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The Heat-Modifiable Outer Membrane Protein of *Actinobacillus actinomycetemcomitans*: Relationship to OmpA Proteins

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The outer membrane of *Actinobacillus actinomycetemcomitans* contains a 29-kDa protein which exhibits heat modifiability on sodium dodecyl sulfate-polyacrylamide gels and represents a major target for immunoglobulin G antibody in sera of periodontitis patients colonized by this organism. In the present study, the N-terminal amino acid sequence of the 29-kDa outer membrane protein was determined and compared with reported sequences for other known proteins. The heat-modifiable outer membrane protein of *A. actinomycetemcomitans* was found to exhibit significant N-terminal homology with the OmpA proteins of other gram-negative bacteria. Moreover, this protein reacted with antiserum raised against the purified OmpA protein of *Escherichia coli* K-12. Whether the heat-modifiable OMP of *A. actinomycetemcomitans* also shares functional properties of OmpA proteins, particularly with respect to bacteriophage receptor activity, is presently under investigation.

Actinobacillus actinomycetemcomitans is a capnophilic gram-negative coccobacillus which has been associated with localized juvenile periodontitis and with some cases of refractory and rapidly progressing periodontitis (23). Immunologic analysis of patients colonized by *A. actinomycetemcomitans* indicates that these individuals often exhibit a marked humoral response to various surface-exposed outer membrane constituents of this organism, including lipopolysaccharide (10) and outer membrane proteins (OMP) (5, 21).

The outer membrane of *A. actinomycetemcomitans* contains an OMP which exhibits heat modifiability (5, 9). This protein has recently been purified and found to represent a major target for immunoglobulin G antibody in sera of patients with localized juvenile periodontitis (22). The isolated OMP migrates on sodium dodecyl sulfate (SDS)-polyacrylamide gels with an apparent mass of 29 kDa following solubilization in sample buffer at ambient temperature, but with an apparent mass of 34 kDa following solubilization at elevated (>50°C) temperatures. Little is known regarding either the structure or function of the OMP or the possible protective effect of antibody directed toward immunoaccessible determinants on this molecule.

The outer membranes of numerous species of gram-negative bacteria, both enteric and nonenteric, contain a major heat-modifiable protein structurally similar to the OmpA protein of *Escherichia coli* K-12 (4). The electrophoretic mobility of the heat-modifiable OMP from *A. actinomycetemcomitans* on SDS-polyacrylamide gels closely parallels that of the OmpA protein (13). The aim of the present study was to determine whether the principal heat-modifiable OMP of *A. actinomycetemcomitans* is structurally related to the OmpA proteins of other gram-negative species. This was accomplished through direct N-terminal amino acid sequencing of the *A. actinomycetemcomitans* OMP and subsequent comparison of this sequence with published sequences for other proteins, including OmpA proteins.

A. actinomycetemcomitans ATCC 43718 (formerly strain Y4) was maintained on 5% sheep blood agar supplemented with 1 g of yeast extract (Difco Laboratories, Detroit, Mich.), 1 mg of 3-phytylmenadione (Sigma Chemical Co., St. Louis, Mo.), and 5 mg of equine hemin III (Sigma) per liter and incubated at 37°C in humidified 5% CO_2. Midlogarithmic phase cultures were prepared in NIH thioglycolate broth (Difco) supplemented with 5 g of yeast extract and 1 g of sodium bicarbonate per liter, as described previously (2). The organisms were suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and subjected to sonic disruption and differential centrifugation to obtain a total membrane fraction. The cytoplasmic membrane was solubilized in 1% sodium *N*-lauroyl sarcosinate for 30 min at ambient temperature, as described previously (11). The sarcosyl-insoluble outer membrane fraction was recovered by centrifugation at 4°C for 1 h at 100,000 × *g*.

The heat-modifiable OMP of *A. actinomycetemcomitans* was isolated according to the procedure employed to obtain the principal heat-modifiable OMP from *Haemophilus influenzae* type b (15). Briefly, the sarcosyl-insoluble outer membrane was incubated for 30 min at ambient temperature in 50 mM Tris, pH 8.0, containing 5 mM EDTA and 1% (wt/vol) octylglucoside (buffer A). Insoluble material was recovered by ultracentrifugation, washed, and resuspended in buffer A supplemented with 0.5 M NaCl. The octylglucoside-NaCl-insoluble fraction contained the heat-modifiable OMP, which was subsequently solubilized by incubation for 30 min at ambient temperature in 20 mM sodium phosphate, pH 7.5, containing 1% SDS. Following centrifugation, the supernatant fraction was applied to a hydroxyapatite column equilibrated with 20 mM sodium phosphate, pH 7.5, containing 0.1% SDS. The heat-modifiable OMP was eluted with a linear gradient of sodium phosphate (50 to 400 mM), pH 7.5, containing 0.1% SDS. The protein was dialyzed against distilled H_2O, precipitated, and dissolved in 20 mM phosphate, pH 7.5, containing 0.1% SDS.

Samples of the heat-modifiable OMP were solubilized at ambient temperature or 100°C for 10 min in sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.003% bromophenol blue in 0.063 M Tris hydrochloride, pH 6.8). The samples were subjected to SDS-polyacrylamide gel electrophoresis on modified Laemmli gels (1) containing a 3% stacking gel and a 14% separating gel. Electrophoretic transfer of the proteins onto 0.45-μm-pore-size polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.) was as described by Matsudaira (14), using a

TABLE 1. Amino acid composition of the 29-kDa OMP of *A. actinomycetemcomitans* Y4

Amino acid	mol% in 29-kDa OMP
Cys	1
Asp	10
Glu	15
Ser	8
Gly	21
His	1
Arg	5
Thr	3
Ala	10
Pro	3
Tyr	3
Val	6
Met	2
Ile	3
Leu	5
Phe	2
Lys	3

semidry electroblotter (Janssen Life Sciences Products, Piscataway, N.J.). The membranes were washed in distilled H_2O and stained with Coomassie brilliant blue R250.

The electroblotted proteins were submitted to the UCLA Protein Microsequencing Facility for determination of amino acid composition and N-terminal amino acid sequence analysis. Amino acid composition was determined by using the Pico-Tag system (Waters/Millipore Corp., Bedford, Mass.) after hydrolysis in 6 N HCl. Cysteine and methionine contents were determined after performic acid oxidation of the samples. N-terminal sequencing was determined by Edman degradation with a Porton PI 2090E protein sequencer.

Samples of sarcosyl-insoluble outer membrane or isolated 29-kDa OMP were also solubilized at ambient temperature or 100°C for 10 min, electrophoresed, and electroblotted onto nitrocellulose membranes (0.2 μm). The membranes were subsequently probed with either normal rabbit serum or rabbit antiserum against the isolated OmpA protein of *E. coli* K-12, as described previously (22). Briefly, following electrophoretic transfer, the nitrocellulose membranes were blocked for 45 min in 1% bovine serum albumin (BSA) (fatty acid-free) in 20 mM Tris, pH 7.5, containing 0.5 M NaCl (TBS), rinsed in TBS–0.2% Tween 20, and then with TBS. The membranes were incubated overnight at 4°C with either normal rabbit serum or anti-OmpA antiserum diluted 1:500 in TBS-BSA. The membranes were rinsed and incubated for 1 h at ambient temperature with a 1:1,000 dilution of biotinylated goat anti-rabbit immunoglobulin G (Calbiochem, La Jolla, Calif.) in TBS with 1% BSA. The blots were rinsed and incubated for 1 h in the dark at ambient temperature with a 1:1,000 dilution of avidin-conjugated horseradish peroxidase (Bio-Rad, Richmond, Calif.), rinsed, and developed with HRP color development reagent (Bio-Rad).

The OMP isolated from the octylglucoside-NaCl-insoluble fraction of the outer membrane exhibited an apparent mass of 29 kDa (as determined by SDS-polyacrylamide gel electrophoresis) following incubation in sample buffer at ambient temperature. In contrast, solubilization at 100°C resulted in a protein which migrated at an apparent mass of 34 kDa. In order to confirm that the 29- and 34-kDa proteins represent a single molecular species exhibiting heat modifiability, amino acid compositional analysis and N-terminal sequencing were performed. The amino acid composition of the 29-kDa OMP is shown in Table 1. The composition of the 34-kDa protein species was found to be virtually identical. Moreover, the N-terminal sequences of these two proteins were identical, providing strong evidence that these two species represent a single OMP whose electrophoretic mobility is heat modifiable.

S. typhimurium OmpA	APKDNTWYAGAKL
S. dysenteriae OmpA	APKDNTWYTGAKL
E. coli OmpA	APKDNTWYTGAKL
A.actinomycetemcomitans 29 kDa OMP	APQANTFYAGAKA

FIG. 1. Comparison of the amino-terminal amino acid sequences of the OmpA proteins of *Shigella dysenteriae*, *E. coli*, and *Salmonella typhimurium* with that of the 29-kDa OMP isolated from *A. actinomycetemcomitans*.

The sequence of the first 13 amino acid residues of the 29-kDa OMP was Ala-Pro-Gln-Ala-Asn-Thr-Phe-Tyr-Ala-Gly-Ala-Lys-Ala. Comparison of this sequence with published sequences by using the National Biomedical Research Foundation protein data base and the sequence analysis programs (version 6.0 [8]) of the Genetics Computer Group of the University of Wisconsin Biotechnology Center (Madison) revealed that the 29-kDa OMP of *A. actinomycetemcomitans* exhibited significant homology with the OmpA proteins of other species of gram-negative bacteria (Fig. 1). The first 12 residues of the *A. actinomycetemcomitans* OMP showed 75% overlap with the *Salmonella* OmpA sequence (12) and 66.7% overlap with the reported sequences for *E. coli* (3) and *Shigella* (6) OmpA proteins.

Further evidence that the 29-kDa membrane protein of *A. actinomycetemcomitans* is related to the OmpA protein of other gram-negative bacteria derived from the results of immunoblot analysis (Fig. 2). Rabbit antiserum against the OmpA protein of *E. coli* K-12 reacted strongly with a 29-kDa species in the sarcosyl-insoluble membrane fraction following solubilization at ambient temperature and with a 34-kDa protein following solubilization at 100°C. An identical pattern of reactivity was observed with the isolated heat-modifiable OMP from *A. actinomycetemcomitans*. Normal rabbit serum exhibited only weak reactivity toward this protein.

The results of this study indicate that the principal heat-modifiable OMP of *A. actinomycetemcomitans* exhibits N-terminal sequence homology and immunologic cross-reactivity with the OmpA proteins of other gram-negative bacteria. OmpA proteins appear to be strongly conserved through evolution. These proteins have been demonstrated to serve as a receptor for certain bacteriophages (7, 20), to stabilize mating aggregates formed during F pilus-mediated conjugation (18, 20), and to confer stability to the outer membrane (19). Whether the heat-modifiable OMP of *A. actinomycetemcomitans* serves a similar function has not been determined.

The presence of bacteriophages capable of infecting *A. actinomycetemcomitans* has been associated with periodontal lesions which were actively breaking down in children with localized juvenile periodontitis (16) and prepubertal periodontitis (17). In contrast, periodontal sites which were clinically stable typically lacked such phages. This has

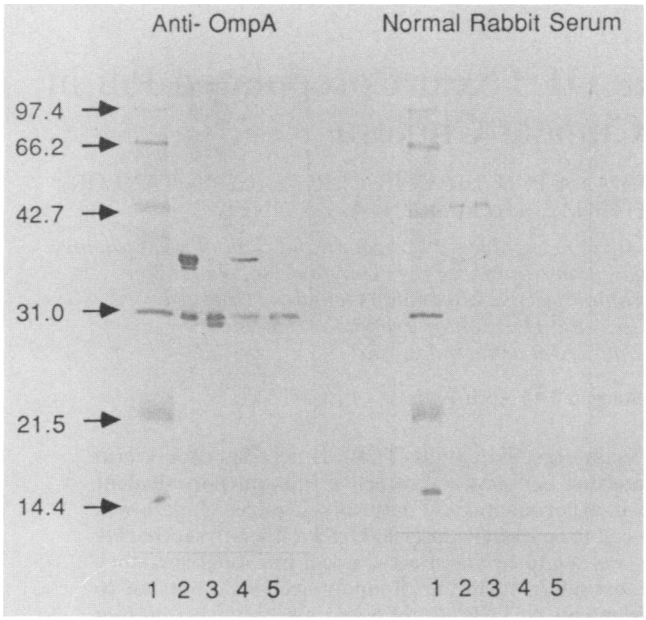

FIG. 2. Reactivity of the heat-modifiable OMP of *A. actinomycetemcomitans* with polyclonal rabbit antiserum against the purified OmpA protein of *E. coli* K-12. The lanes of each panel contained the following: lane 1, biotinylated molecular mass standards; lane 2, sarcosyl-insoluble outer membrane fraction solubilized at 100°C; lane 3, sarcosyl-insoluble outer membrane fraction solubilized at ambient temperature; lane 4, isolated 29-kDa OMP from *A. actinomycetemcomitans* solubilized at 100°C; lane 5, the same protein solubilized at ambient temperature. Molecular masses (expressed in kilodaltons) are indicated on the left.

engendered speculation that phage infection may augment the periodontopathic potential of *A. actinomycetemcomitans*. These observations have prompted current efforts to define the role of the heat-modifiable OMP of *A. actinomycetemcomitans* as a receptor for such bacteriophages.

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