virulence, equivalent amounts of purified outer membrane protein from virulent (LP) and avirulent (HP) strain B31 were probed with hTf-HRP (Fig. 4). Interestingly, the 28-kDa protein was present only in the LP outer membrane fraction, suggesting loss or down regulation of this protein with prolonged in vitro transfer. When HP strain B31 cells were harvested and incubated in media containing 150 μM 2,2′ dipyridyl or media that had been treated with EDDHA, we were unable to restore the levels of expression of the 28-kDa protein observed in LP cells (data not shown). At this time, we do not know if the loss of the 28-kDa protein is reversible or understand how this protein may be regulated.

The 28-kDa protein bound Tf from different hosts. Because *B. burgdorferi* is parasitic in several animal species, having an iron acquisition system that is functional in different hosts

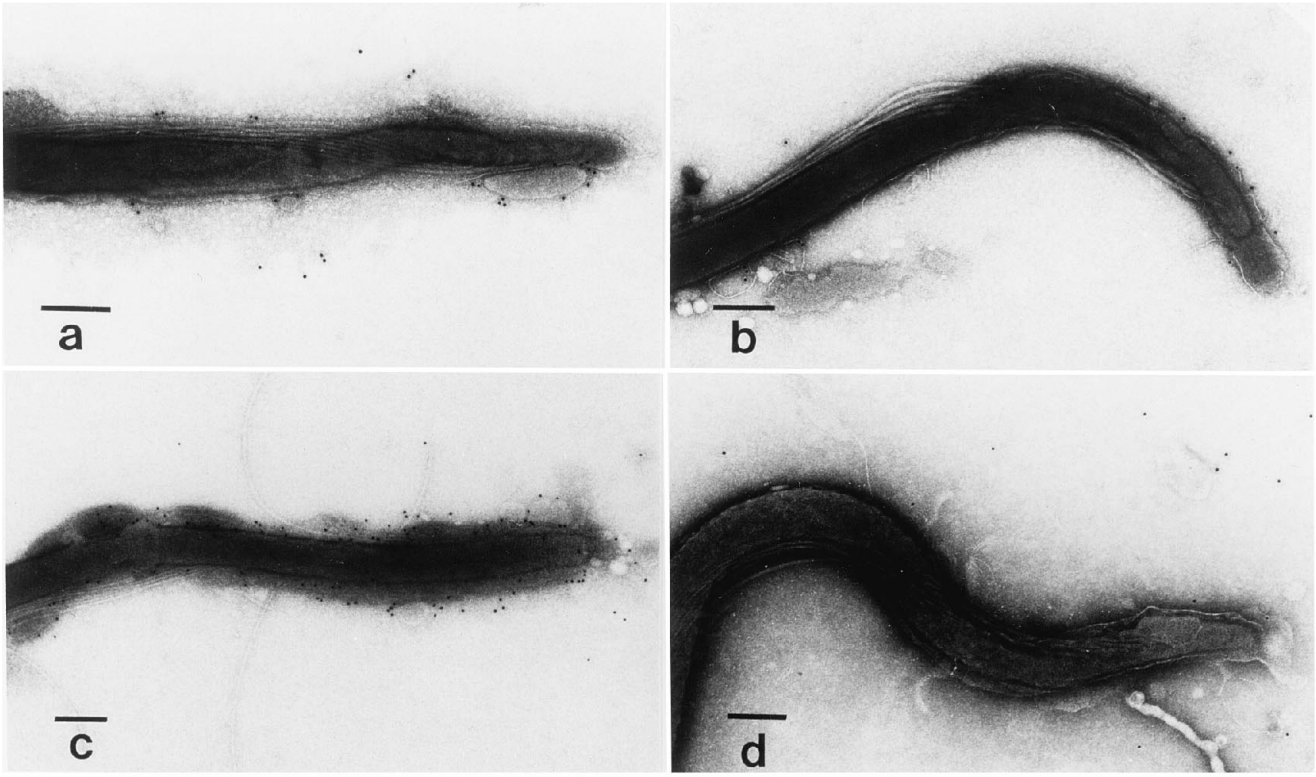

FIG. 1. Electron micrograph of negatively stained *B. burgdorferi* cells probed with hTf-colloidal gold. (a) p2 *B. burgdorferi* B31 cells grown in BSK II medium; (b) p5 B31 cells grown in BSK II medium; p5 B31 cells grown in BSK II with 2,2′ dipyridyl; (d) p100 B31 cells grown in BSK II. All preparations were probed with Tf-colloidal gold conjugate. The bar in each panel represents 0.25 μm.

would be very beneficial and efficient. Other pathogens which survive in several animal hosts and utilize Tf (e.g., *Listeria* and *Staphylococcus* spp. produce a single Tf receptor that binds the Tfs produced by those hosts. To see if this was the case for *B. burgdorferi*, we probed outer membranes from virulent (LP) strain B31 with Tfs isolated from different mammals (Fig. 5). Purified outer membrane proteins were blotted onto nitrocellulose and probed with human (lane a), mouse (lane b), or rat (lane c) Tf-HRP. The same sample was probed with HRP as a control (lane d). The 28-kDa protein bound human, mouse, and rat Tfs, suggesting that *B. burgdorferi* had an iron uptake system that had a broad host range. Outer membrane proteins did not react with HRP alone (lane d).

Properties of the 28-kDa Tbp. In order to better characterize the 28-kDa Tbp, we partially purified it from B31 outer membrane samples by electroelution. The stability of the 28-kDa protein from strain B31 was assessed to determine its ability to bind Tf-HRP during ensuing exposure to various denaturing conditions. As described above, the 28-kDa protein was able to bind Tf after the membrane sample was heated to 100°C for 5 min in the presence of 2% SDS and 1% β-mercaptoethanol (BME) (Fig. 2). When samples were heated for 15 or 30 min, the binding of Tf-HRP to the 28-kDa protein was decreased (Fig. 6, lanes B and C). The ability of the 28-kDa protein to bind Tf was affected when protein samples were solubilized in the presence of 2% SDS, 1% BME, and 9 M urea (Fig. 6, lanes D and E). Heating for 15 or 30 min in the presence of urea totally abolished binding and caused a shift in electrophoretic mobility from 28 to 54 kDa which was seen when blots were stained with Ponceau red S. The effects of urea on the protein suggested that hydrogen bonding in the protein was important for prolonged stability.

The ability of the 28-kDa protein to bind Tf was also affected by the iron bound to Tf (Fig. 7). Commercially produced Tf-HRP (30% saturated) was able to bind to the 28-kDa protein very efficiently (Fig. 7, lane a). However, apo-Tf–HRP was not

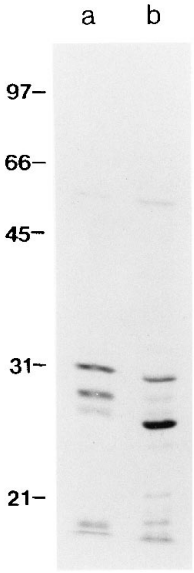

FIG. 2. Comparison of characteristics of Tf binding to total membrane protein samples from p4 *B. burgdorferi* B31 (lane a) and p7 *B. burgdorferi* Sh-2-82 (lane b). Sixty micrograms of protein from total membranes was separated by SDS-PAGE, transferred to nitrocellulose, and probed with hTf-HRP. Reactive bands were visualized by chemiluminescence as described in the text. Numbers on the left represent the relative mobilities of molecular mass standards in kilodaltons.

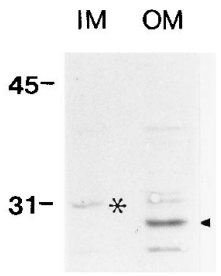

FIG. 3. Localization of Tf-binding proteins in inner and outer membranes from virulent LP *B. burgdorferi* B31. Lanes IM and OM contain 60 μg of inner and outer membranes from p5 strain B31, respectively. The blot was probed with hTf-HRP. The asterisk indicates the 31-kDa protein in the inner membrane fraction, and the arrow indicates the 28-kDa protein in the outer membrane fraction. Standards in kilodaltons are indicated on the left.

bound by the *B. burgdorferi* Tbp (Fig. 7, lane b). Apo-Tf–HRP was generated by lowering the pH of a solution containing 30% holo-Tf–HRP (from 7.4 to 5.5). When this apo-Tf–HRP was reloaded as described in Materials and Methods, the ability to bind was restored (Fig. 7, lane c). This suggested that the 28-kDa Tbp was able to bind only holo-Tf and not apo-Tf.

DISCUSSION

Siderophore-independent receptor-mediated iron uptake systems have been described for such human pathogens as *Neisseria* (23), *Haemophilus* (13), *Listeria* (12), and, more recently, *Staphylococcus* (22) spp. For the gram-negative bacteria, the components that have been identified include an outer membrane TonB-dependent receptor(s) (Tbp), a periplasmic binding protein (ferric binding protein), and a cytoplasmic ferric uptake regulatory protein (fur). The expression of these proteins is derepressed in the absence of iron. To date, the best characterized of these systems are those of *Neisseria* (3, 14) and *Haemophilus* (29, 33) species.

The Tf receptors are the outermost components of the systems that interact with Tf and are usually composed of two outer membrane proteins called Tbp1 and Tbp2. The larger protein subunit of Tf receptors (Tbp1) has been identified as

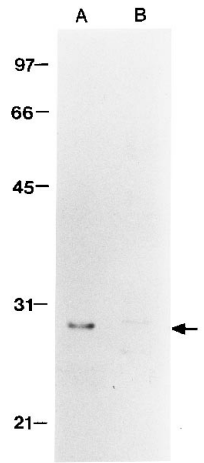

FIG. 4. Comparison of characteristics of Tf binding to outer membrane proteins isolated from virulent LP (lane A) and avirulent HP (lane B) *B. burgdorferi* B31 probed with hTf-HRP. Protein loads were normalized to 60 μg per lane, and reactive bands were visualized as described in the text. The arrow indicates the 28-kDa reactive band. Standards in kilodaltons are indicated on the left.

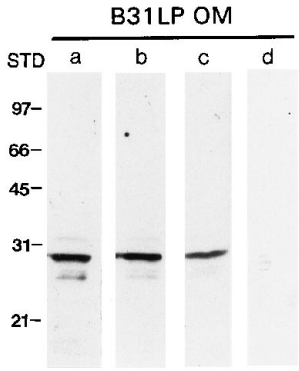

FIG. 5. Binding of Tf-HRP isolated from different mammalian sources to LP *B. burgdorferi* B31 outer membranes. Outer membrane proteins (OM) were separated by SDS-PAGE and transferred to nitrocellulose. The blot was cut into strips, and each strip was probed with a different Tf-HRP combination. Lane a, hTf-HRP; lane b, mouse Tf-HRP; lane c, rat Tf-HRP, lane d, HRP as a negative control. Sixty micrograms of protein was loaded per lane, and reactive bands were visualized as described in the text. Standards (STD) in kilodaltons are indicated on the left.

an integral outer membrane protein with an approximate molecular mass of 96 to 100 kDa in *Neisseria* spp. (30) and 100 kDa in *Haemophilus* spp. (28). Tbp1 proteins will not bind Tf after heating, SDS-PAGE, and electrophoretic transfer. However, Tbp1 will bind Tf that has been covalently bound to an affinity matrix such as Sepharose-4B. Amino acid sequence analysis revealed that Tbp1 proteins are in a class of proteins found in several uptake systems which share a common TonB binding motif (8, 18). Tbp1 mutants in *Neisseria* spp. could be shown to bind Tf on the cell surface, but the organisms lacked the ability to grow in media with holo-Tf as the sole iron source (8).

The smaller subunits (Tbp2) are outer membrane lipoproteins which range in molecular mass from 64 to 85 kDa in *Neisseria* spp. (27, 38) and from 75 to 85 kDa in *Haemophilus* spp. (29). They possess the ability to bind Tf after SDS-PAGE and electrophoretic transfer. They possess the ability to bind Tf after SDS-PAGE and electrophoretic transfer. Tbp2 from *Neisseria meningitidis* is stable when heated to 95°C for 30 min in the presence of 2% SDS–1% BME but loses the capacity to bind Tf after treatment with 3 M urea (37). These results suggest that hydrogen bonding is essential in maintaining the structure responsible for Tbp2 binding. Mutants with mutations in Tbp2 grow more slowly than wild-type *Neisseria* organisms in media with holo-Tf as the only source of iron, indicating that Tbp2 is not essential for Tf binding and utilization (1). The Tbp2s from *Neisseria* and *Haemophilus* spp. are about

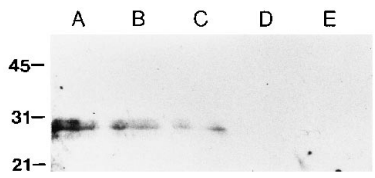

FIG. 6. Stability of binding of the 28-kDa protein from LP *B. burgdorferi* B31. Equivalent quantities of the partially purified 28-kDa protein were heated to 100°C for 5 min (lane A), 15 min (lane B), 30 min (lane C), 15 min with the addition of 9 M urea (lane D), and 30 min with the addition of 9 M urea (lane E). Samples were separated by SDS-PAGE and transferred to nitrocellulose. Reactive bands were visualized as described in the text. Standards in kilodaltons are indicated on the left.

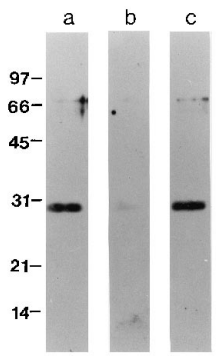

FIG. 7. Binding of apo- or holo-Tf to the 28-kDa protein. Sixty micrograms of outer membrane protein was separated by SDS-PAGE and transferred to nitrocellulose, and the blot was cut into strips. Lane a, sample probed with holo-Tf–HRP; lane b, sample probed with apo-Tf–HRP; lane c, sample probed with reloaded holo-Tf–HRP. Reactive bands were visualized as described in the text. Standards in kilodaltons are indicated on the left.

20.6% identical at the amino acid level and undergo extensive antigenic variation.

More recently a Tf receptor has been described for *Staphylococcus* spp. It consists of a single protein with a molecular mass of 42 kDa which localizes to the cell wall fraction (22). Like Tbp2 from *Neisseria* spp., the 42-kDa Tbp is capable of binding Tf after heating, SDS-PAGE, and electrophoretic transfer but recognizes both holo- and apo-Tf (21) with a dissociation constant (K_d) of 0.27 μM (22). Staphylococcal Tf receptors are apparently iron regulated (except the Tbp from *S. aureus*), and all have the ability to bind Tfs from different sources (rats, rabbits, and humans). To date, a homolog to Tbp1 has not been identified.

Previously, we reported that *B. burgdorferi* Sh-2-82 was able to overcome bacteriostatic, iron-deficient growth conditions by the addition of $Fe(NO_3)_3$ or human holo-Tf (10). As we have demonstrated here, *B. burgdorferi* was readily surface labeled when whole-cell preparations were probed with hTf-colloidal gold and surface labeling of cells grown in Fe^{3+}-chelated medium was apparently enhanced by the addition of 2,2′ dipyridyl. More specifically, we have identified an outer membrane protein from *B. burgdorferi* that exhibits many of the properties of Tf receptors from other pathogenic bacteria and is the most similar to Tbp2s from *Neisseria*, *Haemophilus*, and *Staphylococcus* spp. *B. burgdorferi* B31 and Sh-2-82 express proteins with relative molecular masses of 28 and 26 kDa, respectively, which localize to the outer membrane subcellular fraction and retain the ability to strongly bind Tf after heating, SDS-PAGE, and electrophoretic transfer to nitrocellulose. Because of their similarities to outer surface Tbp2s from several pathogenic bacteria, we have putatively identified the 28- and 26-kDa proteins as Tbp2-type receptors.

The similarities between the 28-kDa Tbp from strain B31 and the 42-kDa Tbp from *Staphylococcus* spp. are striking. First, both proteins are smaller than known Tbp2s from other bacteria. In fact, the Tbp from *B. burgdorferi* is the smallest described to date. Second, the 28-kDa protein from strain B31 was able to recognize and bind Tfs isolated from rats, mice, and humans. In this respect, it is similar to the staphylococcal Tbp receptor and the Tbp from *Listeria* spp. but quite different from the Tbp2s from *Neisseria* and *Haemophilus* spp. As previously stated, the Tbp2s from *Neisseria* and *Haemophilus* spp. have a preference for human Tf, which may reflect their narrow host range. *B. burgdorferi*, on the other hand, has a broad host range and possesses the ability to colonize a number of hosts, including humans, deer, mice, hamsters, reptiles, birds, and ticks (34). Recently, a Tf homolog has even been identified for an insect (15), and it would be interesting to determine if the 28-kDa Tbp could bind this protein as well.

Finally, the 28-kDa protein is extremely stable. Like staphylococcal Tbp and Tbp2 of *Neisseria* spp., it can withstand a number of harsh denaturing conditions and retain the ability to bind Tf. We subjected the partially purified 28-kDa Tbp from strain B31 to 1% SDS–2% BME and heating to 100°C for 30 min. Nine molar urea effectively eliminated binding to Tf and also shifted the mobility of the 28-kDa protein to 54 kDa. A similar aberrant shift in molecular mass (from 69 to 90 kDa) was observed when Tbp2 from *N. meningitidis* was exposed to 3 M urea (37). These effects on the stability of these proteins (37), in addition to the lack of an effect by BME, suggest that disulfide bridges are not necessary to maintain binding to Tf. The stability of these proteins is believed to be the result of strong intramolecular hydrogen bonding.

One striking difference between the 28-kDa Tbp and the staphylococcal Tbp is the former protein's inability to bind apo-Tf. The 42-kDa protein from *Staphylococcus* spp. could bind apo- and holo-Tf equally well (K_d = 0.27 μM) (21, 22). Preliminary data suggest that the Tbp from *B. burgdorferi* would not bind apo-Tf but would bind only holo-Tf. In this respect, it is more similar to the Tf receptor of *Neisseria* spp. (K_d = 0.7 μM), which was thought to bind apo-Tf and holo-Tf with equal affinities (36), but more recent evidence suggests that the neisserial Tf receptor preferentially binds holo-Tf (25). This model is comparable to that for mammalian receptors (K_d = 11.0 nM), which recognize only the holo (diferric) form of Tf (39).

The 28-kDa protein appears to be expressed only by LP (virulent) B31, and it could be a contributing factor in the conversion from virulence to avirulence during in vitro passage. The factors involved in such a conversion have yet to be determined, but the loss of the ability to acquire iron from the host could prove to be a major factor in this process. At this time, a model for iron acquisition by *Borrelia* spp. would be premature. However, as is the case with other pathogenic bacteria that acquire iron by directly binding siderophilins on the cell surface, receptor proteins like the 28-kDa Tbp must play a critical role in that process.

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REFERENCES

1. **Anderson, J. E., P. F. Sparling, and C. N. Cornelissen.** 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. Infect. Immun. **176:**3162–3170.
2. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
3. **Berish, S. A., S. Subbarao, C. Y. Chen, D. L. Trees, and S. A. Morse.** 1993. Identification and cloning of a *fur* homolog from *Neisseria gonorrhoeae*. Infect. Immun. **61:**4599–4606.
4. **Bledsoe, H. A., J. A. Carroll, T. R. Whelchel, M. A. Farmer, D. W. Dorward, and F. C. Gherardini.** 1994. Isolation and partial characterization of *Borrelia burgdorferi* inner and outer membranes by using isopycnic centrifugation. J. Bacteriol. **176:**7447–7455.
5. **Bullen, J. J.** 1981. The significance of iron in infection. Rev. Infect. Dis. **3:**1127–1137.
6. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick borne spirochetosis? Science **216:** 1317–1319.
7. **Carbonetti, N. H., S. Boonchai, S. H. Perry, V. Vaisnen-Rhen, T. K. Korhonen, and P. H. Williams.** 1986. Aerobactin-mediated iron uptake by *Escherichia coli* isolates from human extraintestinal infections. Infect. Immun. **51:**966–968.

8. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
9. **Daskaleros, P. A., and S. M. Payne.** 1987. Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. Infect. Immun. **55:**1393–1398.
10. **Dorward, D. W., V. E. Tamplin, J. A. Carroll, and F. C. Gherardini.** 1994. Detection and possible identification of a transferrin receptor in the outer sheath of *Borrelia burgdorferi*, p. 43–63. *In* R. Cevenini, V. Sambri, and M. La Placa (ed.), Advances in Lyme borreliosis research; proceedings of VI International Conference on Lyme Borreliosis. Societa Editrice Esculapio S.r.l., Bologna, Italy.
11. **Francis, R. T., Jr., W. Booth, and R. R. Becker.** 1985. Uptake of iron from hemoglobin and the heptoglobin-hemoglobin complex by hemolytic bacteria. Int. J. Biochem. **17:**767–772.
12. **Hartford, T., S. O'Brien, P. W. Andrew, D. Jones, and I. S. Roberts.** 1993. Utilization of transferrin-bound iron by *Listeria monocytogenes*. FEMS Microbiol. Lett. **108:**311–318.
13. **Herrington, D. A., and P. F. Sparling.** 1985. *Haemophilus influenzae* can use human transferrin as a sole source for required iron. Infect. Immune. **48:** 248–251.
14. **Holbein, B. E.** 1981. Enhancement of *Neisseria meningitidis* infection in mice by addition of iron bound to transferrin. Infect. Immun. **34:**120–125.
15. **Jamroz, R. C., J. R. Gasdaska, J. Y. Bradfield, and J. H. Law.** 1993. Transferrin in a cockroach: molecular cloning, characterization, and suppression by juvenile hormone. Proc. Natl. Acad. Sci. USA **90:**1320–1324.
16. **Johnson, R. C., N. Marek, and C. Kodner.** 1984. Infection of Syrian hamsters with Lyme disease spirochetes. J. Clin. Microbiol. **20:**1099–1101.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Legrain, M., V. Mazarin, S. W. Irwin, B. Bouchon, M. Quentin-Millet, E. Jacobs, and A. B. Schryvers.** 1993. Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins tbp1 and tbp2. Gene **130:**73–80.
19. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
20. **Mietzner, T. A., and S. A. Morse.** 1994. The role of iron-binding proteins in the survival of pathogenic bacteria. Annu. Rev. Nutr. **14:**471–493.
21. **Modun, B., P. J. Hill, A. Cockayne, R. G. Finch, J. B. Crawley, R. W. Evans, and P. Williams.** 1995. Molecular characterization of a staphylococcal receptor for human transferrin, p. 141. *In* International Conference on Bioiron.
22. **Modun, B., D. Kendall, and P. Williams.** 1994. Staphylococci express a receptor for human transferrin: identification of a 42-kilodalton cell wall transferrin-binding protein. Infect. Immun. **62:**3850–3858.
23. **Nickelsen, P. A., and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. Infect. Immun. **33:**555–564.
24. **Payne, S. M.** 1989. Iron and virulence in *Shigella*. Mol. Microbiol. **3:**1301–1306.
25. **Powell, N. B. L., D. A. Ala'aldeen, A. B. Schryvers, and S. P. Borriello.** 1995. Meningococci express more transferrin binding protein 2 (tbp2) than tbp1, and bind holo-transferrin in preference to apo-transferrin, p. 67. *In* International Conference On Bioiron.
26. **Robinson, E. N., Z. A. McGee, J. Kaplan, M. E. Hammond, J. K. Larson, T. M. Buchanan, and G. K. Schoolnik.** 1984. Ultrastructural location of specific gonococcal macromolecules with antibody-gold sphere immunological probes. Infect. Immun. **46:**361–366.
27. **Rokbi, B., V. Mazarin, G. Maitre-Wilmotte, and M. J. Quentin-Millet.** 1993. Identification of two major families of transferrin receptors among *Neisseria meningitidis* strains based on antigenic and genomic features. FEMS Microbiol. Lett. **110:**51–57.
28. **Schade, A. J.** 1985. Conalbumin and siderophilin as iron-binding proteins: a review of their discovery, p. 3–35. *In* G. Spik, J. Montreuil, R. R. Crichton, and J. Mazurier (ed.), Proteins of iron storage and transport. Elsevier, Amsterdam.
29. **Schryvers, A. B.** 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121–130.
30. **Schryvers, A. B., and B. C. Lee.** 1989. Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*. Can. J. Microbiol. **35:**409–416.
31. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. **2:**281–288.
32. **Schwan, T. G., W. Burgdorfer, M. E. Schrumpf, and R. H. Karstens.** 1988. The urinary bladder: a consistent source of *Borrelia burgdorferi* in experimentally infected white-footed mice (*Peromyscus leucopus*). J. Clin. Microbiol. **26:**893–895.
33. **Sparling, P. F. (University of North Carolina at Chapel Hill).** 1995. Personal communication.
34. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
36. **Tsai, J., D. W. Dyer, and P. F. Sparling.** 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56:**3132–3138.
37. **Vonder Haar, R. A., M. Legrain, H. V. J. Kolbe, and E. Jacobs.** 1994. Characterization of a highly structured domain in tbp2 from *Neisseria meningitidis* involved in binding to human transferrin. J. Bacteriol. **176:**6207–6213.
38. **Williams, P., and E. Griffiths.** 1992. Bacterial transferrin receptors—structure, function, and contribution to virulence. Med. Microbiol. Immunol. **180:**301–322.
39. **Young, S. P., A. Bamford, and R. Williams.** 1984. The effect of the iron saturation of transferrin on its binding and uptake by rabbit reticulocytes. Biochem. J. **219:**505–510.

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