Ultrastructural Analysis of the Rugose Cell Envelope of a Member of the Pasteurellaceae Family

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Bacterial membranes serve as selective environmental barriers and contain determinants required for bacterial colonization and survival. Cell envelopes of Gram-negative bacteria consist of an outer and an inner membrane separated by a periplasmic space. Most Gram-negative bacteria display a smooth outer surface (e.g., Enterobacteriaceae), whereas members of the Pasteurellaceae and Moraxellaceae families show convoluted surfaces. Aggregatibacter actinomycetemcomitans, an oral pathogen representative of the Pasteurellaceae family, displays a convoluted membrane morphology. This phenotype is associated with the presence of morphogenesis protein C (MorC). Inactivation of the morC gene results in a smooth membrane appearance when visualized by two-dimensional (2D) electron microscopy. In this study, 3D electron microscopy and atomic force microscopy of whole-mount bacterial preparations as well as 3D electron microscopy of ultrathin sections of high-pressure frozen and freeze-substituted specimens were used to characterize the membranes of both wild-type and morC mutant strains of A. actinomycetemcomitans. Our results show that the mutant strain contains fewer convolutions than the wild-type bacterium, which exhibits a higher curvature of the outer membrane and a periplasmic space with 2-fold larger volume/area ratio than the mutant bacterium. The inner membrane of both strains has a smooth appearance and shows connections with the outer membrane, as revealed by visualization and segmentation of 3D tomograms. The present studies and the availability of genetically modified organisms with altered outer membrane morphology make A. actinomycetemcomitans a model organism for examining membrane remodeling and its implications in antibiotic resistance and virulence in the Pasteurellaceae and Moraxellaceae bacterial families.

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ifications of the membrane morphology after disruption of the morC gene are accompanied by multiple changes in bacterial physiology (29). Mechanisms dependent on a specific membrane architecture such as toxin secretion (e.g., leukotoxin), sensitivity to salts, hydrophobicity, and normal cell division are the most affected. However, the loss of the rugosity does not seem to influence the presentation of all virulence determinants on the outer bacterial surface. EmaA (extracellular matrix adhesin A), a trimeric autotransporter collagen binding adhesin that forms antenna-like appendages on the surface of the bacterium (13, 22, 30, 31), is present in both wild-type and morC mutant strains, thus indicating that not all outer membrane transport systems are affected. All of these observations raise fundamental questions about the specific role of the outer membrane morphology and the spatial relationship between outer and inner membranes in bacteria with a native rugose phenotype.

In this study, we have characterized the cell surface of A. actinomycetemcomitans from a wild-type and an isogenic morC mutant strain using three-dimensional (3D) electron microscopy of negatively stained whole-mount bacterial preparations and atomic force microscopy. In addition, we determined the spatial relationship between the outer and inner membranes of both strains from ultra-thin sections of high-pressure frozen, freeze-substituted bacterial colonies using a combination of 2D electron microscopy and 3D electron tomography.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A. actinomycetemcomitans strains used in the present study are based on VT1169, an ambrinated strain derived from SUNY 465 (32). The wild-type strain was grown statically in 3% Trypticase soy broth supplemented with 0.6% yeast extract (TSBY; Becton Dickinson, Sparks, MD) at 37°C in a humidified, 10% CO2 atmosphere. The morC mutant strain was grown in TSBYE supplemented with 50 μg of spectinomycin/ml under the same conditions.

2D and 3D transmission electron microscopy of stably stained whole-mount bacterial preparations. A colony of each strain of A. actinomycetemcomitans was grown overnight in 5 ml of culture medium. Cultures were diluted 10-fold and grown for 3 h, which for the wild-type strain corresponds to mid-logarithmic phase (A495 = 0.2). Cells were collected by centrifugation, washed in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride [pH 7.4]), and resuspended in 50 μl of PBS. Then, 5-μl aliquots of the suspension were applied to carbon-coated grids. The grids were rinsed with PBS and subsequently negatively stained with Nano-W (Nanoprobes, Yaphank, NY). For electron tomography experiments, carbon-coated grids were pretreated with 5 μl of polylysine (molecular weight, 2,500 to 4,000, 0.05 mg/ml; Sigma, St. Louis, MO) for 1 min, followed by 5 μl of colloidal gold (SPI, West Chester, PA) for 1 min before the bacterial suspension was added.

Grids were observed using a Tecnai 12 electron microscope (FEI, Hillsboro, OR) equipped with a LaB6 cathode (Kimball Physics, Wilton, NH), a 14-μm 2,048×2,048 pixel charge-coupled device (CCD) camera (TVIPS, Gauting, Germany) and a dual-axis tilt tomography holder (Fischione, Export, PA). The data were recorded on the CCD camera at an acceleration voltage of 100 kV. Tomography tilt series were collected at a nominal magnification of ×42,000, which corresponds to 0.308 nm in the specimen scale, over a ±60° to ±70° angular range in 2° increments. All data were collected under low-dose conditions (10 e−/Å2 for 0° images and below 110 e−/Å2 for each tomography series).

2D and 3D transmission electron microscopy of ultrathin sections from high-pressure frozen, freeze-substituted samples. Bacterial colonies were selected randomly from plates and transferred to planchettes with a diameter of 1.5 mm by 200 μm deep and immediately cryo-immobilized using a Leica EM PACT 2 high-pressure freezing system (Leica, Vienna, Austria) in the absence of cryoprotectants. Planchettes were stored in liquid nitrogen until further use. Frozen samples were freeze-substituted in a Leica EM AFS2 (automatic freeze substitution system), where the substitution was performed in pure acetone containing 1% (wt/vol) osmium tetroxide at −90°C for 72 h. The temperature was gradually increased (5°C/h) to 5°C, held constant for 2 h, and finally increased to room temperature and maintained for 1 h. Samples were washed in acetone at room temperature for 1 h before infiltration with a graded series of Epon-acetone mixtures: 1:3 for 2 h; 2:2 for 2 h, 3:1 for 16 h, and pure Epon 812 (Ted Pella, Inc., Redding, CA) for 30 h. Samples were embedded in fresh Epon and polymerized at 60°C for 48 h. Ultrathin sections (100 to 120 nm) were cut with a diamond knife using an ultramicrotome (Riechert-Jung Ultracut E, Vienna, Austria) and mounted on carbon-coated copper grids. Sections were post-stained with 2% (wt/vol) aqueous uranyl acetate and lead citrate and examined in the electron microscope. The data collection of 2D images and tomography tilt series were performed as described above.

Tomography data analysis. Data were processed using the IMOD software package (33). Briefly, a coarse alignment was first achieved by cross-correlation and a subsequent refined alignment was obtained using fiducial markers. Well-aligned tilt series data were converted to SPIDER format, and 3D tomographic reconstructions were calculated using weighted back projection algorithms in SPIDER (version 5.0, with modifications [34, 35]). Reconstructed tomograms were visualized using the program Chimera (36) and segmented using the Segger package distributed with Chimera (37).

Preparation of bacterial samples for atomic force microscopy (AFM) and cell imaging. Suspensions containing log-phase bacterial cells were centrifuged and gently washed with 150 μl of PBS. The bacteria were resuspended in 50 μl of PBS, and a 5-μl aliquot was applied onto the surface of a freshly cleaved mica disk and allowed to dry overnight at room temperature. Three independent experiments were performed for each bacterial strain. The bacterial cell surfaces were imaged in contact mode using a MFP-3D-BIO AFM (Asylum Research, Santa Barbara, CA) equipped with a piezo scanner (90 by 90 μm). A SiNi cantilever/tip (Bruker, Billerica, MA) with a spring constant of 0.02 N/m, and a resonance frequency of 15 kHz was used for imaging. The data were analyzed with the WSxM 4.0 software package (Nanotec Electronica, Spain) (38).

Statistical analysis. The depth of the grooves on the bacterial surface are presented as means ± the standard deviations (SD) of at least 60 measurements. Statistical analysis of the two means was performed using the Student t test.

RESULTS

Bacterial membranes play an important role in the secretion and presentation of virulence factors on the cell surface. The surface of A. actinomycetemcomitans possesses a rugose appearance when negatively stained whole-mount bacterial preparations are visualized by electron microscopy (Fig. 1A). Convolutions or folds on the outer membrane are easily viewed on higher-magnification images (Fig. 1D). Images of the wild-type strain show grooves on the bacterial surface and also the presence of the collagen binding protein C (MorC), which appears to be located in the inner membrane (29). Inactivation of the morC gene (morC mutant) evoked the loss of convolutions on the bacterial surface and conferred the outer membrane a flat appearance (Fig. 1B). This alteration in membrane morphology affects the secretion of certain virulence factors (e.g., leukotoxin) does not affect the expression of EmaA on the bacterial surface (Fig. 1E). Comple-
mentation of the morC mutant with the full-length morC gene restituted the rugose membrane appearance characteristic of the wild-type strain (Fig. 1C). Compared to the wild-type strain, the complemented strain exhibits an increased number of membrane convolutions (Fig. 1F). Interestingly, the amount of outer membrane vesicles observed in electron micrographs of the different strains correlates with the degree of convolution of the bacterial surface (Fig. 2).

Bacterial membrane convolutions were analyzed by electron tomography of negatively stained whole-mount bacterial preparations and the stain depth was measured in both the wild-type and the morC mutant strains. A total of 18 electron tomography single-axis tilt series were acquired for each of the two strains, and 3D reconstructions or tomograms were calculated (Fig. 3). The view of the wild-type strain tomogram in perspective, along an axis 20° off the imaging axis, evidences the irregular shape of the membrane convolutions on the bacterial surface (Fig. 3A). The tomograms exhibit lighter regions that represent the crests or outer most regions of the convolutions and darker zones that represent the valleys. Dark zones result from stain accumulation in the grooves between the surface convolutions (Fig. 1, 2, and 3).

The depth of the grooves was calculated by examining the Z-slices of the tomogram and recording the position of the Z-slices where stain accumulation starts and finishes. This analysis provided an average groove depth of 16.2 ± 2.2 nm for the wild-type strain. Even though the surface of the morC mutant strain appeared flat in 2D electron micrographs (Fig. 1B and E), convolutions were still observed in the tomograms (Fig. 3B). The curvature of the convolutions was greatly attenuated compared to the wild-type strain, and the measured groove depth for the morC mutant strain was 8.1 ± 1.4 nm. Thus, there is a 2-fold reduction in the groove depth between wild-type and morC mutant strains.

Atomic force microscopy (AFM) is a technique particularly
suited to analyze surface characteristics. In the present study, we used AFM as an independent technique to assess the surface of *A. actinomycetemcomitans* strains (Fig. 4) and at the same time addressed the possibility of preparation artifacts in electron microscopy. 3D images (Fig. 4B) corresponding to the topography images of the wild-type strain (Fig. 4A) revealed the presence of corrugations on the bacterial surface; while the surface of the *morC* mutant strain was almost smooth (Fig. 4A and B). Analysis of section profiles provided detailed information about the vertical and horizontal dimensions of the surface convolutions. In order to measure groove depths, we analyzed 60 and 65 section profiles from the wild-type and the *morC* mutant strains, respectively.

In the wild-type strain, the grooves were 12.4 ± 1.3 nm in depth and about 100 to 150 nm in diameter (Fig. 4C, left panel), and the measured distance between grooves ranged from 65 to 65 nm. In contrast, the distance between grooves of the *morC* mutant strain ranged from 145 to 315 nm, and the groove dimensions were 6.3 ± 0.6 nm in depth and 200 to 250 nm in diameter (Fig. 4C, right panel). These values correspond to approximately half the size of the wild-type strain groove depth and twice the diameter (Fig. 4D). Mean values of the groove depth between the two strains are statistically different with a significance of *P* < 0.001. These data are consistent with the results obtained using electron tomography (Fig. 3).

The structural relationship between the outer and inner membranes still remains an open question. Therefore, we have conducted transmission electron microscopy studies of ultrathin sections of the bacteria after high-pressure freezing fixation and freeze-substitution (HPF-FS) to gain new insights into the topology of the inner and outer membranes. HPF-FS allows for preservation of membranes minimizing possible artifacts that arise when classical chemical fixation protocols are used. 2D micrographs of ultrathin sections (~90 nm) of *A. actinomycetemcomitans* strains show clearly recognizable outer and inner membranes (Fig. 5). Examination of more than 200 micrographs of the wild-type strain revealed a different topology of the inner membrane with respect to the outer membrane. The outer membrane displayed a highly convoluted surface, which is in complete agreement with the results mentioned above (Fig. 5A). Interestingly, the inner membrane did not follow the pattern of the outer membrane convolutions but presented a flat appearance (Fig. 5A). The traits of the outer surface of the *morC* mutant strain (Fig. 5B) correlated well with observations obtained from other techniques used in this study. The topology of the inner membrane shows a smooth appearance most of the time and in rare occasions follows the convolutions of the outer membrane.

2D images of ultrathin sections provide valuable information. However, 3D electron tomography of these samples facilitates a more detailed analysis of the relationship between the outer and inner membranes of *A. actinomycetemcomitans*. We collected 21 to 25 tomography dual-axis tilt series for each strain, from which 3D reconstructions were calculated. It was clear from examining slices of the tomograms that the outer and inner membranes have different topologies in the wild-type strain, showing a convoluted outer membrane and a smooth inner membrane (Fig. 6A). The membranes from sections of the *morC* mutant strain samples analyzed were representatives of the typical topology observed for the outer membrane of this mutant. The inner membrane of this strain shows a smooth appearance (Fig. 6C) reminiscent of the one of the wild-type strain. Computer-assisted manual segmentation of the inner and outer membranes in the tomograms demonstrated not only the topological differences between these two membranes but also revealed connections between the outer and inner membranes in both strains (Fig. 6B and D) that were not apparent in 2D images (Fig. 5). These connections are visible through the series of Z-slices of the region of the tomogram corresponding to the segmented area shown in Fig. 6B (Fig. 7).

Even though the connections are present in both strains, the number of connections per surface area in the wild-type strain is on average 4-fold larger than that in the isogenic *morC* mutant strain. This novel observation has led us to hypothesize that these contact sites may be necessary for maintaining and favoring the presence of convolutions in the *A. actinomycetemcomitans* surface.

**DISCUSSION**

Electron micrographs of the periodontal pathogen, *A. actinomycetemcomitans*, reveal the rugose appearance of the bacterial surface and the propensity of this Gram-negative bacterium to secrete vesicles containing virulence factors such as leukotoxin (22, 29, 39). The high rugosity of the bacterial surface is a trait shared with...
members of the Pasteurellaceae (e.g., *Haemophilus influenzae* [40]) and Moraxellaceae families (e.g., *Moraxella catarrhalis* [23]). Our prior studies demonstrated a strong correlation between membrane convolutions of *A. actinomycetemcomitans* and the presence of morphogenesis protein C (MorC), as revealed by 2D electron microscopy (22, 23, 47). Here, the presence of convolutions on the surface of *A. actinomycetemcomitans* is shown by three independent methods, including HF-FS, currently considered the gold standard for analyzing membranous structures with minimal production of artifacts. Convolutions might be the result of environmental adaptation. Higher organisms develop surface convolutions as a mechanism to increase the surface area for facilitating nutrient uptake necessary for survival (e.g., microvilli). The increase in bacterial surface area due to the convolutions can be assessed by comparing the area of a spherical cap with the area sustained by a disk of the same radius. For the calculation, the diameter of the spherical cap is the distance between grooves and the height is the groove depth. The convolutions observed in *A. actinomycetemcomitans* afford the wild-type bacteria an average increase of 5% in surface area with respect to a smooth surface. This increase can be as small as 0.1% for the morC mutant strain and as large as 14% for the smallest distance between grooves observed for the wild-type strain. This modest increase in surface area might be part of the adaptation mechanism of the bacteria and, as discussed later, may have a strong effect on other physical parameters.

Our electron microscopy studies of thin sections of the wild-type and the morC mutant strains of *A. actinomycetemcomitans* indicated that the inner membrane shows a smooth topology. Thus, the convolutions of the outer membrane not only increase the surface area of the bacteria but, in combination with the smooth inner membrane morphology, increase the volume occupied by the periplasmic space. The decrease in the groove depth between the wild-type and the morC strains by half the size reduces...
the surface area of the outer membrane on average by 4.5%. This change can be as large as 20% when the most extreme values for the distance between the grooves for both strains are compared. Changes in the ratio of volume/area contained within the convolution are less sensitive to the value of the distance between the grooves and are mainly driven by the difference in groove depth. The calculations show that the volume/area ratio contained within the convolution in the morC strain has a 2-fold decrease with respect to the wild-type strain. Thus, the wild-type strain bacteria have twice the amount of periplasm per unit area than the morC mutant strain. This results in a reduction of the space available for ions and proteins with a wide variety of functions (e.g., nutrient binding, degradation, transport, folding, peptidoglycan synthesis, electron transport, and modification of toxic compounds to the cell) in the morC mutant strain. This reduction might be responsible for the increased sensitivity to environmental factors such as salts and other phenotypic changes compared to the wild-type strain, including leukotoxin secretion (29).

Leukotoxin is released via the type I secretion system or secreted in vesicles derived from the bacterial outer membrane (39, 48, 49). The morC mutant strain shows reduced leukotoxin secretion and a diminished production of outer membrane vesicles (Fig. 2). These changes correlate with a decrease in outer membrane curvature and a 2-fold reduction of the volume/area ratio of the periplasmic space. In addition, the wild-type strain exhibits a larger surface area for the outer membrane, which would allow the bacteria to accommodate more lipids in the outer membrane.

Several models have been proposed to account for the effect of membrane curvature on the formation and subsequent release of outer membrane vesicles (for a review, see reference 50). In one of the models, a fast expansion of the outer membrane relative to the peptidoglycan layer causes a dissociation of the latter, which results in bulging of that specific region of the outer membrane (51). A second model emphasizes the effect of turgor pressure on generating membrane curvature. According to this model, the accumulation of peptidoglycan intermediates in the periplasm produces turgor pressure that leads to outer membrane blebbing. In a third model, the asymmetric distribution of lipids (LPS) across the membrane leaflets, responsible for the increase in membrane curvature, finally results in membrane blebbing. The present data cannot exclude any of the proposed models and appear to indicate that all of the models may be involved in the formation and subsequent secretion of outer membrane vesicles. Since membrane biosynthesis and degradation occur during bacterial growth and the membrane is remodeled under different physiological conditions, it is important to further study the mechanism underlying membrane remodeling and its implications in antibiotics resistance and virulence. In the wild-type A. actinomycetemcomitans, a protein like MorC may play an important role in modulating membrane shape by coordinating membrane composition and morphology.

Interactions between different components of the cell envelope have been reported to affect membrane stability and morphology (52; for a review, see reference 53). Detachment and reattachment of these connections have been associated with the release of outer membrane vesicles. These contact sites were originally reported by Bayer (54) and were denoted as “Bayer’s junctions” or “Bayer’s bridges.” However, the existence of contact sites between the two membranes was challenged and considered an artifact of the sample preparation (44). Even though in 1991 Bayer demonstrated their presence by analyzing plasmolyzed E. coli samples from cryofixed and freeze-substituted preparations (41), their existence has

![FIG 5](http://jb.asm.org/)

**FIG 5** Transmission electron micrographs of ultrathin sections from A. actinomycetemcomitans strains prepared by high-pressure freezing and freeze substitution. (A) Wild-type strain; (B) morC mutant. Arrows indicate the inner and outer membranes separated by the periplasmic space. White arrows in the middle column point to the regions of the images that have been magnified four times and are shown in the last column of each row. IM, inner membrane; OM, outer membrane. Bar, 100 nm.
remained controversial since they are not easily observed in 2D images of sectioned material. We have observed connections between the outer and inner membranes during our study of 3D electron tomography of thin sections of *A. actinomycetemcomitans* strains (Fig. 6 and 7). These sites might be similar to the zones of adhesions, the so-called Bayer’s junctions, and thus our results, specifically the visualization and segmentation of the electron tomograms, support the existence of membrane adhesion sites. There might be reasons why the presence of connections has escaped visualization in nonplasmolyzed bacteria since most of the analyses were carried out on 2D projections. The 0° projection of the tilt series does not show clear connections between the inner and the outer membranes. However, they are clearly visible in our 3D studies, thus underlining the importance of 3D electron microscopy (3DEM) as an essential tool for the unambiguous visualization of fine details of membrane ultrastructure.

The connection sites span small regions of the periplasmic space in both wild-type and morC mutant strains of *A. actinomycetemcomitans*. The main difference between the strains is the reduced number of connections observed in the morC mutant strain compared to the wild-type strain. Based on the molecular mass and predicted tertiary structure, MorC could span the periplasmic space bridging the inner and outer membranes, either as a monomer, or in combination with other protein partners. Our results indicate that MorC plays a role in the stability of the connections, assuming the connections are made from the same identical group of proteins. Alternatively, if more than one type of connection exists, MorC could be involved in the connections that are missing in the isogenic mutant. Future experiments combining electron tomography and immunolocalization should be performed to elucidate the role of MorC in outer membrane morphology.

In summary, this is the first time that the surface of a Gram-negative bacterium has been studied using this wide combination of techniques: 3DEM and AFM of whole-mount bacterial preparations and 3DEM of ultrathin sections of HPF-FS samples. This comprehensive study of *A. actinomycetemcomitans* strains has revealed the convoluted appearance of the outer membrane of the Pasteurellaceae and Moraxellaceae families of bacteria. It has also evidenced that the convolutions are not a preparation artifact but represent the morphology of the outer membrane of these bacteria in their native state, which differ from other more commonly studied bacterial species. The present studies and the availability of genetically modified organisms with altered outer membrane morphology make *A. actinomycetemcomitans* a model organism for examining the mechanism of membrane remodeling and its

**FIG 6** Electron tomography and segmentation of the membrane of *A. actinomycetemcomitans* strains in ultrathin sections after high-pressure freezing and freeze substitution. Central slices of a tomogram from the wild-type (A) and morC mutant (C) strains and segmentation of a small area of the inner and outer membranes from the tomograms of the wild-type (B) and morC mutant (D) strains are shown. The membranes clearly separated by periplasmic space are indicated by arrows. IM, inner membrane; OM, outer membrane. Bar, 100 nm.
implications in antibiotics resistance and virulence in the Pasteurellaceae and Moraxellaceae bacterial families.

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