Methyltransferases*§

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**Background:** Methylation of OmpB has been implicated in rickettsial virulence.

**Results:** Native OmpBs purified from Rickettsia contain mono- and trimethyllysine at specific locations that coincide with those catalyzed by methyltransferases in vitro.

**Conclusion:** The number of trimethyllysine clusters in OmpBs correlates with degree of virulence.

**Significance:** This study provides new insight into methylation of OmpB and its correlation with virulence.

Methylation of rickettsial OmpB (outer membrane protein B) has been implicated in bacterial virulence. Rickettsial methyltransferases RP789 and RP027-028 are the first biochemically characterized methyltransferases to catalyze methylation of outer membrane protein (OMP). Methylation in OMP remains poorly understood. Using semi-quantitative integrated liquid chromatography-tandem mass spectroscopy, we characterize methylation of (i) recombinantly expressed fragments of Rickettsia typhi OmpB exposed in vitro to trimethyltransferases of Rickettsia prowazekii RP027-028 and of R. typhi RT0101 and to monomethyltransferases of R. prowazekii RP789 and of R. typhi RT0776, and (ii) native OmpBs purified from R. typhi and R. prowazekii strains Breinl, RP22, and Madrid E. We found that in vitro trimethylation occurs at relatively specific locations in OmpB with consensus motifs, KX[G/A/V/I]N and KT/[L/F], whereas monomethylation is pervasive throughout OmpB. Native OmpB from virulent R. typhi contains mono- and trimethyllysines at locations well correlated with methylation in recombinant OmpB catalyzed by methyltransferases in vitro. Native OmpBs from highly virulent R. prowazekii strains Breinl and RP22 contain multiple clusters of trimethyllysine in contrast to a single cluster in OmpB from mildly virulent R. typhi. Furthermore, OmpB from the avirulent strain Madrid E contains mostly monomethyllysine and no trimethyllysine. The native OmpB from Madrid E was minimally trimethylated by RT0101 or RP027-028, consistent with a processive mechanism of trimethylation. This study provides the first in-depth characterization of methylation of an OMP at the molecular level and may lead to uncovering the link between OmpB methylation and rickettsial virulence.

Rickettsiae are obligatory intracellular infectious Gram-negative bacteria that are responsible for major rickettsiosis, which includes epidemic and endemic typhus, spotted fever, and scrub typhus (1, 2). Like all Gram-negative bacteria, rickettsiae contain several outer membrane proteins (OMPs) that are found in the outer leaflet of the outer membrane. OMPs provide the first line of communication with the extracellular environment, with prominent roles in molecular transport and bacterial infection and pathogenesis (3, 4). Rickettsial OMPs have been shown to participate in host cell attachment, invasion, internalization, and intracellular movement (5–7) and induce strong host humoral and cellular immune responses (8–10).

OMP (outer membrane protein B) is an OMP that is present in all species of Rickettsia and belongs to the family of OMPs called autotransporters. The precursor of OmpB consists of a signal peptide, a large N-terminal passenger domain and a C-terminal β-barrel domain (11, 12). The passenger domain of rickettsial OmpB has been shown to participate in adhesion to mammalian cells in vitro, suggesting this may be the role of OmpB in the virulence of Rickettsia (5, 6, 13).

It has been known for many years that the passenger domain of OmpB of Rickettsia is methylated, and OmpBs from virulent strains are more extensively methylated (14, 15). More recent studies using genetic and biochemical approaches are consistent with the suggestion that methylation of OmpB may contribute to both (i) the host immunogenic response to OmpB itself and (ii) rickettsial virulence (16–18). Methylation of OmpB appears to enhance its antigenicity. For example, rabbit antiserum against recombinant OmpB is less reactive than antiserum against OmpB purified directly from Rickettsia (19). In addition, the immunoreactivity of chemically methylated recombinant OmpB is enhanced against sera from infected patients (20). Thus, methylation of recombinant OmpB could increase the efficacy of diagnostic reagents and help advance vaccine development against Rickettsia (21).

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Methylation Profiles of OmpB in Rickettsia

Protein methylation is a well established post-translational modification known to regulate functions of various proteins (22, 23), frequently through modulating protein–protein interactions (24, 25). The regulatory potential of this modification can be attributed in part to the multiple states of methylation at the \( \varepsilon \)-amino group of lysine, which can be mono-, di-, and trimethylated (26). Unlike the methylation of histones, which has been well established, methylation of outer membrane proteins is not well characterized, particularly at the molecular level.

We have recently characterized two rickettsial lysine methytransferases (MTs) (27). They are RP789 (PKMT1) and RP027-028 (PKMT2) from *Rickettsia prowazekii*. RP027-028, as well as its counterpart in *Rickettsia typhi*, RT0101, catalyzes trimethylation, and RP789 catalyzes monomethylation of OmpB fragments, monitored by incorporation of radioactive [methyl-3H] from [methyl-3H]AdoMet and Western blot analysis, whereas none of these MTs can methylate either histone or *Escherichia coli* protein. These are the first biochemically characterized MTs to catalyze the methylation of OMPs and appear to belong to a novel family of protein lysine MTs. The gene sequences of homologous MTs are found to be conserved in more than 40 rickettsial species and strains. The fact that these MTs recognize OmpB at many diverse sites suggests an unusual substrate recognition mechanism. Characterization of methylation in rickettsial OmpB at the molecular level is essential for elucidating the roles of methylation in bacterial virulence and pathogenesis. Moreover, findings on the rickettsial OmpB methylation may advance our understanding of the structure and function of protein lysine methylation in general. In addition, enzymatic methylation of recombinant OmpB may also provide improved methods for chemical methylation in efforts to advance diagnostic reagents and vaccine candidates.

To better understand OmpB methylation, we used semi-quantitative integrated LC-MS/MS techniques to characterize the location, state and level of methylation. Our study provides the first characterization of methylation in an OMP and lays the foundation for further understanding how methylation of OmpB may contribute to the virulence of Rickettsia.

**MATERIALS AND METHODS**

**OmpB Proteins**—Native OmpB proteins from *R. typhi* and *R. prowazekii* strains Breinl, RP22, and Madrid E were extracted using 10 mM Tris-HCl (pH 7.6) at 45 °C for 30 min and purified as described previously (28). Each of the native OmpB proteins showed a single band at \( M_\text{r} \) 114,000 on SDS-polyacrylamide gel. Recombinant *R. typhi* OmpB fragments OmpB(AN) and OmpB(K), which corresponded to residues Met\(^ {31} \) to Phe\(^ {744} \) and Arg\(^ {745} \) to Gly\(^ {1353} \), respectively, were expressed and purified as previously described (20). Briefly, the bacterial proteins that were expressed in inclusion bodies were dissolved in 8 M urea and 1% deoxycholate, purified by ion exchange chromatography on DEAE-cellulose in 6 M urea. Soluble proteins were obtained by slow dialysis at stepwise decreasing concentrations of urea. No methylated lysine in recombinant OmpB(AN) and (K) were detected by LC-MS/MS. Protein concentrations were determined using Bio-Rad protein assays with bovine serum albumin as the standard.

**Rickettsial MTs**—Purified recombinant *R. prowazekii* RP789 (NP221139) and RP027-028 (ADE29537) and *R. typhi* RT0776 (YP067714) and RT0101 (YP067069) were used in enzymatic methylation of OmpB fragments in the present study. The MTs were prepared by the same method as previously described (27). No methyllysines in recombinant MTs were found as determined by LC-MS/MS.

**Deletions of N-terminal Sequences of RP789 and RT0776**—The plasmids expressing RP789AN (deletion of N-terminal 28 residues) and RT0776ΔN (deletion of N-terminal 27 residues) were constructed in pET28a by subcloning PCR-amplified DNA using the plasmids encoding RP789 and RT0776, respectively, as templates. Primer sequences are available on request. The constructs were confirmed by sequencing. Expression and purification was performed as described previously (27).

**Radioactivity Assay of MT Activity**—The standard assay mixture contained, in 50 \( \mu \)l of final volume, 8.3 mM sodium phosphate (pH 8.0), 0.16 mM [methyl-3H]AdoMet (34 mCi/mmol diluted from 10 Ci/mmol [methyl-3H]AdoMet, which has a >97% purity obtained from PerkinElmer, with the highest purity AdoMet purchased from New England Biolabs) and 2 \( \mu \)M OmpB(AN) or 1 \( \mu \)M OmpB(K). The reactions were initiated by adding MT to a final concentration of 0.26 \( \mu \)M and incubated at 37 °C. Aliquots of the reaction mixture were spotted at the indicated time onto Whatman 3MM cellulose filter paper discs (Fisher Scientific) and soaked briefly with 5% TCA. The paper discs were washed three times with 5% ice-cold TCA, followed by washing with ethanol ether (1:1 by v/v) mixture. The amounts of acid precipitable radioactivity were determined using a PerkinElmer Wallace 1410 liquid scintillation counter. The assay conditions were optimized by varying pH, DTT, KCl, and cations reduce the activity of MT, and none of them was included in the assay.

**Kinetic Analysis of the MTs**—Initial rates of MT-catalyzed reactions were determined from the linear portions of the time courses of methylation at varying concentrations of OmpB(AN) up to 2 \( \mu \)M using the radioactivity assay at 37 °C. These initial rates were used to determine the Michaelis-Menten and catalytic constants. The reactions were initiated by the addition of the specified MT to a final concentration of 0.26 \( \mu \)M. Michaelis-Menten constants and maximum velocities were obtained by direct fit using KaleidaGraph. It should be noted that the initial rates thus determined represent the sum of initial rates of numerous enzymatic methylation reactions at multiple lysine residues in OmpB(AN) to three different methylation states whose rates may vary with wide ranges. The Michaelis-Menten and catalytic constants based on the radioactivity assay can only be considered as apparent Michaelis-Menten and catalytic constants.

**Preparation of Proteins for LC-MS/MS Analysis**—OmpB(AN) (10 \( \mu \)g) and OmpB(K) (5 \( \mu \)g) were methylated separately using 10 \( \mu \)g of specified MT in 50 \( \mu \)l of reaction mixtures containing 3.2 mM AdoMet (New England Biolabs) and 8.3 mM sodium phosphate (pH 8.0). After overnight incubation at 37 °C, the reaction mixture was evaporated to 20 \( \mu \)l using SpeedVac and mixed with SDS sample buffer. The proteins were separated by SDS-PAGE, and OmpB(AN) and (K) protein bands were excised from the gel and subjected to in-gel digestion. Native
OmpB proteins (2 μg each) were separated by SDS-PAGE, excised from the gel, and processed for in-gel digestion. Multiple samples of enzymatically methylated rOmpB and native OmpB proteins were independently prepared and analyzed using LC-MS/MS.

**In-gel Digestion**—In-gel digestion of proteins was carried out as described with modifications (29). Briefly, the excised protein bands from SDS gels were washed using 50% methanol and 5% acetic acid, followed by reduction using 10 mM DTT. The protein samples were alkylated with 100 mM iodoacetamide in the dark. The gel pieces were dehydrated using acetonitrile and rehydrated with 100 mM (NH₄)HCO₃ twice. The gel pieces were mixed with 1 μg of sequencing grade chymotrypsin (Roche Applied Science) in 50 mM (NH₄)HCO₃ and digested overnight at 25 °C. The digested peptides were extracted with 50% (v/v) acetonitrile and 5% (v/v) formic acid. The volume was reduced to less than 20 μl by evaporation, and the final volume was adjusted to 20 μl using 1% formic acid. The samples were purified using Zip-Tip with C₁₈ resin (Millipore, Billerica, MA) according to the manufacturer’s protocol.

**LC-MS/MS**—LC-MS/MS was performed using an Eksigent nanoLC-Ultra two-dimensional system (Dublin, CA) coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific, San Jose, CA). Peptide sample was first loaded onto a Zorbax 300SB-C₁₈ trap column (Agilent, Palo Alto, CA) at a flow rate of 6 μl/min for 9 min and then separated on a reversed phase BetaBasic C₁₈ PicoFrit analytical column (New Objective, Woburn, MA) using a 40-min linear gradient of 5–35% acetonitrile in 0.1% formic acid at a flow rate of 250 nl/min. Eluted peptides were sprayed into the mass spectrometer equipped with a nano-spray ionization source. Survey MS spectra were acquired in the Orbitrap at a resolution of 60,000. Each MS scan was followed by six data-dependent MS/MS scans in the linear ion trap with dynamic exclusion. Other mass spectrometry settings were as follows: spray voltage, 1.5 kV; full MS mass range, m/z 300–2000; and normalized collision energy, 35%.

Data files generated from the mass spectrometer were analyzed using Proteome Discoverer v1.3 software (Thermo Scientific) and the Mascot search engine running on a six-processor cluster at the National Institutes of Health (version 2.3). The search criteria were set to: database, Swiss-Prot (Swiss Institute of Bioinformatics); taxonomy, Bacteria; enzyme, chymotrypsin; maximal cleavages, 3; variable modifications, methylation (K), dimethylation (K), trimethylation (K), oxidation (M), and deamidation (N, Q); fixed modifications, carbamidomethylation (C); peptide precursor mass tolerance, 25 ppm; and MS/MS fragment mass tolerance, 0.8 Da. Peptide-spectrum matches (PSM) were filtered to achieve an estimated false discovery rate of 1%.

Data analysis was performed using Eksigent Proteome Discoverer v1.3 software. The Mascot search engine was used to search the National Institutes of Health (version 2.3) taxonomy database for Bacteria. The enzyme used for the searches was chymotrypsin. The database was searched for a maximum of three missed cleavages and a peptide mass tolerance of 25 ppm. The PSM filter was set to a false discovery rate of 1%.

**RESULTS AND DISCUSSION**

**Kinetics of Methylation by Rickettsial MTs**—The kinetic parameters of OmpB from R. typhi in E. coli were determined using a steady state approach. However, recombinant OmpB (rOmpB) fragments OmpB(AN) and OmpB(K), which correspond to the N- and C-terminal halves of OmpB passenger domain (Fig. 1B), can be successfully purified and refolded to yield soluble protein substrates for analysis. Using the OmpB(AN) as the substrate of MTs, we analyzed the steady state kinetics of two MTs from R. prowazekii (RP789 and RP027-028) and two MTs from R. typhi (RT0766 and RT0101). Table 1 summarizes the steady state kinetic parameters based on the initial rates of the methylation of OmpB(AN) catalyzed by 0.26 μM each of the four MTs by monitoring the incorporation of radioactive [methyl-3H]AdoMet to OmpB(AN). Monomethyltransferases RT0776 and RP789 exhibit an appreciably higher kcat than
those of trimethyltransferases RT0101 and RP027-028. In addition, the enzyme efficiency ($k_{cat}/K_m$) of RP789 is higher than that of RT0776, whereas RT0101 is more active than RP027-028.

To determine methylation profiles in rOmpB, OmpB(AN) and OmpB(K) were enzymatically methylated using 2 μM RT0101 and 3.2 mM AdoMet. After overnight incubation at 37 °C, the methylated OmpB(AN) and OmpB(K) were separated using SDS-PAGE followed by in-gel digestion and LC-MS/MS analysis. The numbers of PSM at four methylation states of all lysine residues in OmpB(AN) and OmpB(K) are shown in supplemental Table S1. The LC-MS/MS analysis revealed a total of 133 trimethyllysine-containing PSM distributed among 16 locations in OmpB. In contrast, nine monomethyllysine-containing PSM and one dimethyllysine-containing PSM were observed (supplemental Table S1). The predominance of trimethyllysine-containing PSM clearly shows that RT0101 is functioned mainly as a trimethyltransferase. The observed mono- and dimethylations could conceivably represent intermediates of trimethylation. Normalized fractions of trimethylation at all lysine residues in rOmpB were calculated for RT0101-catalyzed methylation (Fig. 2C).

The overall schemes of LC-MS/MS analysis (A) and OmpB and recombinant OmpB(AN) and OmpB(K) (B). A, the state, location, and normalized fraction of methylation in native OmpB and in enzymatically methylated rOmpB fragments catalyzed by rickettsial MTs were determined by LC-MS/MS analysis according to the outlined scheme. See “Materials and Methods” for details. B, the full-length R. typhi OmpB precursor consists of a signal peptide (amino acids 1–32) in red, a passenger domain (amino acids 33–1353) in green, and an autotransporter domain (amino acids 1354–1645) in blue. The corresponding residue numbers in R. typhi OmpB for recombinant OmpB(AN) and OmpB(K) are shown.
analysis reveals the presence of two motifs: (i) KX(G/A/V/I)/H9013 (present at Lys205, Lys232, Lys279, Lys667, Lys711, and Lys723) and (ii) KT(I/L/F) (present at Lys226, Lys232, Lys624, Lys635, and Lys667). The sequence at Lys232 and Lys667, KTIN, conforms to both motifs. The remaining sequences do not occur more than three times among the 16 sequences that are trimethylated by
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RT0101. It should be noted that our preliminary results show that RT0101 is unable to catalyze methylation of two synthetic 20-mer peptides, which contain either one or both of these conserved trimethylation motifs (data not shown), indicating that the conformation of OmpB or other unknown factors may also contribute to substrate recognition.

Supplemental Table S1 shows that RT0101 catalyzed methylation yields a disproportionally low number of PSM, which contains mono- and dimethylated lysine at 9 and 1, respectively, relative to 133 PSM for trimethylated lysine. This observation indicates that the trimethylation reaction likely proceeds via a processive instead of a distributive mechanism. With the former mechanism, the monomethyllysine formed remains enzyme-bound, and the release of product occurs when the lysine is fully trimethylated. This notion is supported by the time courses, monitored during the first 2 h, of the methylation of 2 µM OmpB(AN) catalyzed by 2 µM RT0101. The time course showed the PSM of trimethylated peptides increased steadily to 13, 28, and 56 at 30, 60, and 120 min, respectively, whereas the numbers of PSM of monomethylated peptides were 1, 1, and 6 at 30, 60, and 120 min, respectively. Thus, these data indicate that RT0101-catalyzed trimethylation of OmpB may proceed via a processive mechanism.

LC-MS/MS analysis of RT0776-catalyzed methylation of OmpB(AN) and OmpB(K) was also performed. As shown in supplemental Table S2, the total number of PSM containing monomethyllysine is 379 distributed among 34 lysine residues of a total 45 lysines, although the number of PSM containing di- and trimethyllysines is 6 and 2, respectively. The observation of extensive and predominant monomethylation (Fig. 2D) clearly indicates that RT0776 is mainly functioned as a monomethyltransferase that possesses an active site that can accommodate a wide variety of recognition sequences.

R. prowazekii RP789- and RP027-028-catalyzed Methylation of OmpB—Unlike R. typhi, R. prowazekii causes epidemic typhus, which is transmitted by lice. MTs from heterologous species may produce products different from those catalyzed by enzymes from homologous species. To characterize the methylation of OmpB catalyzed by MTs from the heterologous species, we analyzed methylation of R. typhi OmpB using R. prowazekii RP027-028 and RP789, which possess 94 and 93% identities to their corresponding R. typhi orthologs RT0101 and RT0776, respectively. The locations and the PSM numbers of methylated peptides in OmpB catalyzed by RP027-028 are summarized in supplemental Table S1, which are very similar to those observed with the methylation of OmpB catalyzed by RT0101 (Fig. 2C). Similarly, the locations and PSM numbers of methylated peptides in OmpB catalyzed by RP789 are shown in supplemental Table S2, which reveals 743, 47, and 46 PSM with mono-, di-, and trimethyllysines, respectively. These results show that RP027-028 and RP789 function essentially as a monomethyltransferase and monomethyltransferase, respectively, agreeing with the results observed for their R. typhi orthologs. However, unlike RT0776, RP789 catalyzes mono-, di-, and trimethylation to significantly higher normalized fractions than those catalyzed by RT0776, likely because RP789 and OmpB are from two different species, whereas RT0776 and OmpB are from the same species (Fig. 2D and supplemental Table S2). The appre-
of a highly efficient in vitro trimethylation system appeared to be contrary to the observation that a high concentration of MTs is required to achieve an appreciable level of methylated rOmpB. This observation is consistent with the presence of product inhibition because of a slow release of the trimethylated lysine from the MT. However, the presence of cellular proteins or factors, which may interact with the methylated OmpB, would shift the equilibrium toward the methylated form of OmpB by facilitating the release of methylated lysine from its MT complex and/or reducing the population of methylated lysine-MT complex. Alternatively, the reduction of methylated MT population can also be accomplished via subcellular translocation (e.g., cytoplasm or periplasmic space), an effect of allosteric factors that could facilitate the post-translational modification. In addition, it is possible that in vivo methylation could occur before or during folding of the OmpB passenger domain where methylation sites could be more accessible than in the case of the fully folded OmpB.

Methylation in Native OmpBs from Virulent and Avirulent Strains of R. prowazekii—We next examined the methylation in native OmpBs from three strains of R. prowazekii (Madrid E, RP22, and Breinl). R. prowazekii Madrid E is known to be avirulent, whereas R. prowazekii RP22 and Breinl are both highly virulent. PSM numbers of peptides in the three native OmpBs as determined by LC-MS/MS are shown in supplemental Table S5. This table shows that the total numbers of PSM of trimethyllysine-containing peptides in native OmpB from Madrid E, RP22, and Breinl are 0, 271, and 139, respectively (supplemental Table S5). The absence of trimethyllysine in R. prowazekii Madrid E is consistent with the fact that the gene encoding RP027-028 is interrupted by a frameshift mutation, which generates the inactive RP027 and RP028 fragments (17, 27). Fig. 5 shows that the high normalized fraction of trimethylation

occurs at specific locations in OmpBs from R. prowazekii strains RP22 and Breinl, and the high normalized fraction of monomethylation occurs in OmpB from R. prowazekii strain Madrid E, which is devoid of any trimethylation. The location, the state, and the normalized fraction of trimethylation and monomethylation in OmpBs from the two strains RP22 and Breinl are remarkably similar (Fig. 5, A and C). The correlation coefficient of the normalized fractions of trimethylation in native OmpB and those catalyzed by RT0101 is 0.90.

The amino acid sequences at trimethylation sites abide closely to the recognition motifs found earlier based on the in vitro methylation of rOmpB catalyzed by RT0101 and RP027-028. For example, Lys\textsuperscript{130} (KILN), Lys\textsuperscript{204} (KIVN), and Lys\textsuperscript{231} methylation of rOmpB catalyzed by RT0101 and RP027-vitro closely to the recognition motifs found earlier based on the native OmpBs from strains Breinl and RP22 is 0.90.

The amino acid sequences at trimethylation sites abide closely to the recognition motifs found earlier based on the in vitro methylation of rOmpB catalyzed by RT0101 and RP027-028. For example, Lys\textsuperscript{130} (KILN), Lys\textsuperscript{204} (KIVN), and Lys\textsuperscript{231} (KTIN) in OmpB of R. prowazekii conform to the KXG\textsubscript{A/V/I}N motif (Fig. 2C). No other motifs were found using Prosit in ExPASy (33). Similar to R. typhi OmpB, OmpBs from R. prowazekii strains RP22 and Breinl contain a cluster of highly trimethylated lysine residues at Lys\textsuperscript{623}, Lys\textsuperscript{634}, Lys\textsuperscript{666}, Lys\textsuperscript{710}, and Lys\textsuperscript{722}. However, an additional cluster of highly trimethylated sites occurs at Lys\textsuperscript{210} to Lys\textsuperscript{231} and two doublets of highly trimethylated lysine residues in Lys\textsuperscript{309} to Lys\textsuperscript{814} with the trimethylation normalized fraction reaching nearly 100\% at these sites. The amino acid sequences of OmpBs from R. prowazekii and R. typhi differ at these lysine residues and may account for the additional trimethyllysine in R. prowazekii. It is known that R. prowazekii strains RP22 and Breinl are highly virulent, whereas R. typhi is mildly virulent (34, 35).

Both RP789 and RP027-028 are active in RP22 and Breinl as shown by the extensive mono- and trimethylation observed in native OmpBs. The nearly 100\% normalized fraction of trimethylation in OmpB from Breinl and RP22 supports the notion that in vivo OmpB trimethylation and monomethylation are catalyzed by trimethyltransferase, likely via a processive mechanism, and by monomethyltransferase, respectively. In the trimethyltransferase-catalyzed reaction, the reaction intermediates monomethyllysine and dimethyllysine remain enzyme-bound. Under this situation, the monomethyltransferase does not participate in the formation of trimethyllysine.

In comparison to the OmpB from R. prowazekii Madrid E, which is devoid of trimethyllysine, OmpB from R. typhi contains a single cluster of highly trimethylated lysines. The observation that trimethylation occurs in OmpBs from virulent strains but not in OmpB from avirulent strain is in agreement with earlier studies based on amino acid composition analysis (14). However, the present LC-MS/MS analyses show the location, state, and normalized fractions of the modified lysine residues. With this method we found multiple clusters of trimethyllysines in the highly virulent R. prowazekii strains RP22 and Breinl, a single cluster of trimethyllysines in the mildly virulent R. typhi, and none in the avirulent R. prowazekii Madrid E. The number of cluster of highly trimethylated lysines in OmpBs clearly correlates with the degree of virulence of the four strains of Rickettsia.

The correlation between the number of trimethyllysine clusters in OmpBs with the degree of virulence of the four different rickettsial strains suggests that most likely the trimethyllysine clusters in OmpB are associated with rickettsial virulence. It is known that the trimethylation of lysines may enhance cation-\(\pi\) electrostatic (36) and charge independent interactions (37). For example, trimethylation in calmodulin has been shown to modulate NAD kinase (38). Similarly, trimethylation was shown to promote interaction with polynucleotides (39) and participate...
in histone lysine methylation-mediated chromatin remodeling (40). A number of trimethyllysine-binding domains have been found in recent years (41). Association of methylated H3K9 with HP1 mediates the condensation of nucleosomes to heterochromatin in gene silencing (42). Thus, the presence of cluster of trimethyllysine in a given protein would elevate its valency and significantly enhance its affinity for protein-protein interaction. Additionally, multiple trimethyllysine clusters could further enhance protein-protein interactions. Therefore, it will be of interest to identify the putative rickettsial and human proteins that interact with the trimethyllysine clusters in OmpB that in turn may lead to additional molecular links on rickettsial virulence. It should be noted that the three-dimensional structure of OmpB, either from crystallographic study or from molecular modeling analysis, is not known at present. Thus, we cannot address the methylation profile in term of the structural features of OmpB.

**OmpB Purified from the Avirulent Strain Madrid E Is Minimally Methylated by RT0101 and RP027-028**—The observed differences in the normalized fraction of methylation between native OmpBs and in vitro methylated rOmpB prompted us to ask whether the native OmpBs can be further methylated by MTs in vitro. We chose native OmpB of *R. prowazekii* Madrid E as the substrate that contains predominantly monomethyllysine and is devoid of trimethyllysine. We observed that native OmpB from Madrid E is minimally methylated by either RT0101 or RP027-028 (Fig. 6). Both trimethyltransferases can generate only a few trimethyllysines in native OmpB from Madrid E. Our results show that only Lys^{352} in native OmpB was significantly trimethylated by RP027-028. This observation indicates that the preexisting methylation of lysine in the native OmpB prevents further methylation of these lysine residues to form trimethyllysine that is otherwise readily produced in rOmpB catalyzed by RT0101 or RP027-028. Together, these results are consistent with the earlier suggestion that the OmpB trimethylation catalyzed by RT0101 and by RP027-028 may proceed via a processive mechanism in which its reaction intermediates monomethyllysine and dimethyllysine are present as enzyme-bound complexes.

**Concluding Remarks**—Methylation of OMPs has been implicated in contributing to bacterial virulence and pathogenesis. To investigate this further and to characterize methylation of rickettsial OmpB, we carried out an in-depth analysis comparing methylation profiles in rOmpB catalyzed by four different MTs and in native OmpB purified directly from virulent and avirulent bacteria, showing their profiles agree closely in loca-
tion and state of methylation. Our results suggest that mono-
methylation of OmpB is carried out by monomethyltras-
ferases (RT0776 and RP789), and trimethylation is carried out
by trimethyltransferases (RT0101 and RP027-028). The near
quantitative trimethylation of specific lysine residues found in
native OmpB of virulent Rickettsia is consistent with the exist-
ence of an efficient cellular system of trimethylation of OmpB
in vivo. Unlike typical lysine MTs, rickettsial MTs can recognize
and methylate a diverse set of amino acid sequences. Our study
reveals recognition motifs of rickettsial trimethyltransferases,
which could be used to predict methylation sites in other
OMPs. Additionally, heterologous MTs and N-terminal trunc-
ations of MTs significantly alter methylation profiles pro-
duced in OmpB. Interestingly, the number of cluster of tri-
methyllysines in OmpB correlates with the increasing virulence
of four rickettsial strains. Furthermore, our study reveals that
trimethylation may proceed via a processive mechanism such
that monomethylation in OmpB could have an antagonistic
effect on trimethylation.

In summary, our study provides the first characterization of
methylation of OMPs from Gram-negative bacteria. The new
findings on OmpB methylation may bring forward the develop-
ment of new approaches of investigating the plausible link be-
 tween OMP methylation and bacterial virulence and raise
the possibility of targeting MT (43, 44) to advance new ther-
apeutic strategy against Rickettsia.

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