The Stability of Cytadherence Proteins in Mycoplasma pneumoniae Requires Activity of the Protein Kinase PrkC\\(^{\text{\\(\text{W}\)}}\)^{\text{\textdagger}}

Sebastian R. Schmidl, Katrin Gronau, Claudine Hames, Julia Busse, Dörte Becher, Michael Hecker, and Jörg Stülke^{\text{\textasteriskcentered}}

Department of General Microbiology, Institute of Microbiology and Genetics, Georg-August University Göttingen, Germany, and Institut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität, Greifswald, Germany

Received 21 August 2009/Returned for modification 8 October 2009/Accepted 17 October 2009

Mycoplasma pneumoniae belongs to the mollicutes, a group of bacteria that have strongly reduced genomes but that are nevertheless capable of independent life. With only three transcription factors, the regulatory features of these bacteria are very limited. Thus, posttranslational regulation might be important for M. pneumoniae. In addition to the highly specific HPr kinase, the M. pneumoniae prkC gene encodes the serine/threonine protein kinase C. In order to study the function(s) of this kinase, we isolated an M. pneumoniae mutant affected in PrkC. This mutation resulted in nonadherent growth and loss of cytotoxicity. Examination of the phosphorylation profile of the prkC mutant suggested that phosphorylation of cytadherence proteins was affected by the loss of this kinase. In contrast, inactivation of the prpC gene affecting the protein phosphatase that antagonizes PrkC-dependent phosphorylation resulted in more intensive phosphorylation of the cytadherence proteins HMW1 and HMW3 of the major adhesin P1 and of the surface protein MPN474. Moreover, loss of PrkC affects not only the phosphorylation state of the cytadherence proteins but also their intracellular accumulation. However, the expression of the corresponding genes was not affected by PrkC, suggesting that PrkC-dependent phosphorylation results in stabilization of the cytadherence proteins. The HMW proteins and P1 are part of the so-called terminal organelle of M. pneumoniae that is involved in gliding motility, cell division, and adhesion to host epithelial tissues. Our observations suggest that the posttranslational modification of cytadherence proteins by PrkC is essential for the development and function of the M. pneumoniae terminal organelle.

\[^{\}\text{\textdagger}\text{\textdagger}}\text{Supplemental material for this article may be found at http://iai.asm.org/}\

\[^{\text{\textasteriskcentered}}\text{Corresponding author. Mailing address: Department of General Microbiology, Institute of Microbiology and Genetics, Georg-August University Göttingen, Grisebachstr. 8, 37077 Göttingen, Germany. Phone: 49 551 393781. Fax: 49 551 393808. E-mail: jstuelek@gwdg.de.}\

\[^{\text{\textdagger}}\text{Published ahead of print on 26 October 2009.}\\]
HP r(Ser-P) in M. pneumoniae requires the protein phosphatase PrpC, the product of the MPN247 gene (18). This gene is clustered with another gene that potentially encodes a protein kinase (PrkC). This gene cluster is conserved in all firmicutes, i.e., in gram-positive bacteria with a low GC content of their genomic DNA (this group includes the mollicutes). In other firmicutes, PrkC has been shown to be a protein kinase involved in many functions, such as phosphorylation of glycolytic enzymes, virulence, and germination (11, 25, 26, 30). However, the potential protein kinase PrkC of M. pneumoniae has so far not been the subject of any studies.

To date, phosphorylation of HP r by the HP r kinase is the best-studied protein phosphorylation event in M. pneumoniae (3, 16, 18, 27, 31). However, in addition to HP r, several other proteins are phosphorylated in these bacteria. Among these proteins are HMW1 and HMW2, large cytadherence proteins that were shown to be phosphorylated on serine and threonine residues (9, 24). Recently, a proteomic approach was used to identify phosphorylated proteins in M. genitalium and M. pneumoniae (33). This study identified 18 phosphoproteins in M. pneumoniae, among them the surface protein MPN474, the cytadherence protein HMW3, and several metabolic enzymes. Unfortunately, none of the previously identified phosphoproteins was found by the phosphoproteome approach. Until now, it has not been known which kinases are responsible for these phosphorylation events. Moreover, the function of these phosphorylations has not been addressed.

In this work, we have studied the function of PrkC in M. pneumoniae by genetic and proteomic methods. The analysis of a prkC mutant revealed that this kinase is required for adherent growth of the cells on solid surfaces and for cytotoxicity toward eukaryotic cells. Moreover, we provide evidence that PrkC-dependent protein phosphorylation is crucial for the stability of a group of high-molecular-weight cytadherence proteins.

MATERIALS AND METHODS

Bacterial strains, oligonucleotides, and growth conditions. The M. pneumoniae strains used in this study were M. pneumoniae M129 (ATCC 29342) in the 32nd broth passage and its isogenic mutant derivatives GPM11 (prkC::mini-Tn, Gmr) (18), GPM70 (MPN247::mini-Tn, Gmr) (19). The oligonucleotides used in this study are listed in Table S1 in the supplemental material. Flamingo signals were detected using a Typhoon 9400 image scanner. Relative quantification of the phosphosignals intensities was performed using Image J software version 1.42 (1).

Protein identification by MS. Gel pieces were washed twice with 200 μl 20 mM NH₄HCO₃/30%/ (vol/vol) acetonitrile for 30 min at 37°C and dried in a vacuum centrifuge (Concentrator 5301; Eppendorf). Trypsin solution (10 ng/ml trypsin in 20 mM ammonium bicarbonate) was added until gel pieces stopped swelling, and digestion was allowed to proceed for 16 to 18 h at 37°C. Peptides were extracted from gel pieces by incubation in an ultrasonic bath for 15 min in 20 μl high-performance liquid chromatography-grade water and transferred into micro vials for mass spectrometric analysis. Peptides were separated by liquid chromatography and measured online by electrospray ionization mass spectrometry (MS) using a nanoAcquity ultra-performance liquid chromatography system (Waters, Milford, MA) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were desalted onto a trap column (Symmetry C18; Waters) with a binary gradient of buffer A (0.1% [vol/vol] acetic acid) and buffer B (100% [vol/vol] acetonitrile, 0.1% [vol/vol] acetic acid) over a period of 80 min with a flow rate of 400 nL/min. The LTQ Orbitrap was operated in data-dependent tandem MS (MS-MS) mode using multistage activation for phospho-relevant masses. Proteins were identified by searching all MS-MS spectra in protein identification, and their genomic DNA (this group includes the mollicutes). In other mollicutes, i.e., in gram-positive bacteria with a low GC content of their genomic DNA (this group includes the mollicutes). In other mollicutes, i.e., in gram-positive bacteria with a low GC content of their genomic DNA.
promoter sequence (see Table S1 in the supplemental material). The quantification was performed using Image J software version 1.42 (1).

HeLa cell cytotoxicity assay. HeLa cells were grown in 24-well plates with 2.5 × 10^4 cells per well in 700 μl Dulbecco modified Eagle medium for 24 h at 37°C and 5% CO2. The M. pneumoniae cultures were grown for 96 h at 37°C. The M. pneumoniae cells were then washed three times with 67.6 mM HEPES, pH 7.3; 140 mM NaCl; and 7 mM MgCl2. The M. pneumoniae cells were resuspended with a 0.4- by 20-mm needle. Depending on the size of the pellet, they were resuspended in 5 to 8 ml buffer. The cell suspensions were adjusted to an A550 of 0.1 and centrifuged for 5 min at 10,000 rpm at 4°C. The pellet was resuspended in 125 μl of modified Hayflick medium with a 0.4- by 20-mm needle. The cells were then pipetted onto the lawn of HeLa cells and incubated for 2 h at 37°C and 5% CO2. Then the supernatant was removed and replaced by 700 μl Dulbecco modified Eagle medium, and the cells were incubated. The cytotoxicity assays were performed in triplicate.

RESULTS

Isolation of an M. pneumoniae prkC mutant. To get more insights into the function of the protein kinase PrkC (MPN248), we attempted to isolate prkC mutants. This was done using “haystack mutagenesis” (18). This strategy is based on an ordered collection of pooled random transposon insertion mutants that can be screened for junctions between the transposon and the gene of interest due to transposition insertion. Sixty-four pools containing 2,976 individual mutants (18) were used in a PCR to detect junctions between the prkC gene and the minitransposon using the oligonucleotides CH35 and SH29 for prkC and the minitransposon, respectively (Fig. 1A). For one pool that gave a positive signal, colony PCR with the 50 individual mutants resulted in the identification of one prkC mutant. The presence of the transposon insertion in prkC was verified by Southern blot analysis using a prkC-specific probe (Fig. 1B). To test whether this strain contained only a unique transposon insertion, we did another Southern blotting using a probe specific for the aac-aphD resistance gene present on the minitransposon. As shown in Fig. 1B, only a single band hybridizing with this probe was detected; moreover, this fragment had the same size as that of the XhoI fragment hybridizing to the prkC probe (Fig. 1B). The isolated prkC mutant strain was designated GPM11. The position of the transposon insertion in the prkC gene of M. pneumoniae GPM11 was determined by DNA sequencing. The prkC gene was disrupted between nucleotides 313 and 314, resulting in a truncated protein of 106 amino acids, with one additional amino acid and the following stop codon encoded by the inserted minitransposon. The disruption destroyed the kinase domain of PrkC (amino acids 80 to 256), suggesting that no active PrkC is present in the mutant strain.

Implication of PrkC in adherent growth and cytotoxicity. For an initial characterization of the prkC mutant strain GPM11, we observed growth of this strain in modified Hayflick medium. A comparison of the growths of the wild type and the prkC mutant strain revealed that both strains grow at the same rate in the presence of glucose as the carbon source (data not shown). However, in contrast to its isogenic wild type, GPM11 was unable to attach to the surface of the culture flask, and the cells grew as a suspension in liquid medium.

This loss of adherent growth suggested that the prkC mutant might also be impaired in cytotoxicity. To assess the cytotoxicity of the prkC mutant strain, we infected confluently grown HeLa cell cultures with M. pneumoniae cells (multiplicity of infection of 2). The cytotoxicity of the prkC mutant was compared to that of wild-type strain M129 and M. pneumoniae GPM68, which is affected in prpC, the protein phosphatase that is thought to be the antagonist of PrkC. As shown in Fig. 2, the HeLa cells underwent lysis after 6 days upon infection with wild-type M. pneumoniae. In contrast, a large portion of viable cells was observed after infection of the cell culture with prkC mutant GPM11. In contrast, cytotoxicity induced by prpC mutant strain GPM68 was equivalent to that of the wild-type strain (Fig. 2B). These data clearly demonstrate that PrkC is required for host cell damage, whereas PrpC is not. This suggests that PrkC-dependent protein phosphorylation is an important factor for cytotoxicity.

Identification of proteins that are subject to PrkC-dependent phosphorylation. The loss of adherent growth and cytotoxicity in the prkC mutant suggested that proteins that are phosphorylated by PrkC might be involved in these functions. To identify such proteins, we separated whole-cell extracts of M. pneumoniae by SDS-PAGE. The proteins were consecu-
tively stained with Pro-Q Diamond, a dye for the detection of Ser/Thr/Tyr-phosphorylated proteins, and with Flamingo fluorescence stain for the detection of the total proteins. For this analysis, we compared the wild-type strain M129 and the prkC mutant strain GPM11. As a control, we used the prpC mutant strain GPM68. Since PrpC is thought to be the antagonist of PrkC, we expected to observe a more intense phosphorylation of GPM68. Since PrpC is thought to be the antagonist of PrkC, we decided to analyze specifically the phosphorylation pattern of large proteins by using 6% SDS-polyacrylamide gels. A comparison of the total protein patterns in the wild type and the prkC mutant strain revealed that the quantity of four proteins was severely reduced (Fig. 3A). These protein bands were identified by MS as the three high-molecular-weight proteins HMW1, HMW2, and HMW3 and the major adhesin P1. The amounts of these four proteins in the prpC mutant were indistinguishable from those in the wild type, suggesting that PrkC is necessary for their accumulation in the cell. The quantitative analysis of the Pro-Q Diamond signal intensities for the detection of phosphorylated proteins revealed that the bands of these proteins were absent or much weaker in the prkC mutant (Fig. 3B and C). In contrast, the phosphorylation signals of HMW1, HMW2, and P1 were more intense in the prpC mutant than in the wild type (Fig. 3C). This observation indicated the accumulation of phosphorylated protein that cannot be dephosphorylated by the cognate protein phosphatase. In turn, this suggests that these proteins are targets of PrkC-dependent phosphorylation and that phosphorylation plays a role in their accumulation. In addition, one protein with an apparent molecular mass of 160 kDa accumulates in the prpC mutant and shows also a new very strong phosphorylation signal in this mutant (Fig. 3B). This protein is the acidic surface protein MPN474.

Impact of PrkC on the accumulation of cytadherence proteins. It has been shown that HMW2 mutants of M. pneumoniae are impaired in the accumulation of other cytadherence proteins, such as HMW1, HMW3, and MPN309 (P65) (6). Since the amounts of the HMW proteins were reduced in the prkC mutant, we determined the cellular amounts of other cytadherence proteins by use of specific polyclonal antibodies raised against these proteins (Table 1 provides a summary of the results). As controls, we used the elongation factor G (FusA) and the MPN567 (P200) protein, which is required for gliding motility (23). The amounts of both proteins were virtually identical in all strains tested in this study (Fig. 4C).

First, we studied the proteins encoded by one gene cluster containing the gene encoding HMW2 (the so-called P65 operon). As shown in Fig. 4A, the cellular amounts of all these proteins are significantly reduced in the prkC mutant. In fact, the HMW2 protein and HMW2-S (also called P28), a second translation product of the same mRNA (7), are nearly completely absent in the prkC mutant strain. The accumulation of all the proteins encoded by the gene cluster is not affected by a disruption of the prpC gene (Fig. 4A). Thus, the phosphorylation of these proteins seems to be essential for their accumulation. The so-called HMW gene cluster encodes the HMW1, HMW3, and MPN453 (P30) proteins. As observed for HMW2, the two high-molecular-weight proteins encoded by this gene cluster are much less abundant in the prkC mutant, whereas none of the other tested mutations had any effect. The accumulation of MPN453 was also somewhat reduced, but to a lesser extent (Fig. 4B). Finally, we assayed the amounts of the

FIG. 2. Cytotoxicity of M. pneumoniae toward HeLa cell cultures. (A) Infection assay with the M. pneumoniae prkC::Tn strain GPM11 and the prpC::Tn strain GPM68. HeLa cell culture without M. pneumoniae (left top; control); HeLa cell culture incubated with wild-type M. pneumoniae (right top; wt); HeLa cell culture incubated with the prkC or prpC mutant (bottom; prkC::Tn or prpC::Tn, respectively). After 6 days, HeLa cell cultures were stained with crystal violet and photographed. Scale bar, 0.1 mm. (B) Quantification of HeLa cells after infection with wild-type M. pneumoniae or the prkC or prpC mutant. The cell count of surviving cells is indicated as the number of viable cells per field of view, quantified by crystal violet staining after 6 days of incubation. An uninfected HeLa cell culture served as a control. Error bars indicate the standard deviation (based on three independent experiments).
major adhesins MPN141 (P1) and MPN142 (P40) and of the surface protein MPN474, which was shown to be phosphorylated by PrkC (Fig. 3). As shown for the large cytadherence proteins, many fewer adhesins were detected in the prkC mutant than in the wild type or all other strains. In contrast, the cellular amounts of MPN474 were not affected by the prkC mutation. As expected, the antibodies do not cross-react with any protein in the MPN247 mutant strain GPM70.

Transcription of the genes encoding cytadherence proteins in mutants affecting protein phosphorylation. The virtual absence of cytadherence proteins in the prkC mutant can be explained by two alternative scenarios: (i) the expression of the corresponding genes and the accumulation of their mRNAs might require some kind of PrkC-dependent protein phosphorylation and (ii) the proteins might be translated to a lesser extent or be subject to rapid degradation. To distinguish between these possibilities, we compared the amounts of the mRNAs of the cytadherence genes in the wild-type strain M129 and in our set of mutants that are affected in protein (de)phosphorylation. For this purpose, we isolated RNA from cultures grown in modified Hayflick medium supplemented with glucose and performed slot blot analyses.

For the P65 operon, we observed similar amounts of mRNA for the two proximal genes of the operon, MPN309 (encoding P65) and hmw2 (encoding HMW2) in the wild type and in the kinase and phosphatase mutants (Fig. 5A). This observation is in sharp contrast to the strongly reduced amounts of the corresponding proteins in the prkC mutant (Fig. 4A). For the two distal genes of the operon, MPN311 (P41) and MPN312 (P24), we observed a slight reduction of mRNA amounts in the prkC
Table 1. Summary of Western blot analyses with a prkC::Tn mutant (GPM11)

<table>
<thead>
<tr>
<th>M. pneumoniae locus tag</th>
<th>SwissProt accession no.</th>
<th>Protein</th>
<th>Signal in Western blot (%)</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPN141</td>
<td>P11311</td>
<td>P1</td>
<td>35</td>
<td>Adhesin</td>
</tr>
<tr>
<td>MPN142</td>
<td>Q50341</td>
<td>P40</td>
<td>40</td>
<td>Involved in cytodherence</td>
</tr>
<tr>
<td>MPN227</td>
<td>P75544</td>
<td>FusA</td>
<td>100</td>
<td>Elongation factor G</td>
</tr>
<tr>
<td>MPN309</td>
<td>P53663</td>
<td>P65</td>
<td>9</td>
<td>Adhesin-related protein</td>
</tr>
<tr>
<td>MPN310</td>
<td>P75471</td>
<td>HMW2</td>
<td>0</td>
<td>Cytodherence high-molecular-wt protein 2</td>
</tr>
<tr>
<td>MPN311</td>
<td>P75470</td>
<td>P41</td>
<td>8</td>
<td>Adhesin-related protein</td>
</tr>
<tr>
<td>MPN312</td>
<td>P75469</td>
<td>P24</td>
<td>41</td>
<td>Adhesin-related protein</td>
</tr>
<tr>
<td>MPN447</td>
<td>Q50365</td>
<td>HMW1</td>
<td>20</td>
<td>Cytodherence high-molecular-wt protein 1</td>
</tr>
<tr>
<td>MPN452</td>
<td>Q50360</td>
<td>HMW3</td>
<td>22</td>
<td>Cytodherence high-molecular-wt protein 3</td>
</tr>
<tr>
<td>MPN453</td>
<td>P75330</td>
<td>P30</td>
<td>34</td>
<td>Adhesin</td>
</tr>
<tr>
<td>MPN474</td>
<td>P75310</td>
<td>MPN474</td>
<td>104</td>
<td>Coiled-coil protein, putative structural protein involved in cytoskeleton</td>
</tr>
<tr>
<td>MPN567</td>
<td>P75211</td>
<td>P200</td>
<td>96</td>
<td>Involved in cytodherence</td>
</tr>
</tbody>
</table>

The protein amount in the prkC mutant relative to that in the wild-type strain (100%) is indicated.

Discussion

Protein phosphorylation is a major mechanism to control the activities of proteins. This posttranslational modification has been observed with all organisms for which it has been studied, including the minimal bacteria M. genitalium and M. pneumoniae (33; this work). However, the physiological consequences of protein phosphorylation events in bacteria, as well as the responsible kinases, have remained largely unknown.

So far, only a few classes of serine/threonine protein kinases have been identified and characterized for bacteria. M. pneumoniae contains two of these protein kinases, HPrK and PrkC (31; this work). While HPrK is generally thought to phosphorylate only one specific substrate, the HPr protein of the phosphotransferase system, a variety of substrates have been proposed for PrkC and its homologues in different Gram-positive bacteria. In Bacillus subtilis, PrkC was shown to phosphorylate the elongation factors Tu and G, the small-ribosome-associated GTPase CpgA, and the potential stressosome component YezA (2, 30). PrkC is required for germination of B. subtilis spores in response to muropeptides (30). The extracellular PASTA domain of PrkC senses these muropeptides (30). Since M. pneumoniae neither sporulates nor possesses a peptidoglycan cell wall, it is not surprising that the PrkC protein of this bacterium does not contain a PASTA domain. Thus, it is safe to assume that M. pneumoniae PrkC has a function(s) that differs completely from that described for B. subtilis. Indeed, this work provides evidence that PrkC is required for adhesive growth of M. pneumoniae, and a prkC mutant has lost cytoxicity. This is reminiscent of observations that have been made with several other pathogenic firmicutes. In Streptococcus pyogenes, the homolog of PrkC is required for adherence to host cells and for invasion (21). Similarly, the kinase of Enterococcus faecalis is implicated in persistence in the intestine of mice (25). In contrast, PrkC of Staphylococcus aureus was reported to phosphorylate glycolytic enzymes (26).

While the importance of PrkC-dependent protein phosphorylation is well established for many bacteria, the molecular consequences of the primary phosphorylation events have not been studied so far. Most often, protein phosphorylation results in a shift of a protein’s activity (22).

In this work, we observed that PrkC-dependent phosphorylation of large cytodherence proteins is required for the stability of these complexes. It is unknown which of these proteins is responsible for the destabilization of cytodherence proteins in the prkC mutant, but our studies revealed that HMW1, HMW3, the major adhesin P1, and the surface protein MPN474 are subject to PrkC-dependent phosphorylation. This conclusion is supported not only by the absence of these proteins in cell extracts of the prkC mutant but also by the increased intensity of phosphorylation signals for these proteins in the prpC mutant strain. PrpC is the protein phosphatase that reverses PrkC-dependent protein phosphorylation. Thus, our work confirms and extends earlier reports on the phosphorylation of cytodherence proteins (24, 33). We can not exclude, however, the possibility that an additional kinase(s) might phosphorylate HMW3 and P1 (Fig. 3C). It is well established that the cytodherence proteins show reciprocal dependency in their stabilities, i.e., mutations in one of the corresponding genes result in destabilization and delocalization of the other proteins of the attachment structure (5, 28, 35). Specifically, HMW1 was shown to be required for HMW2 and P1 localization and stability. In turn, HMW2 is necessary for the stabilization of HMW3 and P65. As shown in this work, it has been suggested previously that the interdependence of the proteins of the attachment organelle does not involve reduced transcription (28). Taken together, these findings establish that phosphorylation of the HMW proteins by PrkC is a prerequi-
FIG. 4. Western blot analyses of expression profiles of *M. pneumoniae* cytadherence proteins. Whole-cell extracts of the *M. pneumoniae* wild type and different mutant strains were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Cytadherence proteins were detected by using polyclonal rabbit antibodies raised against proteins of the P65 operon (A), the HMW gene cluster (B), and major adhesins (C). A polyclonal rabbit antibody raised against *M. pneumoniae* elongation factor G (FusA) was used as the control. Ten μg of extract was applied to each lane. The names of antibodies are given next to each blot. α, anti. HMW1, HMW2, HMW3, MPN474, P1, and P200 were analyzed by 6% SDS-PAGE, and the other proteins by 12% SDS-PAGE. The graphs show the quantification of Western blot signals relative to that for the *M. pneumoniae* wild type. Error bars indicate the standard deviation (based on three independent experiments). The organization of the different operons is shown by arrows. Note that there are four genes between *hmw3* and *hmw1*, which is indicated (panel B). Moreover, MPN474, P200, and fusA are not part of operons (panel C).
site for their stability and perhaps for their proper localization in the attachment structure. This is the first time that protein phosphorylation was shown to increase the stability of a protein. *M. pneumoniae* has only limited regulatory potential. These bacteria may use, in addition to three potential transcription regulators, protein phosphorylation to adapt to changes in environmental conditions. An analysis of the *M. pneumoniae* genome revealed the presence of two genes for serine/threonine kinases, i.e., *hprK* and *prkC*, encoding the HPr kinase and protein kinase C (PrkC), respectively. Based on our present knowledge, it appears that these two proteins represent the total serine/threonine kinome of *M. pneumoniae*. It will be interesting to study how PrkC activity is triggered and whether other proteins are targets of this pleiotropic protein kinase.

**ACKNOWLEDGMENTS**

We are grateful to Richard Herrmann for the gift of antibodies. Hinnerk Eilers is acknowledged for helpful discussions.

This work was supported by Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. S.R.S. was supported by a personal grant from the Studienstiftung des Deutschen Volkes.

**REFERENCES**

7. Boonmee, A., T. Ruppert, and R. Herrmann. 2009. The gene mpn310 (hmw2) from *Mycoplasma pneumoniae* encodes two proteins, HMW2 and...


Editor: A. Camilli