Prediction of Bacterial Proteins Carrying A Nuclear Localization Signal and Nuclear Targeting of HsdM from *Klebsiella pneumoniae*

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(Received July 16, 2009 / Accepted August 4, 2009)

Nuclear targeting of bacterial proteins is an emerging pathogenic mechanism whereby bacterial proteins can interact with nuclear molecules and alter the physiology of host cells. The fully sequenced bacterial genome can predict proteins that target the nuclei of host cells based on the presence of nuclear localization signal (NLS). In the present study, we predicted bacterial proteins with the NLS sequences from *Klebsiella pneumoniae* by bioinformatic analysis, and 13 proteins were identified as carrying putative NLS sequences. Among them, HsdM, a subunit of *Kpn*AI that is a type I restriction-modification system found in *K. pneumoniae*, was selected for the experimental proof of nuclear targeting in host cells. HsdM carried the NLS sequences, $_7$KKAKAKK$_{13}$, in the N-terminus. A transient expression of HsdM-EGFP in COS-1 cells exhibited exclusively a nuclear localization of the fusion proteins, whereas the fusion proteins of HsdM with substitutions in residues lysine to alanine in the NLS sequences, $_7$AAAKAAA$_{13}$, were localized in the cytoplasm. HsdM was co-localized with importin α in the nuclei of host cells. Recombinant HsdM alone methylated the eukaryotic DNA *in vitro* assay. Although HsdM tested in this study has not been considered to be a virulence factor, the prediction of NLS motifs from the full sequenced genome of bacteria extends our knowledge of functional genomics to understand subcellular targeting of bacterial proteins.

Keywords: nuclear localization signal, DNA methylation, pathogenesis, *K. pneumoniae*

Subcellular targeting of bacterial proteins to the nuclei and the subsequent pathology of host cells are an emerging pathogenic mechanism of bacteria. Several type III secretion system effector proteins, including IpaH9.8 and OspF of *Shigella* species (Okuda *et al.*, 2005; Arbibe *et al.*, 2007), SspH1 of *Salmonella enterica* (Haraga and Miller, 2003), and YopM of *Yersinia* species (Benabdillah *et al.*, 2004), have been known to target the nuclei of host cells. In addition, cytolethal distending toxin from several Gram-negative bacteria (Lara-Tejero and Galán, 2000) and outer membrane protein A of *Acinetobacter baumannii* (Choi *et al.*, 2008) also target the nuclei of host cells. As bacterial proteins are translocated into the nuclei of host cells, they can interact with nuclear molecules, resulting in the alteration of cell physiology or even cytotoxicity (Lara-Tejero and Galán, 2000). Although few bacterial proteins have been found to target the nuclei of host cells so far, bioinformatic analysis predicts many bacterial proteins with the putative nuclear localization signal (NLS) sequences from *Escherichia coli* (Cokol *et al.*, 2000). NLSs are characterized by arginine- and/or lysine-rich sequences and mediates the nuclear translocation of cytoplasmic proteins with the size of >40 kDa (Izaurralde and Adam, 1998; Moroianu, 1998). A complex of cytoplasmic proteins carrying the NLS sequences and importins is transported to the nuclei through the nuclear pore complex (Pemberton *et al.*, 1998).

Klebsiella pneumoniae is a normal commensal, but is an important opportunistic pathogen that causes various types of extraintestinal infections. Clinical isolates of *K. pneumoniae* are usually multi-drug resistant to antimicrobial agents and cause a serious therapeutic problem in clinical settings. This microorganism produces several virulence factors, including antiphagocytic capsular polysaccharides (Cotres *et al.*, 2002), lipopolysaccharides (Shankar-Sinha *et al.*, 2004; Lawlor *et al.*, 2005), and iron acquisition systems (Nassif and Sansonetti, 1986). However, the subcellular targeting of bacterial proteins and subsequent host cell events have not been recognized in *K. pneumoniae*. In the present study, we predicted proteins with the putative NLS sequences from the open reading frames (ORFs) of *K. pneumoniae*, and investigated nuclear targeting and a possible nuclear event of the selected protein HsdM.

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Materials and Methods

Bacterial strains and DNA manipulations

K. pneumoniae was isolated from sputum from a patient who was diagnosed as pneumonia in a Korean hospital and was phenotypically identified by API 20E kit (bioMérieux, France). Bacteria were cultured on blood agar plates or in a Luria-Bertani (LB) broth at 37°C. *E. coli* DH5α and BL21 (DE3) were grown on an LB agar plate or in a LB broth at 37°C. Routine DNA manipulations were performed as described previously (Sambrook *et al.*, 1989) or performed as recommended by the manufacturers of the reagents used. Polymerase chain reaction (PCR) reagents were purchased from Promega (USA). All PCR products were sequenced to verify the fidelity of the PCR using an ABI Prism 3100 Analyzer (Applied Biosystems, USA).

Cell culture

COS-1 cells from African green monkey kidney were purchased from American Type Culture Collections. Cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA), 2 mM L-glutamine, 1,000 U/ml penicillin G, and 50 mg/ml streptomycin at 37°C in 5% CO_2. Cells were seeded in 35 mm dishes or 24-well tissue culture plates to transfect plasmids or to treat recombinant HsdM (rHsdM) proteins.

Construction and expression of HsdM-EGFP fusion proteins

The 1,620 bp of *hsdM* gene was amplified using the primer pairs H-1; 5'-CTGGAATTCATGGCCAAAGCACCAACCA AA-3' and H-2; 5'-CCGTCGACCCTCCCCATAACCCAAC GCCTC-3'. The genomic DNA was isolated from clinical *K. pneumoniae* isolate and used as a PCR template. The PCR products were ligated into a pGEM-T easy vector for PCR cloning (Promega) and transformed into *E. coli* DH5α. The plasmid DNA, from a clone that harbors the full-length of *hsdM* gene, was digested with *Eco*RI and *Sal*I. The *hsdM* gene fragments were ligated into the pEGFP-N2 (Clontech, USA) that had been already digested with the same enzymes. The resulting plasmid DNA was used to transform *E. coli* DH5α. COS-1 cells that were seeded at a density of 5×10^5 per dish were transiently transfected the following day with 1~2 mg/ml of plasmid DNA using Fugene 6 (Roche Applied Science, USA). Cells were fixed with 4% paraformaldehyde and the expression of fusion proteins examined using fluorescence microscopy (Nikon, Japan).

Production of rHsdM proteins and polyclonal anti-mouse HsdM sera

The pGEM-T easy plasmid carrying the full length of *hsdM* gene was digested with *Eco*RI and *Sal*I. The plasmid samples were run on 2% agarose gels and the eluted *hsdM* gene was ligated into the pET28a expression vector (Novagen, USA). *E. coli* BL21 (DE3)/pET28a harboring the *hsdM* gene were grown in a LB medium at 37°C, and recombinant proteins were overexpressed with 1 mM IPTG at 30°C for 4 h. Bacterial cells were sonicated and the supernatant con-

taining the soluble form of rHsdM was collected by centrifugation. The protein samples were loaded onto a 5 ml HiTrapTM FF column (Amersham Biosciences, USA) that was equilibrated with a binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 5 mM imidazole). His-tagged HsdM was eluted by an elution buffer (20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole). After the massive dialysis with the elution buffer without imidazole, the samples were dialyzed with a phosphate-buffered saline (PBS, pH 7.4) to reduce the salt concentrations. The rHsdM was incubated with an endotoxin removal resin (Sigma Chemical Co., USA) overnight and concentrated by Centricon (2000 MW cut-off; Millipore, USA). Polyclonal anti-HsdM antiserum was raised in mice by routine immunogenic procedures (Hanly *et al.*, 1995).

Site-directed mutagenesis

The oligonucleotide mutations in the NLS sequences were performed using a site-directed mutagenesis kit (Stratagene, USA). The mutagenic oligonucleotides in the NLS sequences were GCAGCAGCCAAAGCAGCGGCA encoding AAKAAA, which were generated by a PCR using the primers M-1; 5'-GCCAAAGCACCAACCGCAGCAGCCAAAGCAGCG GCAGGC-3' and M-2; 5'-GTCTTCAAAGCCTGCCGCTG CTTTGGCTGCTGCGGTTGG-3'.

DNA methyltransferase assay

The *in vitro* DNA methyltransferase activity was measured as the incorporation of tritium into the unmethylated DNA using a modified SssI protocol (Suzuki *et al.*, 2006). The CpGenome™ Universal unmethylated DNA (100 ng) (Chemicon, USA) was incubated with 2.5, 5, and 10 μg of rHsdM at 37°C for 4 h in 50 μl of buffer containing 0.75 μM S-adenosyl-S-[methyl-^{3}H]methionine (80 Ci/mM, Amersham Bioscience), 0.75 μM unlabeled S-adenosyl methionin, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. As a positive and negative control for the methylation reaction, the unmethylated and methylated DNA was treated with 4 U of SssI (New England BioLabs, USA) in the same reaction buffer, respectively. Reactions were inactivated by 20 min incubation of the mixtures at 75°C. The methylated DNA was purified using a Geneclean kit (Qbiogene, USA). Purified DNA was dissolved in 200 μl of water and performed scintillation counting. In parallel, a DNA methylation assay was performed using DNA adenine methyltransferase (Dam), which methylates the adenine moieties in GATC sequences.

Confocal microscopy

COS-1 cells were transfected with HsdM-EGFP plasmid on a glass coverslip the day before the assay. After incubation for 24 h, cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized for 10 min with 0.25% Triton X-100 in PBS. HsdM was labeled with polyclonal anti-mouse HsdM antibody, followed by Alexa 568-conjugated goat anti-mouse IgG antibody (Molecular Probes, USA). Importin α was stained with anti-mouse antibody (Molecular Probes). The cellular distribution of HsdM and importin α were observed using a Carl Zeiss confocal microscope (Germany).

Table 1. Proteins carrying the putative nuclear localization signals found in *K. pneumoniae*

NCBI accession no.	Annotation (No. of amino acids)	Putative NLS sequences
AAK92016	Acid shock protein (Asr) (139)	$_{82}$KKHKK$_{86}$, $_{101}$KKHHK$_{105}$
AAC02705	DNA replication terminus site-binding protein (Tus) (310)	$_{226}$RLKIKRPVK$_{234}$
AAB70709	Methyltransferase subunit of type I restriction-modification system (HsdM) (539)	$_{7}$KKAKAKK$_{13}$
AAR07888	Hypothetical ORF in IS2 (301)	$_{39}$DRRRKRK$_{45}$
AAR07861	Hypothetical protein LV008 (122)	$_{82}$KRRRRRK$_{88}$
AAR07809	Hypothetical protein LV177 (214)	$_{39}$DRRRKRK$_{45}$
CAD10421	Cell division protein (MukB) (1479)	$_{1049}$GKKRAR$_{1054}$
AAS55462	ORF1 (248)	$_{202}$KKKKQQIKEKKISK$_{215}$
CAJ13509	ORF3 (297)	$_{133}$DKLKKR$_{138}$
AAO32358	Putative replication protein (RepC) (244)	$_{200}$KKRRQTARK$_{208}$
CAJ29547	Putative transposase (316)	$_{51}$RLRRRPR$_{57}$
CAA08925	Replication protein (RepA) (287)	$_{181}$RKKQKLK$_{187}$
AR07675	Tellurite resistance protein (TerB) (171)	$_{42}$GRYKNKK$_{48}$

Prediction of NLS sequences

The PredictNLS (http://cubic.bioc.columbia.edu) software and modules were installed in Redhat Linux-based workstation computer (Redhat, USA). In addition, we used Perl scripts (O'Reilly Media Inc, USA) and Emboss package software (http://emboss.sourceforge.net) for the automated handling of large sequences. All amino acid sequences related with *K. pneumoniae*, *A. baumannii*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella sonnei*, and *Streptococcus pneumoniae*, were obtained from the NCBI taxonomy browser database (http://www.ncbi.nlm.nih.gov/Taxonomy) and the duplicated data were removed. Rearrangement of amino acid sequences were processed by using Perl script and PredictNLS software. All predicted proteins with the putative NLS sequences were collected.

Results and Discussion

Prediction of bacterial proteins with the putative NLS sequences

The translocation of bacterial proteins to the nuclei of host cells has been first predicted by the presence of NLS sequences in *E. coli* (Cokol *et al.*, 2000). Thereafter, several bacterial proteins were shown to be translocated to the nuclei of host cells, where they induced cytotoxicity through the interactions with nuclear molecules (Lara-Tejero and Galán, 2000; Benabdillah *et al.*, 2004; Okuda *et al.*, 2005; Choi *et al.*, 2008). In this study, we identified 13 *K. pneumoniae* proteins with the predicted NLS sequences out of a total of 5,306 ORFs. Of these, six were sequences of proteins with known functions, whereas the remaining seven were hypothetical ORFs (Table 1). In light of the prediction of proteins with the putative NLS sequences in *K. pneumoniae*, we used the putative NLS sequences to search for proteins in six different pathogenic bacterial species. Bacterial proteins with the putative NLS sequences were most commonly identified in *S. aureus* (n=123), whereas only three proteins were predicted to carry the putative NLS sequences in *S. sonnei*. Other bacterial species, including *A. baumannii* (n=90), *P. aeruginosa* (n=85), *S. pneumoniae* (n=72), and *H. pylori* (n=52), also carried proteins with the putative NLS sequences. These results suggest that the pathogenic bacteria tested carry the potential nuclear-targeting proteins.

Nuclear targeting of HsdM in eukaryotic cells

Of the 13 proteins with NLS sequences from *K. pneumoniae*, we selected HsdM, comprised of 539 amino acids, and proved its nuclear targeting in host cells. It could not be determined if eleven proteins with molecular sizes <40 kDa were targeting the nucleus, because they passively diffused through the nuclear pore complex (Izaurralde and Adam, 1998; Moroianu, 1998). To investigate whether HsdM carried the putative NLS sequences ($_{7}$KKAKAKK$_{13}$) targeting the nucleus of host cells, the full-length hsdM gene was cloned into the pEGFP-N vector in the frame N-terminus of EGFP. The plasmid construct was transfected into COS-1 cells for 24 h and the distribution of fusion proteins was examined by confocal microscopy. The control EGFP proteins were localized in both the cytoplasm and nucleus of host cells (Fig. 1A), whereas the fusion proteins of HsdM-EGFP were exclusively localized in the nuclei of COS-1 cells (Fig. 1B), suggesting that HsdM targets the nuclei of
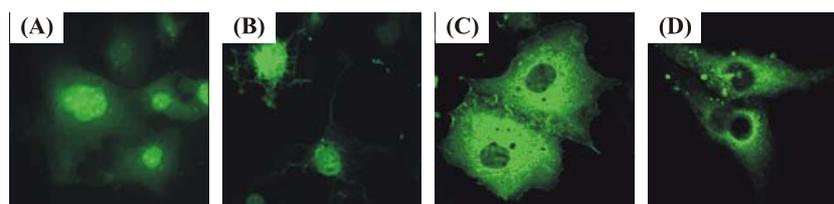
Fig. 1. Nuclear localization of the fusion proteins of HsdM-EGFP. COS-1 cells were transfected with (A) pEGFP-N vector, (B) pEGFP-HsdM$_{1-539}$, (C) pEGFP-HsdMΔNLS, and (D) pEGFP-HsdM-NLS-M ($_{7}$AAAKAAA$_{13}$) for 24 h. Magnification ×200.
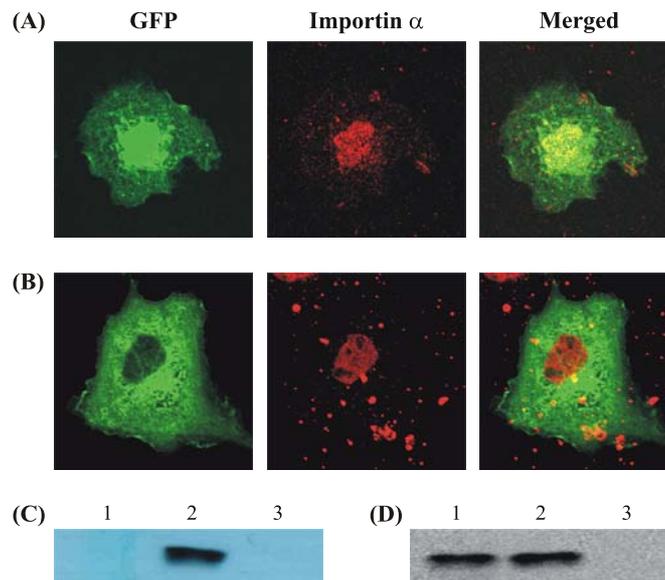
Fig. 2. Importin α mediates nuclear translocation of HsdM. (A and B) Co-localization of HsdM and importin α in the nuclei of COS-1 cell. Cells were transfected with (A) pEGFP-HsdM$_{1-539}$ and (B) pEGFP-HsdM-NLS-M for 24 h, incubated with importin α antibody, followed by Alexa 568-conjugated anti-mouse IgG antibody. Magnification ×200. (C and D) COS-1 cells were transfected with pEGFP-HsdM$_{1-539}$ for 24 h. (C) Cell lysates were immunoprecipitated with anti-importin α antibody and the precipitates were immunoblotted anti-mouse GFP antibody. (D) Cell lysates were subjected to immunoprecipitate with a mouse GFP antibody and the resulting precipitates were immunoblotted anti-importin α antibody. Lanes: 1, the lysates of COS-1 cells were directly loaded in the gels; 2, lysates of COS-1 cells transfected with pEGFP-HsdM; 3, lysates of COS-1 cells transfected with pEGFP.

host cells. To investigate the direct contribution of the NLS sequences to the nuclear targeting of HsdM, the cellular distribution of HsdMΔNLS was observed. The fusion proteins of HsdMΔNLS-EGFP were localized in the cytoplasm of COS-1 cells (Fig. 1C). Site-directed mutagenesis was performed in which four lysine residues were replaced with alanine residues (NLS-M, $_7$AAAKAAA$_{13}$) in the NLS sequences. The fusion proteins of NLS-M-EGFP were localized in the cytoplasm of host cells, but not in the nuclei (Fig. 1D), suggesting that lysine residues in the NLS sequences are responsible for the nuclear targeting of HsdM.

Importins play a role in shuttle for the nuclear translocation of cytoplasmic proteins between the cytoplasm and nucleus (Moroianu, 1998). The nuclear transporter receptors importins recognize the NLS region of cytoplasmic proteins. To determine whether the nuclear translocation of HsdM was mediated by the binding of NLS region of HsdM to importin α, COS-1 cells were transfected with HsdM-EGFP and stained with anti-importin α antibody, followed by Alexa 568-conjugated anti-mouse IgG antibody. The confocal microscopy exhibited the co-localization of HsdM and importin α in the nuclei of host cells (Fig. 2A), whereas the co-localization of the fusion proteins of HsdM-NLS-M-EGFP with importin α was not observed in the merged images (Fig. 2B). To determine whether HsdM was bound to importin α in the cytoplasm of host cells, the lysates of COS-1 cells transfected with HsdM-EGFP were subjected to immunoprecipitate with anti-GFP and anti-importin α antibodies and then the resulting precipitates were immunoblotted with anti-importin α and anti-GFP antibodies, respectively. This revealed that the fusion proteins of HsdM-EGFP were bound to importin α in the cytoplasm (Fig. 2C and D).

Collectively, our results indicated that the nuclear transport of HsdM is mediated by the NLS of HsdM recognized by importin α.

DNA methylation activity of rHsdM

HsdM is a subunit of *Kpn*AI that is a type I restriction-modification system found in *Klebsiella* species (Lee *et al.*, 1997). *Kpn*AI is composed of three different subunits encoded by *hsdS*, *hsdR*, and *hsdM*. A complex of three subunits produces a potent restriction endonuclease activity, whereas a complex of HsdS and HsdM binds to prokaryotic
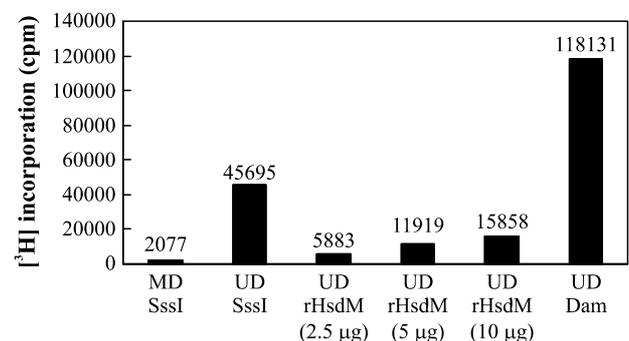
Fig. 3. Quantitative analysis of DNA methylation by rHsdM. Unmethylated DNA (UD) was incubated with various concentrations of rHsdM at 37°C for 4 h in the buffer containing S-adenosyl-S-[methyl-^{3}H] methionine and incorporation of [^{3}H] was determined by scintillation counter. As a positive control, UD was incubated with SssI and Dam in the same reaction buffer. Methylated DNA (MD) was used for negative control. Representative data are shown from three independent experiments which showed similar patterns.

DNA and methylates adenine nucleotides (Lee *et al*., 1997). To investigate whether HsdM could induce molecular or cellular change after nuclear targeting, DNA methylation activity of rHsdM was determined *in vitro* by assessing the incorporation of [^{3}H] methyl groups from S-adenosyl-S-[methyl-^{3}H] methionine into unmethylated DNA. Dam methylase for adenine methylation and Sss1 methylase for cytosine methylation were used as positive controls. Incorporation of [^{3}H] methyl groups increased in the samples of unmethylated DNA incubated with rHsdM in a concentration-dependent manner (Fig. 3). However, incorporation of [^{3}H] methyl groups into unmethylated DNA incubated with rHsdM was not reach to the levels of Sss1 or Dam.

It has been known that a complex of HsdS and HsdM recognizes and methylates 5'-GA^{m6}A(6N)TGCC-3' or 5'-GGC^{m6}A(6N)TTC-3' (Lee *et al.*, 1997; Obarska-Kosinska *et al.*, 2008). However, our results showed that rHsdM alone had the ability to transfer a methyl group in eukaryotic DNA. The WST1 assay determined that the cytotoxicity of host cells by the fusion proteins of HsdM-EGFP was slightly increased at 24 h compared to host cells transfected with the pEGFP-N2 vector, but not significantly different between experimental and control cells (data not shown). These results suggest that HsdM translocated to the nuclei can methylate eukaryotic DNA, but does not induce acute cell damage.

This study is the first experimental proof for the nuclear targeting of bacterial proteins with the NLS sequences predicted by bioinformatics. Although HsdM has not been considered to be a virulence factor of *K. pneumoniae*, the cytosolic HsdM targeted the nuclei of host cells and altered the DNA methylation patterns *in vitro*. HsdM is a cytoplasmic protein in *K. pneumoniae* and its release or secretion is essential to nuclear targeting. HsdM does not carry signal peptides and the secretion of HsdM through general secretion system was not identified. However, it is possible that the HsdM released from bacteria during bacterial lysis binds to the surface of host cells or is phagocytosed by phagocytic cells. In the present study, we could not identify any potential virulence factor with the NLS sequences in *K. pneumoniae*. To extend our understanding of bacterial pathogenesis regarding the nuclear targeting of bacterial proteins, secretion or release of bacterial proteins into extrabacterial milieu, internalization of bacterial proteins in the host cells, and acute or chronic cellular damage should be characterized *in vitro* and *in vivo*. The prediction and identification of bacterial proteins that target the nuclei of host cells can open up a new field of bacterial pathogenesis.

Acknowledgements

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