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Short Communication

Identification of *Chlamydia pneumoniae* candidate genes that interact with human apoptotic factor caspase-9

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***Chlamydia pneumoniae* is an obligate intracellular pathogen responsible for respiratory diseases, including pneumonia and bronchitis, and is highly involved in chronic diseases, including atherosclerosis, asthma, and Alzheimer's disease. We previously showed that the host apoptotic factor caspase-9 played a crucial role for chlamydial multiplication and host apoptosis inhibition by chlamydial infection. To identify chlamydial genes interacting with human caspase-9, yeast two-hybrid screening was performed and 5 chlamydial genes, including Cpj0838 and *pmpG* were isolated from the *C. pneumoniae* genomic library. Pull-down experiments showed that caspase-9 physically bound to the Cpj0838 product and chlamydial cells, which contain PmpG proteins. This study could provide a clue to understanding host-*Chlamydia* interactions, especially the apoptosis repression by *Chlamydia* infection.**

Key Words: apoptosis; caspase-9; *Chlamydia pneumoniae*; Glycogen synthesis; host-parasite interaction; yeast two-hybrid

Abbreviations: EB, elementary body; RB, reticulate body; IFN-γ, interferon-gamma; STS, staurosporine; TNF-α, tumor necrosis factor alpha; kbp, kilobase pair; kDa, kilodalton; Y2H, yeast two-hybrid; Pmp, polymorphic membrane protein; GST, glutathione-S-transferase; PBS, phosphate buffered saline; DTT, Dithiothreitol

Chlamydia pneumoniae is an obligate intracellular bacterium firstly described as a pathogen causing acute respiratory diseases (Grayston et al., 1986). *C. pneumoniae* was isolated from chronic bronchitis patients (Blasi et al., 1998), and may be also involved in other chronic inflammatory processes, such as atherosclerosis (Rosenfeld et al., 2000), asthma (Hahn et al., 1991), and Alzheimer's disease (Balin et al., 1998). *Chlamydia* species show different morphologies and functions in intra- and extra-cellular environments, referred to as a biphasic developmental cycle, i.e., the infectious but metabolically inert elementary body (EB), and the metabolically active but non-infectious reticulate body (RB) (Moulder, 1991). EBs invade into host cells by inducing phagocytosis and differentiate into RBs within inclusions derived from phagosomes. RBs multiply by binary fission with nutrients acquired from the host cell. At the end of the developmental cycle, the RB converts into an EB and the EB is released from the host cell, ready for subsequent infection. In addition, during persistent infection caused by the exposure to interferon-gamma (IFN-γ) or antibiotics, RBs differentiate into aberrantly large and non-multiplying RBs (Belland et al., 2003a).

Apoptosis is a crucial process for the maintenance of homeostasis in multicellular organisms. When mitochondrion-dependent apoptosis is activated by internal stresses such as exposure to staurosporine (STS) and tumor necrosis factor alpha (TNF-α), anti-apoptotic Bcl-2 family proteins, including Bcl-2 and Mcl-1, are inactivated, and pro-apoptotic Bcl-2 family proteins, including Bax, Bid, and Bak, are activated (Tafani et al., 2002). Subsequently, cytochrome *c* is released from mitochondria into the cytoplasm and triggers the activation of caspase-9 by forming an apoptosome with Apaf-1. Activated caspase-9 in turn activates the effector caspase-3 to promote apoptosis (Chowdhury et al., 2006). The elimination of pathogen-infected cells is an important outcome of apoptosis as a primary defense mechanism against intracellular bacteria; however, intracellular pathogens have developed a variety of strategies to evade the immune system by the inhi-

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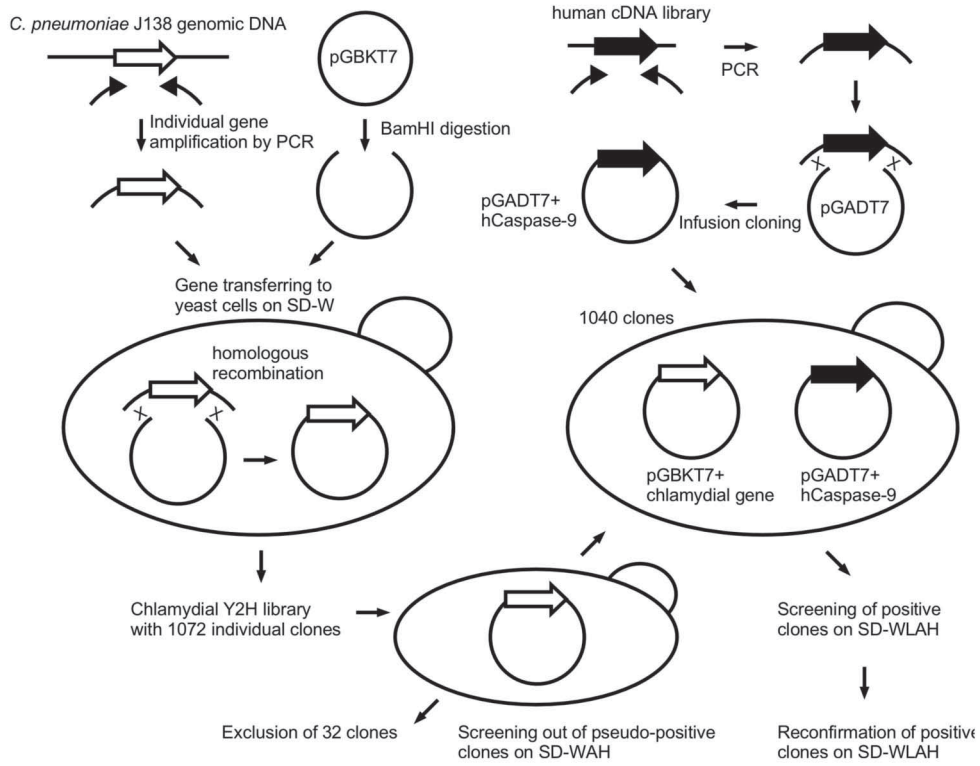

Fig. 1. Scheme of establishment of chlamydial genome library for Y2H and screening of chlamydial genes interacting with human caspase-9.

bition of host cell apoptosis (Gao and Kwaik, 2000). *C. pneumoniae* has been previously reported to inhibit apoptosis induced by STS and TNF-α (Geng et al., 2000; Rahman et al., 2015).

Investigation carried out to clarify the molecular mechanisms modulating host apoptosis using *C. trachomatis* has shown that *Chlamydia* infection prevents cytochrome *c* release and caspase-3 cleavage (Fan et al., 1998). This prevention was explained by specific degradation of the pro-apoptotic BH3-only proteins such as Bad by a chlamydial protease/proteasome-like activity factor (CPAF) (Fischer et al., 2004). However, subsequent studies have shown that the proteolysis of reported CPAF substrates was due to the enzymatic activity in cell lysates rather than in intact cells (Chen et al., 2012).

Instead of degradation of pro-apoptotic factors, a variety of explanations for chlamydial apoptotic repression have been presented, including mislocalization of pro-apoptotic factors and stabilization of anti-apoptotic factors. For example, it was shown that pro-apoptotic Bad was sequestered to *C. trachomatis* inclusion via a host factor 14-3-3β and its inclusion protein IncG (Verbeke et al., 2006), and a pro-apoptotic effector, protein kinase Cδ (PKCδ), was diverted to the inclusion (Tse et al., 2005). *C. trachomatis* deubiquitinating enzyme Cdu1 on the inclusion membrane was shown to interact with, and stabilize, anti-apoptotic Mcl-1 by deubiquitination at the chlamydial inclusion for protection agaisnt host defenses (Fischer et al., 2017). It was shown that infection with *C. trachomatis* and *C. pneumoniae* led to up-regulation and stabilization of the cellular inhibitor of apoptosis 2 (cIAP2), resulting in the inhibition of apoptosis (Wahl et al., 2003). However, *C. pneumoniae* species contain no genes significantly similar to the *C. trachomatis* Cdu1 (data not shown), and the 14-3-3β was not recruited onto the *C. pneumoniae* inclusion (Verbeke et al., 2006). Using cells deficient in anti-apoptotic factors such as cIAP2 and Mcl-1, it was shown that the functions of these proteins are not essential for apoptosis-protection by *C. trachomatis* (Ying et al., 2008).

In our previous study, we found that during *C. pneumoniae* infection, caspase-9 was sequestered to, and accumulated into, the inclusion and, interestingly, it was activated even under conditions lacking Apaf-1, and caspase-9 activation supported infection by *C. pneumoniae* (Rahman et al., 2015). It is possible that there might be many paths involved in the control of host apoptosis by chlamydial infection and each *Chlamydia* species evolutionally alter different paths for their tissue or host tropism. In this research, we constructed a whole genome library of *C. pneumoniae* for yeast two-hybrid (Y2H) screening, and pursued chlamydial genes interaction with caspase-9.

To construct a chlamydial genome library for Y2H screening, DNA fragments coding chlamydial genes were individually amplified by PCR using *C. pneumoniae* J138 genomic DNA as a template (Miura et al., 2008) (Figs. 1 and 2a). In addition to the 20-base gene specific sequences, forward and reverse primers contained extra 20-base sequences, 5′-AGGCCGAATTCCCGGGGATC-3′ and 5′-CCGCTGCAGGTCGACGGATC-3′, respectively, for *in vivo* homologous recombination with pGBKT7 vector at its *Bam*HI site (Clontech/Takara, Mountain View, CA). PCR products and the pGBKT7 vector digested with

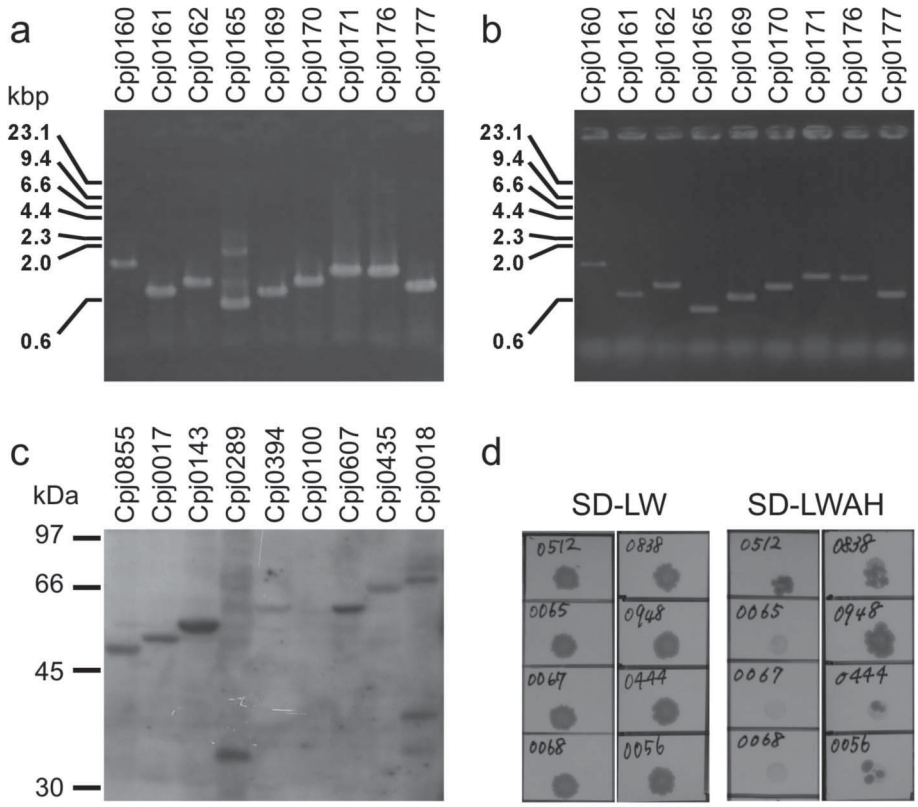

Fig. 2. Preparation of the chlamydial genome library for Y2H.

a) Nine products of PCR using the genomic DNA of *C. pneumonia* J138 as a template are shown as representative findings from the experiment. DNA size markers (kbp, kilo base pairs) are shown on the left. b) PCR products of colony PCR confirmed the insertion of DNA in the pGBKT7 vector in the 1072 yeast strains. Here, the yeast strains containing the same genes as used in panel a are shown. c) Yeast strains were randomly selected and gene expression was analyzed by western blotting using anti-cMyc antibody. Results from only 9 samples are shown as representative findings from the experiment. Protein size markers (kDa, kilo Dalton) are shown on the left. d) Growth of 1040 strains carrying pGADT7-hcaspase-9 and pGBKT7-chlamydial genes was evaluated on a SD medium without Leu and Trp (SD-LW), and without Ade, His, Leu and Trp (SD-LWAH). Here 8 strains are shown.

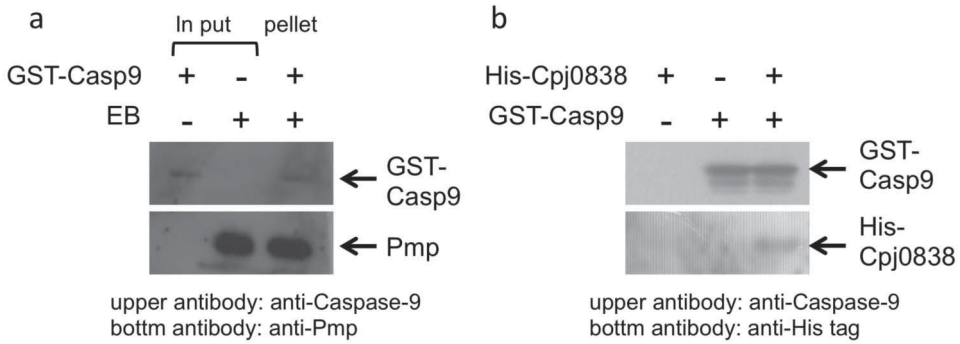

Fig. 3. Direct interaction between human apoptotic factor caspase-9 and *C. pneumoniae* EB and Cpj0838 protein.

a) After mixing with GST-Casp9, EBs were retrieved and analyzed by western blotting, shown as "pellet". Input samples of GST-Casp9 and EBs were separately analyzed, shown as "input". Pmp, a polymorphic membrane protein, on the EBs was detected using the anti-*Chlamydia* Pmp antibody. b) GST-Casp9 and His-Cpj0838 were mixed together and pulled down with glutathione beads. GST-Casp9 and His-Cpj0838 were detected separately.

*Bam*HI were transformed into *Saccharomyces cerevisiae* AH109 cells (Fukunaga et al., 2013). Each yeast strain carrying pGBKT7+chlamydial gene was isolated on SD medium without Trp (SD-W). The sizes of the chlamydial genes in the 1072 yeast strains were confirmed by colony PCR using primers, sequences of which were the same as the extra 20-base sequences for *in vivo* homologous recombination (Fig. 2b). Thirty-two yeast strains were eliminated from the 1072 clones because of the growth on SD medium without Trp, Ade, and His (SD-WAH) as pseudo-

positive clones. Twenty randomly selected strains were subjected to western blotting analysis using an anti-c-Myc mouse monoclonal antibody (Clontech). All samples showed target proteins at expected sizes at detectable levels (Fig. 2c). Here, the chlamydial genome library for Y2H screening has been established.

To construct a bait vector, pGADT7+caspase-9, a human caspase-9 DNA fragment was amplified by PCR using human aorta cDNA library as a template and two primers, 5′-CATCGATACGGGATCATGGACGAAGCGGATCGGC-3′ and 5′-CGAGCTCGATGGATCGGGGCCCTGGCCTTATGAT-3′. The DNA fragment was cloned into a *Bam*HI site of pGADT7 vector using the infusion cloning method (Clontech). After confirmation of the DNA sequence, pGADT7+caspase-9 was individually transformed into the 1040 yeast strains. The growth of transformants was tested on an SD medium without Leu, Trp, Ade and His (SD-LWAH). As a result, 8 chlamydial genes were isolated as candidates. After retrieving the plasmids and re-transformation, 5 genes were confirmed as positive by Y2H (Fig. 2d). The 5 genes are Cpj0444 (*pmpG6* coding a polymorphic membrane protein (Pmp) G, homologous to *C. trachomatis* CT871), Cpj0838 (*mnmE/TrmE* coding tRNA modification GTPase, CT698), Cpj0056 (*pgcA/pgm* coding phosphoglucomutase, CT295), Cpj0948 (*glgA* coding glycogen synthase, CT798), and Cpj0512 (a hypothetical protein, CT425). The 5 gene products do not contain any caspase-9 cleavage sites or domains such as caspase recruitment domains, and their amino acid sequences are not significantly conserved among one another (data not shown).

Each chlamydial genome encodes a variable number of Pmp genes (Azuma et al., 2006). *C. trachomatis* PmpG, the most immunogenic and a major constituent of outer membrane complex (Mygind et al., 2000), is expressed throughout all stages of the developmental cycle (Belland et al., 2003b). Cpj0444 is a paralogue of 15 *pmpG* family genes in the genome of *C. pneumoniae* J138 (data not shown). To clarify whether caspase-9 can bind to the chlamydial outer membrane, a pull-down experiment was carried out using recombinant caspase-9 and the EBs of *C. pneumoniae* J138. Human caspase-9 DNA was amplified by PCR using pGADT7+caspase-9 as a template and two primers, 5′-TTTGGATCCCATATGGACGAAGCGGATCGGCG-3′ and 5′-AAAGTCGACGGGGCCCTGGCCTTATGATG-3′. The PCR product was cloned into the *Bam*HI and *Sal*I sites of pGEX(2T-P) after removing the *SRP1* gene from pNOY3198 (Azuma et al., 1995). After confirmation of the DNA sequence of pGEX(2T-P)+caspase-9, glutathione-S-transferase (GST) fused caspase-9 proteins (GST-Casp9) were purified from the transformed *E. coli* DH5α. Briefly, cells were lysed using the lysis buffer (1% Triton X-100 and 1 × phosphate buffered saline (PBS), pH 7.4 (Sigma/Merch, Darmstadt, Germany)) by ultrasonication on ice and the supernatant was collected by centrifugation at 7,740 × *g* at 4°C for 15 min. GST-Casp9 was purified using Glutathione Sepharose 4B beads (Amersham/GE Healthcare, Marlborough, MA). GST-Casp9 and purified EBs of *C. pneumoniae* J138 (Rahman et al., 2015) were mixed in lysis buffer and incubated at 37°C for 15 min. EBs were washed with lysis buffer three times and collected by centrifugation at 4°C for 5 min at 21,500 × *g*. GST-Casp9 was then analyzed by western blotting using anti-pro-caspase-9 mouse monoclonal antibody (Santa Cruz, Dallas, TX), followed by a second detection using anti-*Chlamydia* Pmp mouse monoclonal antibody (Cp-11, HITACHI, Tokyo, Japan). In both detections, alkaline phosphatase conjugated anti-mouse IgG goat polyclonal antibody (Santa Cruz) was used as a secondary antibody, and target proteins were visualized using CDP-star (Roche, Basel, Switzerland). The results indicate caspase-9 binding to EBs (Fig. 3a). This supports the previous observation that caspase-9 accumulates in inclusions (Rahman et al., 2015), as well as the finding of the Y2H assay. Further investigation is required to clarify the EB factor(s) involved in the binding of caspase-9 and its significance.

MnmE is well conserved in all three kingdoms of life and is involved in the modification of uridine bases (U34) at the first anticodon position of tRNAs. However, no data exists regarding the localization and functions of chlamydial MnmE. To confirm the interaction between Cpj0838 and caspase-9, a pull-down experiment was performed. To prepare Cpj0838 protein, a Cpj0838 DNA fragment was amplified by PCR using two primers, 5′-CGCGCGGCAGCCATATGCTAAAGCACG-ATACCATT-3′ and 5′-GGATCCTCGAGCATACTA-TTTTCCAATGCAAAATTTAC-3′, and cloned into a *Nde*I site of pET-15b vector (Clontech). After confirmation of the DNA sequence, *E. coli* BL21(DE3) was transformed with the pET-15b+Cpj0838. Cells were lysed using lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8.0) and His-tagged Cpj0838 (His-Cpj0838) was purified using a Ni-NTA spin column (Qiagen, Venlo, The Netherlands). GST-Casp9 and His-Cpj0838 were mixed in the pull-down buffer (1 × PBS, 1 mM DTT, 0.5% Triton X-100, and 10 mM $MgSO_4$, pH 7.4) at 25°C for 10 min, and GST-Casp9 was retrieved with glutathione beads. After washing the beads three times with the pull-down buffer, the proteins on the beads were analyzed by western blotting using anti pro-caspase-9 mouse monoclonal antibody (Santa Cruz) and alkaline phosphatase conjugated anti-6X His tag monoclonal antibody (Abcam, Cambridge, UK). The result of the pull-down experiment showed that Cpj0838 bound to caspase-9 (Fig. 3b). This indicates that Cpj0838/MnmE might function pleiotropically not only for the modification of tRNA but also for *C. pneumoniae* infection.

C. trachomatis was reported to accumulate glycogen, while *Chlamydia psittaci* and *C. pneumoniae* could not (Gilkes et al., 1958; Moulder, 1991). However, during *C. psittaci* infection, glycogen production in HeLa cells was increased (Ojcius et al., 1998), and all chlamydial genomes encode the genes necessary for both glycogen biosynthesis and catabolism (data not shown). It is possible that all chlamydia species can accumulate glycogen within the chlamydial inclusion or host cytoplasm. Interestingly, *C. trachomatis* glycogen synthase, GlgA, was shown to be secreted into the host cell cytoplasm (Lu et al., 2013). The products of Cpj0948/*glgA* might play an important role, possibly in conjunction with caspase-9. Additionally, the glucose metabolism enzyme, phosphoglucomutase (PgcA),

is known to be involved in the production of polysaccharides, including glycogen, and the pathogenicity in bacterial pathogens (Buchanan et al., 2005). However, interactions among caspase-9, GlgA, and PgcA were shown only by Y2H, it is possible that the product of Cpj0056/*pgcA* is located in inclusions and caspase-9 is involved in the glycogen metabolism accompanied by two additional enzymes, GlgC (Cpj0607) and GlgB (Cpj0475) (Supplementaly Fig. S1).

The hypothetical protein encoded by *C. trachomatis* CT425, which is homologous to Cpj0512, was shown to be immunogenic in humans infected with *C. trachomatis* (Barker et al., 2008). However, this protein contains a histidinol phosphatase domain, which is conserved among *Chlamydia* species and other bacteria. Further investigation is required to predict its functions. In conclusion, this study could serve as a clue to understanding molecular interactions between host and chlamydial factors, and to develop therapeutic agents to interfere with *Chlamydia* infection.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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