The surface (S-) layer is a virulence factor of *Bacteroides forsythus*


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*Bacteroides forsythus* has emerged as a crucial periodontal pathogen with possible implications for systemic disease. The aim of this study was to isolate the S-layer from *B. forsythus* and examine its virulence potential as a part of efforts to characterize virulence factors of *B. forsythus*. The role of the S-layer in the haemagglutinating and adherent/invasive activities was evaluated. It was observed that the S-layer alone was able to mediate haemagglutination. In adherent and invasive studies, transmission electron microscopy clearly revealed that *B. forsythus* cells were able to attach to and invade KB cells, showing the formation of a microvillus-like extension around adherent and intracellular bacteria. The quantitative analysis showed that five different *B. forsythus* strains exhibited attachment (1:9–2:3 %) and invasion (0:4–0:7 %) capabilities. It was also observed through antibody inhibition assays that adherent/invasive activities of *B. forsythus* are mediated by the S-layer. Furthermore, an *in vivo* immunization study adopting a murine abscess model was used to prove that the S-layer is involved in the infectious process of abscess formation. While mice immunized with purified S-layer and *B. forsythus* whole cells did not develop any abscesses when challenged with viable *B. forsythus* cells, unimmunized mice developed abscesses. Collectively, the data obtained from these studies indicate that the S-layer of *B. forsythus* is a virulence factor.

**INTRODUCTION**

It has been established that the aetiology of human periodontal disease is dental plaque, a biofilm consisting of more than 500 different bacterial species and their products (Kroes et al., 1999; Paster et al., 2001). Epidemiological data, both longitudinal and cross-sectional, strongly suggest that a population shift toward Gram-negative anaerobic species in dental plaque is responsible for the initiation and progression of the periodontal disease (Kroes et al., 1999; Moore & Moore, 1994; Socransky & Haffajee, 1994). Therefore, understanding microbial pathogenesis of periodontal disease requires a detailed analysis of the complicated interaction between the host and Gram-negative anaerobic bacterial species in the periodontal environment.

During the past several decades, important data on this interaction have been presented for major periodontal pathogens, including *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. However, so far, such information has not been available for a newly emerged periodontopathic micro-organism, *Bacteroides forsythus*. *B. forsythus* is a Gram-negative, filament-shaped, non-pigmented and non-motile anaerobe, and has been associated with advanced forms of periodontal disease, including severe and refractory periodontitis (Dzink et al., 1988; Liljenberg et al., 1994; Listgarten & Lai, 1999; Tran et al., 2001). After routine periodontal therapy, the level of *B. forsythus* was significantly reduced (Listgarten et al., 1993; Winkel et al., 1997). In addition, *B. forsythus* has also been implicated in periodontitis of smokers (Zambon et al., 1996) and in the transition from periodontal health to disease (Tanner et al., 1998). Also, the presence of *B. forsythus* in periodontal pockets has been implicated as a great risk factor for cardiovascular disease (Noack et al., 2001). These data strongly suggest that *B. forsythus* is a crucial periodontal pathogen that may exert a long-term impact on systemic health. Recently, *B. forsythus* has been designated as one of the three strong candidates for aetiological agents of periodontal diseases (Zambon et al., 1996). However, microbial pathogenesis of *B. forsythus* infection has yet to be determined, since the putative virulence factors involved in the steps of the pathogenicity have not yet been adequately identified and characterized. Only a few potential virulence factors of *B. forsythus* have been described, including a trypsin-like protease (Loesche et al., 1990; Saito et al., 1997; Tanner et al., 1985) and a sialidase (Moncla et al., 1990). Saito et al. (1997) reported the cloning of a trypsin-like protease gene (*prtH*) and it was found that the presence of

**Abbreviations:** HA, haemagglutination assay; NaDoc, sodium deoxycholate; TEM, transmission electron microscopy.
the prtH gene was strongly associated with diseased sites in periodontitis patients (Tan et al., 2001). In addition, interactions between B. forsythus and host cells have been reported. Apoptotic activity of B. forsythus was found against HL-60 and other human leukaemic cells (Arakawa et al., 2000). B. forsythus has also been shown to adhere to host cells, including red blood cells, PMNs and fibroblasts (Munemasa et al., 2000). Sharma et al. (1998) reported the cloning of bspA, encoding a cell-surface antigen, which is involved in binding to fibronectin and other extracellular matrix components. Subsequently, a BspA-deficient mutant was constructed and found to exhibit decreased binding activity, implicating BspA as a virulence factor (Honma et al., 2001). This is the only B. forsythus mutant study reported, to the best of our knowledge. More information on the interaction between B. forsythus and the host is required for a better understanding of the periodontal pathogenesis mediated by B. forsythus.

Most bacteria and archaea are known to possess a unique surface structure called the S-layer, one of the most common surface structures and one of the most abundant cellular proteins in prokaryotic cells. S-layers are composed of regularly aligned protein or glycoprotein subunits ranging in size from 40 to 200 kDa (Sara & Sleytr, 2000). Even though the exact functions of S-layers have not been determined for most micro-organisms, S-layers are considered to provide micro-organisms with a protective coating, act as molecular sieves and ion traps, or promote cell adhesion and surface recognition by directly interfacing with the environment (Beveridge et al., 1997; Sara & Sleytr, 2000). For example, it was determined that the S-layer protein of Lactobacillus crispatus is required for binding to collagen and laminin, anchoring to the bacterial cell wall and self-assembly. Campylobacter rectus, a putative periodontal pathogen, exhibits an S-layer that determines cell surface hydrophobicity and is involved in interaction with the host (Borinski & Holt, 1990). Campylobacter fetus, an opportunistic pathogen in immunocompromised patients, also possesses a unique S-layer that is involved in resistance to phagocytosis (Blaser & Pei, 1993). In Aeromonas salmonicida, a crucial fish pathogen, it was found that the S-layer is involved in adherence and invasion (Garduno et al., 2000), and mutants lacking the S-layer are avirulent (Ishiguro et al., 1981). These data strongly suggest that the S-layer is an important virulence factor.

The existence and ultrastructure of the S-layer of B. forsythus were first reported by Kerosuo (1988). It was determined that the S-layer consisted of serrated structural subunits (about 10 nm wide and 10 nm high) and the type of lattice was either oblique or tetragonal. However, so far, little effort has been made on the isolation and characterization of the S-layer of B. forsythus and therefore its function is not known. In this study, the S-layer was first isolated and purified, and the virulence of the S-layer was investigated. In vitro functional activity of the S-layer was tested by haem-agglutination, adherence and invasion assays. In addition, the in vivo role of the S-layer in B. forsythus virulence was assessed by an immunization protection study by adopting a mouse abscess model.

**METHODS**

**Bacterial strains and growth conditions.** B. forsythus 43037 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and B. forsythus clinical isolates 9610, 31229, 50109 and 51118 were obtained from patients with rapidly progressive periodontitis at the Oral Research Clinic, University of Washington, Seattle, WA, USA. All clinical and reference strains of B. forsythus and other bacterial strains used in this study and their origins are listed in Table 1.

B. forsythus cells were grown and maintained anaerobically on tryptic soy agar (Difco) supplemented with 0·4 % (w/v) yeast extract (Difco), 0·4 % (w/v) phytone (Difco), 5 % sheep erythrocytes (Waltz Farm, Smithburg, MD, USA) and 0·001 % (w/v) N-acetylglucosamine acid (Sigma). P. gingivalis 381 was grown anaerobically on tryptase soy broth (Difco) enriched with 5 % yeast extract (Difco), 5 µg haemin ml−1 (Sigma), 1 µg menadione ml−1 (Sigma) and 5 % (w/v) sheep blood (Waltz Farm). Escherichia coli DH5α was grown aerobically at 37 °C on Luria–Bertani (LB) medium containing 1 % (w/v) Bacto-tryptone (Difco), 1 % (w/v) NaCl and 0·5 % (w/v) Bacto-peptone extract (Difco).

**Isolation of the S-layer.** The S-layer of B. forsythus was isolated using sodium deoxycholate (NaDoc) (Sigma) followed by CaCl iso-tpic gradient centrifugation. Briefly, B. forsythus cells were grown as described above for 4–5 days. The bacterial cells were washed three times in Tris/HCl (pH 7·4) at 8000 g for 10 min. Then, 0·5 g B. forsythus cells were resuspended in 50 ml 2 % NaDoc in Tris/HCl (pH 7·4) and stirred for 3 h at 4 °C. The bacterial suspension was centrifuged and washed twice with Tris/HCl (pH 7·4) at 8000 g for 10 min at 4 °C. Then, the supernatants were centrifuged at 100 000 g for 1 h at 4 °C and resuspended in 20 ml 50 % CaCl in Tris/HCl (pH 7·4). The gradients were centrifuged at 100 000 g for 18 h, revealing two major bands. Each band was examined by electron microscopy and SDS-PAGE. After being removed from the tube using an insulin syringe, the purified S-layer was washed twice in 30 ml Tris/HCl (pH 7·4) and stored at −20 °C for further analysis.

**Electron microscopy.** Bacterial cells were grown for 3 days, harvested by centrifugation at 8000 g for 10 min and resuspended in Tris/ HCl (pH 7·4) (to approximately 1010 cells ml−1). Ten microlitres of a bacterial cell suspension was negatively stained with 1 % sodium phosphotungstate (Fisher Scientific), pH 6·8. The specimens were contrasted with phosphomolybdic acid (Sigma) and the specimens were examined on a JEOL JEM 1230 transmission electron microscope.

**Table 1. Bacterial strains used in the study**

<table>
<thead>
<tr>
<th>Species/strains</th>
<th>Designated name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. forsythus 43037</td>
<td>BF 43037</td>
<td>ATCC</td>
</tr>
<tr>
<td>B. forsythus 9610</td>
<td>BF 9610</td>
<td>University of Washington</td>
</tr>
<tr>
<td>B. forsythus 50109</td>
<td>BF 50109</td>
<td>University of Washington</td>
</tr>
<tr>
<td>B. forsythus 31229</td>
<td>BF 31229</td>
<td>University of Washington</td>
</tr>
<tr>
<td>B. forsythus 51118</td>
<td>BF 51118</td>
<td>University of Washington</td>
</tr>
<tr>
<td>P. gingivalis 381</td>
<td>Pg 381</td>
<td>University of Florida</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>DH5α</td>
<td>Promega</td>
</tr>
</tbody>
</table>
examined with a JEOL JEM-1200EX II transmission electron microscope at an accelerating voltage of 80 kV. Images were recorded on Kodak EM imaging films.

For ultrathin-sectioning, bacterial cells were grown for 4 days, harvested by centrifugation at 8000 g for 10 min, and the pellet was washed twice with Tris/HCl (pH 7.4) and resuspended in 1% glutaraldehyde in 0.1 M cacodylate buffer (Electron Microscopy Sciences) on an ice bath for 30 min. The bacterial cells were post-fixed with 1% osmium tetroxide at room temperature overnight, dehydrated in a graded series of ethanol and embedded in Epon (Electron Microscopy Sciences). Ultrathin sections were obtained using a Reichert-Jung (Ultracut E) ultramicrotome. The sections were stained with saturated uranyl acetate, pH 4-6, followed by lead citrate. The stained sections were examined by transmission electron microscopy (TEM) and appropriate micrographs were recorded.

Immunogold labelling of LR White resin (Electron Microscopy Sciences) sections was performed as described by Coleman et al. (1990). The resulting ultrathin sections were placed on nickel grids floated onto 20 μl drops of 1:40 diluted rabbit antisera prepared against the 200/210 kDa proteins and incubated in a moist chamber at 37 °C for 1 h. Specimens were then washed 10 times with PBS and the grids were floated onto 20 μl 1:40 diluted goat anti-rabbit antibody conjugated with 5 or 15 nm gold particles (Amersham Biosciences). After incubation for 30 min at 37 °C and washing, the specimens were stained with uranyl acetate and lead citrate, and examined by TEM.

For determination of adherence and invasive activity, B. forsythus cells were incubated with KB cells. After incubation with B. forsythus ATCC 43037 cells (1 h for adherence and 5–6 h for invasion assay), the KB cell monolayer was trypsinized and washed three to five times with PBS. Detached KB cells were washed three times with Tris/HCl (pH 7.4), resuspended in 1 ml 0.1 M cacodylate buffer and fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer on ice for 30 min. Following postfixation, the specimens were prepared for TEM and examined as described above.

SDS-PAGE and Western blot analysis. A discontinuous gel system, consisting of 5–7.5% SDS-polyacrylamide gels for protein resolution and 3–5% for stacking samples, was used to separate the whole-cell and purified S-layer samples as described by Laemmli (1970). Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Immobilon-NC; Millipore) and Western blot analysis was performed as described by Towbin (1979) using rabbit antisera prepared against the 200/210 kDa proteins. The membrane was incubated with goat anti-rabbit immunoglobulin G alkaline phosphatase-conjugated secondary antibodies (Bio-Rad) and visualized by adding BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (Nitro Blue Tetrazolium salt).

Haemagglutination assay (HA). HA was performed according to a method described by Falkler & Hawley (1977). Human (A, AB, O, B), sheep and chicken red blood cells were obtained from University of Maryland, Waltz Farm, Smithburg, MD, USA, and Turslow Farm, Chestertown, MD, USA, respectively. The bacterial cells (OD600 = 1.5) and the purified S-layer (10–20 μg ml⁻¹) were adjusted in PBS and mixed with 1–2% red blood cells for 1 min using a Micro-Shaker (Dynatech). The plates were then incubated at 37 °C for 1 h followed by 2 h at 4 °C. Final results were recorded by visual inspection as follows. A positive result was assigned when a diffuse blanket of red blood cells was formed at the bottom of the microtitre plate, whereas a negative result was assigned when a dense button of red blood cells was formed. For a negative control, E. coli DH5α was used. The minimum haemagglutination dose was defined as the least OD600 of bacterial cells that gave visible haemagglutination.

The effect of heat on haemagglutinating activity was also examined. B. forsythus whole cells (OD600 = 1.5) or 20 μg purified S-layer were subjected to 15 min incubation at 22, 37, 50, 80 and 100 °C, followed by HA. The effect of carbohydrate on haemagglutinating activity was also examined. Six different sugars, including D-galactose, lactose, D-mannose, D(+)-glucose, N-acetylglucosamine and α-methylmannoside, were tested for their ability to inhibit haemagglutination of B. forsythus strains with red blood cells from different origins.

Antibody inhibition assays were performed using antibodies prepared against the whole cell and the 200/210 kDa S-layer proteins. Serial dilutions of rabbit antisera and preimmune sera (Sims et al., 1998) were incubated with whole cells and the S-layer anaerobically for 1 h at 37 °C before 2% sheep red blood cells were added. After incubation, the bacterial cells were washed with PBS and the red blood cells were added as described above.

Epithelial cells culture. The KB-cell lines (ATCC CCL-17) were obtained from the ATCC and grown at 37 °C for 2–3 days in 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 50 μg gentamicin ml⁻¹. The cells were subcultured into 25 cm² tissue culture flasks and grown overnight before adherence, invasion and electron microscopy studies.

Adherence and invasion assays. The adherent activity of B. forsythus was measured as described by Han et al. (2000). Bacterial cells were grown, washed with PBS and resuspended in DMEM at a concentration of 10¹⁰ ml⁻¹. KB cells were grown to near confluence of 10¹⁵ cells per flask. The cells were washed with PBS to remove unattached cells and bacteria were added at an m.o.i. of 50–100. For adherence, bacteria and KB cells were incubated under 5% CO2 at 37 °C for 1 h. For an invasion assay, the epithelial cells were first incubated with bacterial cells for 5–6 h and subjected to incubation in 5 ml DMEM containing 300 μg gentamicin ml⁻¹ and 50 μg metranidazole ml⁻¹ for 30 min to kill non-invaded adherent bacterial cells. After incubation, the cells were washed and lysed with sterile water for 15 min at room temperature. The lysates were removed and then diluted for viable counting of bacteria on appropriate media plates. P. gingivalis 381 and E. coli DH5α were used as positive and negative controls, respectively. The levels of attachment and invasion were expressed as the percentage of bacteria retrieved following cell lysis relative to the total number of bacteria initially added. All experiments were performed in triplicate and repeated at least three times.

Inhibition assays were performed using rabbit antibodies prepared against the whole cell and the 200/210 kDa S-layer proteins. KB cells were seeded into tissue flasks and allowed to reach a density of 10¹⁰ cells per flask. Bacterial cells (10⁹) were incubated with 1:400 dilutions of rabbit anti-whole cell or anti-200/210 kDa proteins antibodies anaerobically for 1 h at 37 °C. After the incubation, the bacterial cells were washed and resuspended to a final concentration of 10¹² ml⁻¹. Then, bacterial cells were added to the tissue flask containing KB cells, incubated for 1 h and subjected to the procedure for adherence assay as described above. Pre-immune rabbit sera were used as negative control.

Mouse immunization study. Optimal conditions for induction of localized subcutaneous abscesses in mice by B. forsythus ATCC 43037 were determined as follows. Bacterial cells were grown, washed and resuspended in sterile PBS containing 0.02% N-acetylmuramic acid. N-acetylmuramic acid was added in injection inocula to ensure optimal in vivo growth of B. forsythus. A total of 24 female BALB/C mice purchased from Harlan were divided into six groups. Different numbers of bacterial cells (1·5 × 10⁹, 1·6 × 10⁹, 1·4 × 10⁹, 4·1 × 10⁸, 1·1 × 10⁹ and 3 · 10⁸) were injected subcutaneously into two sites 1 cm lateral to the dorsal midline following a localized
depilation of the mice in each group. The size and the shape of the abscess lesion at the injection site were observed at 24 h and up to 7 days after the injection.

Using a mouse abscess model, an immunization protection study was performed to assess the *in vivo* role of the S-layer. A total of 28 female BALB/c mice, 6–8 weeks old, were divided into four groups. Each group was immunized with formalin-killed *B. forsythus* 43037, *E. coli* DH5α, purified S-layer and PBS, respectively. At baseline, after determining antibody titres against *B. forsythus*, 0.1 ml of the immunogen preparations was injected into the mice subcutaneously in the neck area. Fifteen days after the first immunization, all mice received a booster injection. At this time, blood was drawn from their tails, and mice sera obtained were stored at −20 °C and later used for ELISA for the 200/210 proteins. Fifteen days after the booster injection, all groups were challenged with *B. forsythus* cell suspensions to induce a localized abscess. The mice were monitored for their health and abscess formation. The sizes and shapes of the abscesses were measured every day for up to 4 days. Seven days after bacterial challenge, mice were killed and blood was collected for ELISA.

**RESULTS**

**Isolation of the S-layer of *B. forsythus***

The ultrastructure of *B. forsythus* ATCC 43037 and other isolates was characterized by electron microscopy using both ultrathin-section and negative staining techniques as described above. The results revealed that all *B. forsythus* isolates tested possessed outermost fuzzy surface layers external to the outer membrane (Fig. 1a) as described by Kerosuo (1988). The S-layers were isolated from different *B. forsythus* isolates.

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![Figure 1](image_url)

**Fig. 1.** (a) TEM of an ultrathin section of a *B. forsythus* ATCC 43037 whole cell. The S-layer (S), the outermost surface layer, is shown outside of the outer membrane (Om). The plasma membrane (Pm) is also shown. (b) A negatively stained suspension of the isolated S-layer. Bars, 0.1 μm.
strains using NaDoc, followed by CsCl gradient centrifugation, and the ultrastructure of the purified S-layer is shown in Fig. 1(b), exhibiting a structure resembling organized arrays, characteristic of S-layers described in other bacteria (Sara & Sleytr, 2000). When the S-layer preparations were subjected to SDS-PAGE analysis, it was shown that the S-layers isolated from different strains of \textit{B. forsythus} exhibited similar molecular masses. It was also observed that S-layer proteins are major cellular components of \textit{B. forsythus} as predicted, since they exhibited heavily stained bands among the total proteins of \textit{B. forsythus} (data not shown). As shown in Fig. 2(a), it was found that the isolated S-layers presented two major protein bands with molecular masses of 200 and 210 kDa, termed the '200/210 kDa proteins'. Besides the 200/210 kDa proteins, a minor protein band of about 15 kDa was observed in all the S-layer preparations.

Western blot analysis was performed using rabbit polyclonal antisera prepared against the 200/210 kDa proteins of \textit{B. forsythus} 43037 to examine any cross-reactivity among the purified S-layers and to verify the purity of the isolated S-layer preparation. The results showed that the 200/210 kDa bands reacted strongly with the antibodies in all tested \textit{B. forsythus} strains (Fig. 2b). This result indicates that there is a strong antigenic relationship among the purified S-layers of \textit{B. forsythus} strains. However, the minor 15 kDa protein did not react with the antibodies. These results confirmed that the S-layer preparation consists mainly of the 200/210 kDa proteins. The 200/210 kDa proteins were further purified from the S-layer preparation by gel-excision and the purity of each protein was checked again by SDS-PAGE.

The S-layer proteins are immunogens in periodontitis patients

In a previous study by Sims \textit{et al.} (1998), the major cellular protein components of \textit{B. forsythus}, designated as the 200/210 kDa proteins, elicited strong humoral antibody responses in some patients with rapidly progressive periodontitis. As described above, we isolated the S-layer from \textit{B. forsythus} and found that the sizes of the major proteins of the S-layer were 200 and 210 kDa. Therefore, to determine that these two 200/210 kDa proteins were actually the same proteins described by Sims \textit{et al.} (1998), we performed an immunogold labelling study using rabbit antisera prepared against the gel-excised 200/210 kDa proteins as described above. The results of immunogold electron microscopy, as shown in Fig. 3(a), revealed that the 200/210 kDa proteins are located on the outer surface of \textit{B. forsythus}. It was also found by immunogold electron microscopy that the labelled antibodies were bound to the S-layer preparation (Fig. 3b). These results indicated that the 200/210 kDa proteins are actually components of the S-layer of \textit{B. forsythus}, and that the S-layer of \textit{B. forsythus} was able to induce a humoral immune response in patients with rapidly progressive periodontitis.

The S-layer is a haemagglutinin

HA was performed to assess putative functions of the S-layer of \textit{B. forsythus}. It was found that all \textit{B. forsythus} strains tested were able to agglutinate sheep, human and chicken red blood cells (data not shown). To test whether the purified S-layer alone is able to mediate haemagglutination, the purified S-layer was used in the HA with sheep red blood cells. The results indicated that the S-layer was able to induce the haemagglutination. In addition, haemagglutination inhibition studies were performed to determine the effects of antibodies prepared to \textit{B. forsythus} whole-cell and S-layer proteins on haemagglutination activity. A minimum amount (\(\mu\)g) of antibody effective in haemagglutination inhibition was measured. It was found that 1·25 \(\mu\)g of rabbit

![Fig. 2.](http://mic.sgmjournals.org)
antibodies to the S-layer proteins and *B. forsythus* whole cells was able to inhibit the haemagglutinating activity of *B. forsythus* whole cells and the purified S-layer, respectively. However, rabbit pre-immune sera did not inhibit the haemagglutination activity.

The heat treatment (at >80 °C) of *B. forsythus* cells led to the abolition of the haemagglutinating activity (Fig. 4a). When the total cellular lysates were separated on the SDS-PAGE gel, it was found that the 210 kDa protein disintegrated (Fig. 4b). This result indicates that the 210 kDa protein is responsible for the haemagglutinating activity.

It was also observed that N-acetylglucosamine was the only carbohydrate capable of inhibiting the haemagglutination activity at concentrations of 100 and 200 mM. The other five sugars did not inhibit the haemagglutination activity of *B. forsythus*. No differences in inhibition of haemagglutination activity was observed using erythrocytes from different origins.

**The S-layer is involved in the adherent activity**

The ability of *B. forsythus* to adhere to KB cells (ATCC CCL-17) was also observed by electron microscopy. The results clearly showed that all tested *B. forsythus* strains were able to adhere to and subsequently invade KB cells. It was observed that invading *B. forsythus* cells are surrounded by the cell membrane of the KB, triggering internalization (Fig. 5a), eventually leading to invasion. The invaded *B. forsythus* cells were located near the nucleus of the KB cell (Fig. 5b).

The adherent activity was also quantified as described by Han *et al.* (2000). All human clinical isolates showed almost identical adherence capability, varying between 1.9 and 2.3% (Table 2). No major difference in adherence activity was observed among different strains of *B. forsythus*. These results indicated that *B. forsythus* can adhere to KB cells.
An adherence inhibition study was performed using rabbit anti-200/210 kDa proteins, anti-whole cells and the pre-immune sera. The results showed that the adherence activity of B. forsythus cells incubated with the specific antibodies was significantly decreased (Table 3), indicating that the S-layer is involved in the adherence of B. forsythus to KB cells.

### The S-layer is involved in the invasive activity

The ability of B. forsythus strains to invade oral epithelial cells was determined by quantitative invasion analysis using a standard antibiotic protection assay as described above. The results indicated that 0.4–0.7% of B. forsythus cells were able to invade KB cells. TEM studies clearly showed that B. forsythus could invade KB cells and that invasive B. forsythus cells were located near the nucleus of the KB cell. An invasion inhibition study was also performed as in the adherence inhibition assay, using rabbit anti-200/210 kDa proteins, anti-whole cells and pre-immune sera. The results indicated that invasive activities were significantly decreased by addition of antibodies against the S-layer (Table 3). This indicates that invasive activities might be mediated by the S-layer.

### Table 2. Adherence and invasion of KB cells by B. forsythus

The assays were carried out as described in the text. Representative mean values are shown. The adherence and invasive activities were expressed as the percentage of bacteria recovered relative to the total number of bacteria initially added. The assay was carried out in duplicate and repeated at least three times. ND, Not done.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Adherence (%)</th>
<th>Invasion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pg 381</td>
<td>9.6 ± 0.02</td>
<td>3.4 ± 0.02</td>
</tr>
<tr>
<td>E. coli DH5za</td>
<td>0.3 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>BF 43037</td>
<td>1.9 ± 0.02</td>
<td>0.5 ± 0.02</td>
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<tr>
<td>BF 9610</td>
<td>2.2 ± 0.02</td>
<td>0.7 ± 0.02</td>
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<tr>
<td>BF 51118</td>
<td>2.3 ± 0.02</td>
<td>0.4 ± 0.02</td>
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<td>BF 50109</td>
<td>2.0 ± 0.02</td>
<td>0.6 ± 0.02</td>
</tr>
<tr>
<td>BF 31229</td>
<td>1.9 ± 0.02</td>
<td>0.4 ± 0.02</td>
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</tbody>
</table>

### Table 3. Effect of antibodies on adherence and invasion of KB cells by B. forsythus 43037

The adherence and invasive activities were expressed as the percentage of bacteria recovered relative to the total number of bacteria initially added. The assay was carried out in duplicate and repeated at least three times.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Adherence (%)</th>
<th>Invasion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.90 ± 0.13</td>
<td>0.5 ± 0.012</td>
</tr>
<tr>
<td>Preimmune sera</td>
<td>0.47 ± 0.11</td>
<td>–</td>
</tr>
<tr>
<td>Anti-whole cell</td>
<td>0.00007 ± 0.00</td>
<td>&lt;0.00001*</td>
</tr>
<tr>
<td>Anti-S-layer</td>
<td>0.00004 ± 0.00</td>
<td>&lt;0.00001*</td>
</tr>
</tbody>
</table>

*Culture dilution less than 10^{-5} was not tested.
The S-layer is involved in the abscess formation in mice

It was found that the minimum number of B. forsythus cells inducing a subcutaneous abscess in mouse was \(1 \times 10^6\), inducing a localized abscess within 2 days after the injection. This finding indicates that B. forsythus ATCC 43037 is not invasive. It was also observed that the abscess lesions could last up to 4 days and shrink, sometimes disappearing completely 6–7 days after the bacterial injection.

In the present study, an immunization study was performed adopting a murine abscess model to test if the S-layer is involved in the infectious process. It was found that B. forsythus whole cells and the S-layer were effective immunogens in mice, eliciting significant antibody responses as measured by ELISA (data not shown). Accordingly, a group immunized with purified S-layers did not develop any abscesses when challenged with viable B. forsythus cells. Also, a group immunized with a formalin-killed B. forsythus whole-cell showed little or no abscess formation, of which the mean size was less than 3 mm\(^2\). On the contrary, a group immunized with E. coli and a non-immunized control group exhibited normal abscesses, showing mean sizes of 34 and 42 mm\(^2\), respectively. The results of these studies strongly suggest that the immunization with S-layer proteins confers a protection against subsequent B. forsythus infection, supporting the idea that the S-layer is involved in the abscess formation.

DISCUSSION

The purpose of this study was to isolate and examine the virulence function of the B. forsythus S-layer. We were able to isolate an intact S-layer from B. forsythus ATCC 43037 by NaDoc treatment and subsequent isopycnic density-gradient centrifugation. It was found that this purification procedure did not change the antigenic properties of the S-layer, since the purified S-layer was able to mediate specific antigen–antibody interactions as shown by ELISA using antibodies prepared against the B. forsythus (data not shown).

It was determined in this study that the S-layer proteins induced an immune response in humans. Even though this finding does not demonstrate the role of the S-layer as a virulence factor, it verifies that the S-layer interacts with the host to elicit a humoral immune response. Despite recent data confirming that B. forsythus is a predominant microorganism isolated from periodontal lesions (Chandad et al., 1997; Lai et al., 1987; Meurman et al., 1997; Moncla et al., 1991), it has been observed that not all B. forsythus strains induce strong immune responses in patients (Califano et al., 1997; Persson et al., 2000). The decreased immune reactivity to B. forsythus observed in some patients may not be attributed to the weak immunogenicity of B. forsythus, since B. forsythus was able to mount significant immune responses in rabbit and mice (Bird et al., 2001). While the reason for varied immune responses to B. forsythus is not completely understood, it can be postulated that B. forsythus under a specific host environment can modulate antigen expression. In a specific environment, B. forsythus may down-regulate the expression of other antigens, except the 200/210 kDa proteins. Therefore, strong immune responses to the 200/210 kDa proteins may suggest a role(s) for these proteins in the host–parasite interaction in periodontitis patients.

As mentioned above, it was determined by SDS-PAGE analysis of the purified S-layer (Fig. 2a) that the major components of the S-layer are the 200 and 210 kDa proteins. Higuchi et al. (2000) described the identification of major, high-molecular-mass proteins of 230 and 270 kDa in B. forsythus ATCC 43037. They suggested that these 230/270 kDa proteins might be the components of the S-layer, based upon the results of an immunoelectron microscopic study. However, we hereby report that the S-layer consists of the 200 and 210 kDa proteins. In their study, the 230/270 kDa proteins were obtained by excision from the SDS-PAGE gel after running samples containing the envelope fraction of B. forsythus ATCC 43037. These proteins were then injected into rabbits and the antisera prepared against these proteins were thus acquired. Subsequently, they performed an immunoelectron microscopic study using rabbit antisera and found that these proteins were located on the surface of B. forsythus ATCC 43037. While we have shown by negative staining electron microscopy (Fig. 1b) that our S-layer preparation exhibited a typical two-dimensional array structure of the S-layer, Higuchi et al. (2000) did not show that the 230/270 kDa proteins were indeed the components of the S-layer. They confirmed only that the 230/270 kDa proteins were located on the surface of B. forsythus. It is possible that the 230/270 kDa proteins are components of other surface structures in this micro-organism.

To explore the putative function of the S-layer in the host–parasite relationship, we first tested the haemagglutinating activity of B. forsythus, since surface bacterial structures have the potential to function as adhesins. It has been well established that haemagglutination activity is associated with virulence of pathogens, since haemagglutinin often serves as adhesin for many bacterial genera such as Bordetella (Makov et al., 1994; Menozzi et al., 1994), Vibrio (Booth et al., 1984), Salmonella (Lee et al., 1996), Staphylococcus (Meyer et al., 1996) and Escherichia (Adlerberth et al., 1995; Duguid et al., 1979; Nowicki et al., 1989). In this study, it was found that the S-layer is a haemagglutinin of B. forsythus. Recently, Murakami et al. (2002) reported that they observed haemagglutinating activity in the envelope fraction of B. forsythus ATCC 43037. In addition, they also found that the 230/270 kDa proteins, which were previously identified as major surface components of B. forsythus ATCC 43037 by Higuchi et al. (2000), did not play a role in haemagglutination activity, since the antibodies prepared against these proteins did not inhibit the haemagglutination. On the contrary, we hereby report that antibodies prepared against the 200/210 kDa proteins were able to inhibit haemagglutination activity. This result strongly
suggests that the 200/210 kDa proteins and the 230/270 kDa proteins described by Higuchi et al. (2000) are different proteins, and that the 200/210 kDa proteins are involved in haemagglutination, implicating that the S-layer is a haemagglutinin.

Attachment to epithelial cells and subsequent invasion is an important step in the pathogenesis of many bacterial infections, including Brucella, Listeria, Salmonella and Shigella spp. (Falkow et al., 1992; Finlay & Falkow, 1997). The ability to survive inside host cells allows bacteria to evade the immune system and to disseminate and proliferate, which is vital for the virulence of these pathogens. It has been observed that periodontal pathogens such as A. actinomycetemcomitans and P. gingivalis can adhere to and subsequently invade gingival epithelial cells and vascular endothelial cells (Dorn et al., 2000; Lamont et al., 1995; Meyer et al., 1997; Schenkein et al., 2000). It has been suggested that these micro-organisms can penetrate deeper structures of connective tissues, possibly by degrading epithelial cell–cell junction complexes (Katz et al., 2002; Papapanou et al., 1994), enter the blood stream and travel to other parts of the body. The results of an electron microscopic study have shown that B. forsythus adheres to the KB cell surface, triggering internalization and eventually leading to invasion. It is most likely that this process is a multi-stage process from the initial phase of electrostatic attraction to the later phase of the formation of fibrils and microvilli. Invading B. forsythus cells were surrounded by cell membranes of KB cells, suggesting that the invasion activity may be associated with receptor-mediated endocytosis. To the best of our knowledge, this is the first report demonstrating the invasive activity of B. forsythus. Quantitative assays have shown that all tested B. forsythus strains were able to adhere to and invade KB cell surfaces, even though the adherent and invasive activities were not as strong as those of P. gingivalis 381.

In the present study, we showed that the S-layer is involved in abscess formation by using an immunization protection study. Since the anti-S-layer antibodies provided protection against future abscess formation following B. forsythus infection of mice immunized with the S-layer, it was concluded that the S-layer was involved in abscess formation. However, the validity of the S-layer as a virulence factor in periodontal disease should ultimately be tested in more relevant animal models for periodontitis, such as a gnotobiotic rat model (Malek et al., 1994) or a mouse model for alveolar bone resorption (Baker et al., 1994), since the mouse subcutaneous abscess model does not truly represent a model for periodontitis. The use of a B. forsythus isogenic mutant deficient in the S-layer in these animal models will definitively test whether the S-layer is involved in the initiation and/or development of periodontitis.

In summary, we have shown that B. forsythus possesses potential virulence functions as a pathogen, including activities of inducing haemagglutination, adherence/invasion and mouse abscess formation. We also have shown that the S-layer is involved in these activities, implying that the S-layer is a virulence factor of B. forsythus. To better understand roles of this virulence factor in B. forsythus pathogenicity, genetic and molecular analyses of the S-layer are currently under way.

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**REFERENCES**


Bacteroides forsythus S-layer


