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Full Length Research Paper

An efficient secretion of the protein fused to the AgfA signal sequence in *Salmonella*

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Signal sequence (SS) of surface or secreting proteins plays an important function for protein secretion in bacterial system. The SS of various proteins may mediate different level of protein excretion yield of the proteins. In order to examine the effect of SS types in protein secretion, signal sequences of Bla (β– lactamase), AgfA (thin aggregative fimbriae A), StfA (*Salmonella typhimurium* fimbriae A) and OmpW (outer membrane protein W) were selected for the secretion of PspA protein which was used as a test protein. The PCR-amplified DNAs corresponding to each SS were cloned into the plasmid pYA3342. A primer used in PCR was designed to insert a His_6-tag at the C- terminal of SS for the convenient detection of expressed SS. The 0.8 kb *Eco*RI-*Hin* dIII *pspA* gene was cloned into the recombinant plasmids, resulting pMMP66, pMMP67, pMMP68 and pMMP70. The *S. typhimurium* strains harboring the recombinant plasmids expressed the His_6-tagged PspA, demonstrating in-frame fusion of PspA to each Depending upon the type of SS, cell lysate as well as secreted PspA of each *Salmonella* samples was found different. Relatively, *S. typhimurium* containing pMMP67 (carrying AgfA SS) secreted the highest level of PspA than others, and suggested that the AgfA SS mediates efficient translocation of the PspA. Conclusively, the AgfA SS mediated secretion system in pMMP67 can be used in variety fields required for the high level of protein secretion, especially antigen delivery in recombinant attenuated *Salmonella* vaccines.

Key words: β–Lactamase, thin aggregative fimbriae A, outer membrane protein W, *Salmonella typhimurium* fimbriae A, signal sequence, secretion.

INTRODUCTION

The conventional vaccines against pathogenic bacteria have been produced to majorly kill these bacteria or act as subunit vaccines (Meeusen et al., 2007). However, the manufacturing of these vaccines are very complicated from isolation up to the final step. Additionally, the prepared vaccines require stable storage to avoid any side effects or denaturation. These vaccines are most likely administrated to host through syringe, thereby making the child to be afraid. One way to resolve these

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Table 1. Bacterial strains and plasmids used for this study.

Strain or plasmid	Genotype or phenotype	Reference or source
Bacterial strains		
E. coli		
DH5	endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 (lacZYA-argF)U169 (80lacZ M15)	New England Biolabs
6212	F^{-} $^{-}$ 80 (lacZYA-argF)endA1recA1hadR17deoRthi-1glnV44gyrA96 relA1 asdA4	Nakayama et al. (1988)
Salmonella		
8554	hisG asdA16	Kang et al. (2002)
Plasmids		
pGEM-T vector	Cloning vector	Promega
pYA3342	Asd pBRori	Kang et al. (2002)
pYA3493	Derivative -lactamase signal sequence-based periplasmmic secretion plasmid	Kang et al. (2002)
pYA3494	A pYA3493 derivative containing pspA gene	Kang et al. (2002)
pMMP58	A derivative of T vector containing Bla signal sequence (SS) and His-tag	This study
pMMP59	A Derivative of T vector containing agfA SS and His-tag	This study
pMMP60	A derivative of T vector containing OmpW SS and His-tag	This study
pMMP61	A derivative of T vector containing stfA SS and His-tag	This study
pMMP62	A derivative of pYA3342 containing ompW SS and His-tag	This study
pMMP63	A derivative of pYA3342 containing stfA SS and His-tag	This study
pMMP64	A derivative of pYA3342 containing Bla SS and His-tag	This study
pMMP65	A derivative of pYA3342 containing agfA SS and His-tag	This study
pMMP66	A derivative of pMMP63 containing pspA gene	This study
pMMP67	A derivative of pMMP65 containing pspA gene	This study
pMMP68	A derivative of pMMP64 containing pspA gene	This study
pMMP70	A derivative of pMMP62 containing pspA gene	This study

inconveniences is the development of attenuated bacterial vaccines. Recombinant attenuated *Salmonella* vaccines have been adapted to elicit mucosal and humoral immunities after oral administration. The elicited immune responses were augmented when the delivered antigen was translocated into cell envelop or out of the cells (Kang and Curtiss, 2003; Kang et al., 2002; Kim et al., 2007). Multiple methods were applied to translocate the foreign antigen from cytosol into at least cell envelop or complete secretion (Kim et al., 2007; Wong et al., 1995). Researchers inserted the target antigen or epitope into surface structure molecules such as flagella or fimbriae to present the antigen on cell surface (Majander et al., 2005a, b; Spreng et al., 1999). However, a limited size of the antigen can be inserted into surface molecule for stable conformation. Another more convenient way is the secretion of the foreign protein into extracellular matrix through the use of a signal sequence (SS) (Koshland and Botstein, 1980; Li et al., 2008).

A previous study demonstrated that the PspA antigen was translocated into periplasmic space or outside of the cell through the fusion of -lactamase signal sequence (Kang et al., 2002) . Although this system has a merit to secret the protein to the extracellular matrix, a large portion (50%) of the expressed proteins exists still in the cytosol. Due to the fact that more secreted antigen elicits the better immune responses, it is needed to develop a new efficient protein secretion system in live vaccine mediated antigen delivery.

This study aimed to develop an efficient protein secretion system in *Salmonella*.

MATERIALS AND METHODS

Bacterial strains and agents

The bacterial strains, plasmids and oligonucleotides used for this study are listed in Tables 1 and 2. *Salmonella enterica* serotype Typhimurium and *Escherichia coli* were grown at 37°C using Luria-Bertani (LB) or M9 minimal medium supplemented with 1.5% agar (Bertani, 1952). The antibiotics were added in the following

Table 2. Synthetic oligonucleotides used for this study.

Oligonucleotide name	Oligonucleotide sequence (5' 3')
SagfA-F-BspHI	TCATGAAACTTTTAAAAGTGGCAG
SagfA-R-His/EcoRI	GAATTCATGGTGATGGTGATGATGGCCGCCGTTATGATTACCGC
Sbla-F-BspHI	TCATGAGTATTCAACATTTCCGTG
Sbla-R-His/EcoRI	GAATTCATGGTGATGGTGATGATGTTCAGCATCTTTTACTTTCA
SompW-F-BspHI	TCATGAAAAAATTTACAGTGGCGG
SompW-R-His/EcoRI	GAATTCATGGTGATGGTGATGATGTCCGGCTTCGTGCGCGAACG
SstfA-F-BspHI	TCATGAATACAGCAGTAAAAGCTG
SstfA-R-His/EcoRI	GAATTCATGGTGATGGTGATGATGACCGGTAAAAGTCACCGTAC

concentrations for each culture condition: ampicillin, 100 g/ml; streptomycin, 50 g/ml.

DNA manipulations

General DNA manipulations were conducted as described by Sambrook et al. (1989). Plasmids were introduced into *E. coli* competent cells by heat-shock with $RbCl_2$ treatment (Hanahan, 1983) and were introduced into *Salmonella* competent cells by electroporation with 10% glycerol treatment (Sambrook et al., 1989). Nucleotide sequencing was conducted by using an ABI 373 automatic sequencer (PE Applied Biosystems).

Cloning of genes for signal sequence

The DNA regions corresponding to signal peptide of Bla, AgfA, OmpW and StfA was PCR-amplified from pYA3493 or *S. typhimurium* χ3339 chromosome as a template. The oligonucleotide primers used in PCR-amplification were designed to insert restriction sites for *Bsp*HI and *Eco*RI (Table 2). The primers were also designed to include His_6-epitope at the C-terminal of each signal peptide, which allows the detection of expressed signal peptides by immunoblot with anti-His_6-epitope antibody. PCR reaction conditions were as follows: denaturation at 95°C for 30 s, primer annealing at the temperature of primer's melting temperature (T_m) for 30 s, polymerization at 72°C for 30 s ~ 3 min depending on the length of DNA fragment and a final extension at 72°C for 10 min. The amplified DNA fragments were cloned into T-vector (Promega) as a temporary step. The *Bsp*HI -*Eco*RI DNA segment isolated from the recombinant T-vector was cloned into a plasmid pYA3342 digested with *Nco*I and *Eco*RI.

SDS-PAGE (polyacrylamide gel electrophoresis) and immunoblot

Bacterial culture broth was centrifuged for 10 min at 5,000 x *g* to separate the cells from the culture supernatant. The supernatants was collected and further used for the analysis of secreted proteins. The precipitated cell pellet was washed twice with 0.85% NaCl and then resuspended in 10 mM Tris HCl, pH 7.5. The suspended cells were mixed with SDS gel loading buffer and lysed thermally. The 65 μg proteins of total cell lysates were then separated through SDS-PAGE and the separated bands were visualized by Coomassie staining (Sambrook et al., 1989). For the analysis of secreted proteins, the cultural supernatant was concentrated for 1 h in ice-cold TCA (trichloroacetic acid) solution (Hoong et al., 1995; Ma et al., 1996) and then 8 μg secreted proteins were separated by SDS-PAGE. For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred onto the nitrocellulose membranes. The immunoblotting was conducted in accordance with the protocols established by Sambrook et al. (1989). The PspA protein and His_6-tagged signal peptides were specifically recognized by the use of Xi126 monoclonal antibody (McDaniel et al., 1984) and His_6-tag monoclonal antibody (Southern Biotech), respectively.

Goat anti-mouse IgG, horseradish peroxidase (HRP) conjugated antibody was used as secondary antibody and the 4-choloro-1-naphthol was used for the substrate of HRP.

RESULTS AND DISCUSSION

Background of construction for new foreign antigen-carrying systems and the results

Most of the bacteria operate general secretory pathway (GSP) to translocate proteins from cytoplasm to peri-plasmic space, outer membrane or extracellular matrix. The SS located at N- terminus of the secreting protein leads the translocation of the protein to membrane. In a previous study (Kang et al., 2002) a plasmid system (pYA3493) was generated for the secretion of proteins through GSP. Main idea of the pYA3493 based expression system is the fusion of - lactamase SS at the N-terminus of expressed proteins. In this study, we examined protein secretion capacity by the replacement of -lactamase SS with the SS of other proteins in pYA3493. The plasmid pYA3493 carries an aspartate semialdehyde dehydrogenase (*asd*) gene, which is required for the biosynthesis of m- diaminopimellic acid (m-DAP), a component of bacterial cell wall. The "*asd*" gene in pYA3493 complements "Asd" deficiency of bacterial hosts, *E. coli* χ6212 and *S. typhimurium* χ8554 (Table 1). Multicloning sites (MCS) is positioned at the downstream sequence of SS sequence to fuse signal peptide with the protein of cloned gene. To examine the effect of the type of signal peptide for protein secretion, various DNA fragments corresponding to the signal peptide of -lactamase (Bla), AgfA, OmpW and StfA were localized at the upstream of MCS: "bla SS" in pMMP62, "agfA SS" in pMMP63, "ompW SS" in pMMP64 and "stfA SS" in pMMP65. The C-terminus of each signal peptides

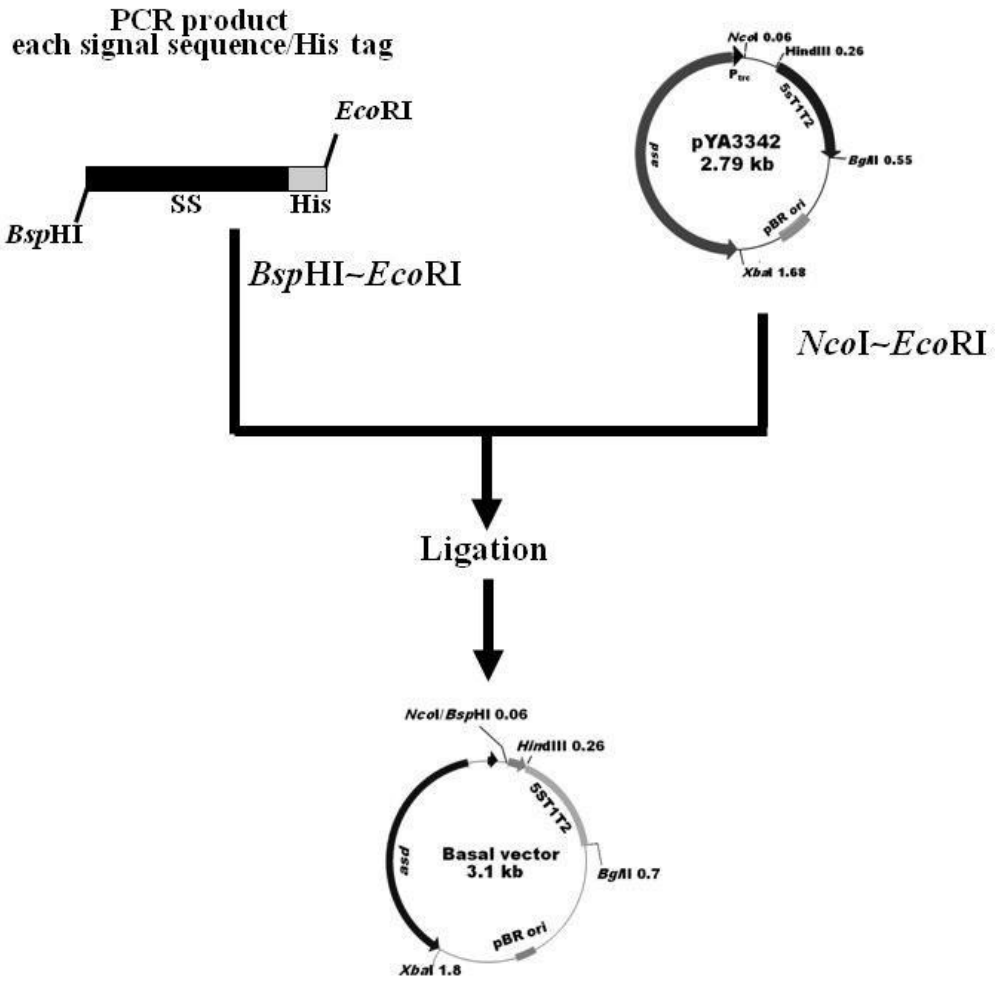

Figure 1. Schematic diagram of strategy for cloning of signal sequence (SS) DNA. The signal sequences of Bla (β–lactamase), AgfA (thin aggregative fimbriae A), StfA *S. typhimurium* fimbriae A) and OmpW (outer membrane protein W) were used in this study. Each PCR- amplified *Bsp*HI-*Eco* RI SS DNA was cloned into pYA3342 digested with *Nco*I and *Eco*RI. Gray box indicates a His_6-tag contained at the C-terminus of each SS.

exhibited His_6 residues. It is possible to speculate that expression of each SS and His_6 coding residue were regulated by P_{trc} promoter. A strategy for preparation of these vectors is shown in Figure 1. In *E. coli*, the -lactamase is secreted into periplasmic space through type II Sec-dependent secretion system, but in independent manner of SecB (Beha et al., 2003). AgfA and StfA, major fimbrial proteins of *S. typhimurium*, Agf and Stf fimbriae are secreted into extracellular matrix by type IV Sec-dependent secretion system.

The mechanism of Agf fimbrial protein secretion is unknown, whereas Stf fimbrial proteins are secreted by similar manner to type II Sec-dependent pathway (Humphries et al., 2003). The OmpW (outer membrane protein W) is translocated through type II Sec- and SecB-dependent pathway (Driessen et al., 2001; Koch et al., 2003).

Identification of foreign antigen expression by PspA protein

The PspA protein (*Streptococcus pneumoniae* surface protein A) was selected as a testing protein, for protein secretion through the system developed in this study (Briles et al., 1998). A 765 bp *Eco*RI- *Hin*dIII *pspA* gene isolated from pYA3494 was ligated with the plasmids pMMP62, pMMP63, pMMP64 or pMMP65 digested with *Eco*RI and *Hin*dIII (Figure 2). The recombinant DNAs were transformed into *E. coli* x6212 (Kang et al., 2002). Furthermore, the presence of recombinant plasmids were confirmed by analyses of restriction enzyme digestion

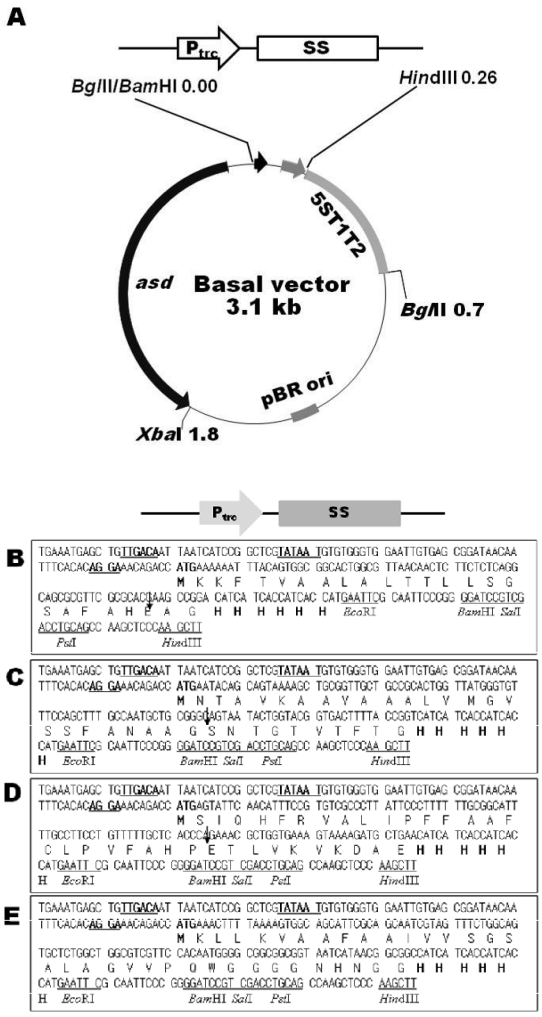

Figure 2. Genetic maps of recombinant plasmids. The physical maps of each recombinant plasmid are indicated with their representative restriction enzyme sites: (A) Basal vector, (B) pMMP62, (C) pMMP63, (D) pMMP64 and (E) pMMP65. The P_{trc} promoter region, ribosome binding site (RBS), each SS, His_6-epitope and multi-cloning sites (MCS) are revealed in enlarged box. Unique enzyme sites in MCS are designated by underlined bold letters. P_{trc}, *trc* promoter; 5ST1T2, 5S rRNA terminator; "asd", aspartate semialdehyde dehydrogenase gene; SS, OmpW SS (B), StfA SS (C), Bla SS (D) and AgfA SS (E).

and nucleotide sequencing, and were finally designed as pMMP66, 67, 68 and 70 (Table 1). The level of expressed PspA protein in the *S. typhimuium* χ8554 host was examined by immunoblotting. A monoclonal antibody Xi126 (McDaniel et al., 1984) was used to detect PspA protein specifically. An anti-His_6-epitope antibody (SantaCruz Biotechnology, Inc.) raised in mouse was used to detect expressed protein against His_6-tagged signal peptide. An anti-mouse IgG conjugated with HRP (horseradish peroxidase) (Stressgen Co.) obtained from goat was used as a secondary antibodies to recognize primary antibodies. The *S. typhimuium* χ8554 carrying pYA3494, pMMP66, pMMP67, pMMP68 or pMMP70 were cultured to OD_{600} 0.8 in LB broth at 37°C. Total cell lysate of each sample containing 65 μg of protein was subjected for SDS-PAGE analysis. As seen in Figure 3, all samples exhibited a major protein bands with approximately 37 kDa size. A little size differences of the

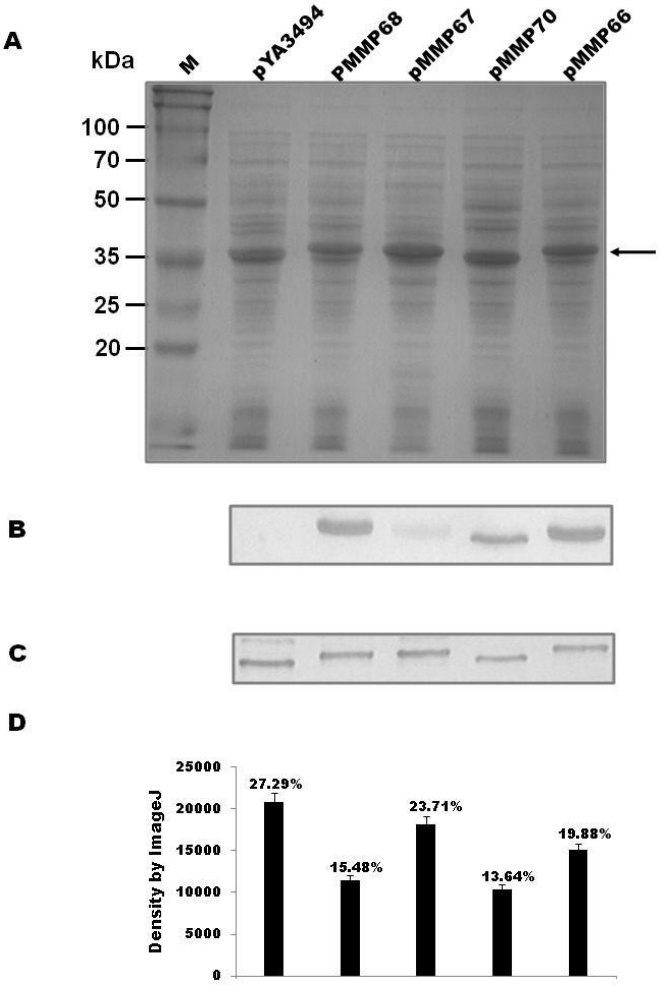

Figure 3. Expression of PspA in *S. typhimurium* containing recombinant plasmids. *S. typhimurium* strains harboring pYA3494, pMMP66, 67, 68 and 70 were grown in LB broth until exponential phase (0.8 at OD_{600}). Equal amounts of cell lysates were subjected to SDS-PAGE followed by immunoblot analyses with specific antibodies for each component. The goat anti-mouse conjugated with HRP was used as the 2nd antibodies. (A) SDS-PAGE of total lysates. Separated bands were visualized by Coomassie staining. (B to C) immunoblot of total lysates. His_6-tagged SS was detected by monoclonal antibody specific for His_6-tag (B), and expressed PspA protein was detected by monoclonal antibody specific for PspA (C). (D) Quantitative analysis of PspA protein. Relative immuno-reactive intensities were analyzed by imageJ program. Arrow indicates the PspA protein.

major bands between samples can be explained by the size difference of signal peptide which was fused to the PspA. The 37 kDa proteins were reacted with anti-His_6-epitope antibody, which confirms that each 37 kDa protein contains signal peptide designed in this study. The protein of *S. typhimuium* χ8554 harboring pMMP67 exhibited a faint immune-reactive band, hypothesizing the access difficulty of H_6-antibody due to protein conformation. As we expected, there was no His_6-antibody reaction band in sample of *S. typhimuium* χ8554 harboring pYA3494, which does not carry His_6-tagged signal peptide.

Immunoblot analyses with anti-PspA antibody demonstrated that the 37 kDa protein seen in all samples

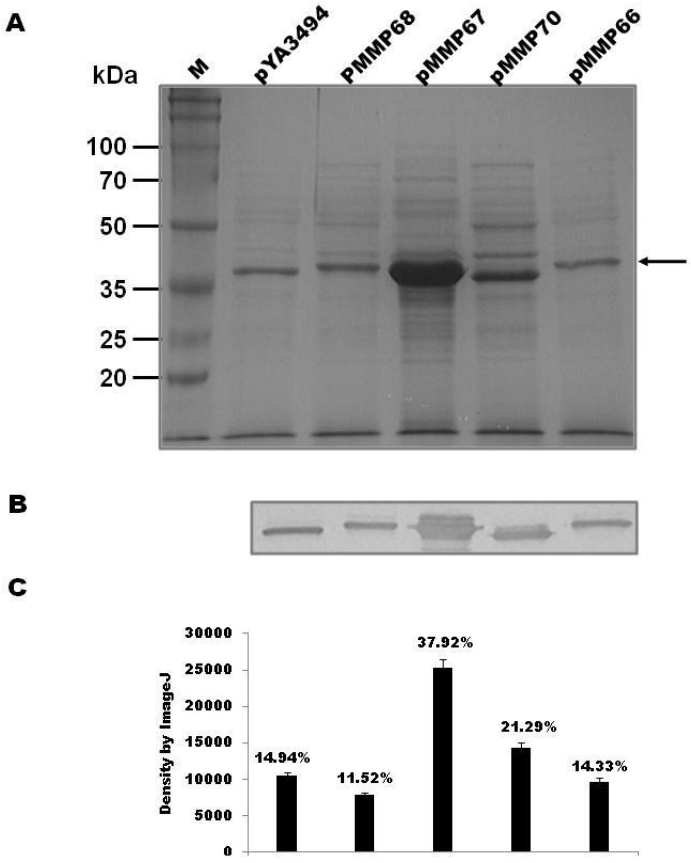

Figure 4. Immunoblot of secreted PspA proteins. *S. typhimurium* χ8554 harboring plasmids were cultured in LB broth until exponential phase (0.8 at OD_{600}). Equal amount of cell-free culture supernatant were subjected to SDS-PAGE and immunoblot analyses. (A) SDS-PAGE analysis. Separated bands were visualized by Coomassie staining. (B) Immunoblot of secreted proteins. The PspA protein was detected by monoclonal antibody specific for PspA. (C) Quantitative analysis of PspA protein. Relative immuno-reactive intensities were analyzed by imageJ program. Arrow indicates the PspA protein.

is the PspA protein. The highest amount of cell associated PspA protein was detected in *Salmonella* containing pYA3494, which was almost 2 fold higher levels than that in *Salmonella* containing pMMP70 (carrying OmpW SS) . The *Salmonella* containing pMMP67 or pMMP66 expressed PspA 1.5 or 1.3 folds higher than *Salmonella* containing pMMP68. An advantage of the system developed in this study is the detection of the expressed protein with commercially available His_6-epitope antibody, without specific antibody for the expressed protein.

Identification of secretion ability according to signal sequences

The protein expression system developed in this study is designed for the secretion of the PspA protein. Each signal peptide fused to PspA may lead to the translocation of the cytoplasmic PspA to the cell envelop or to the outside of the cell. To examine the level of protein secretion, the *S. typhimuium* χ8554 carrying pYA3494, pMMP66, pMMP67, pMMP68 or pMMP70 were cultured to OD_{600} 0.8 in LB broth at 37°C. Cell-free supernatant of each culture was examined for the secreted protein fractions. To eliminate the presence of *Salmonella* cells entirely, culture supernatant after centrifugation was filtered through passing 0.2 μm filter. The proteins in 1 ml of supernatant were precipitated using 10% TCA as described earlier. The precipitated pellets after washing twice were resuspended in 60 μl of SDS loading buffer. 8 μg protein of each sample was subjected to SDS-PAGE. Parts of samples were

employed for immunoblot analysis after SDS-PAGE. All samples exhibited a major protein bands with approximately 37 kDa size likely as seen in total lysates (Figure 4).

The 37 kDa proteins were reacted with both anti-His_6-epitope antibody and anti-PspA antibody, demonstrating PspA protein of the 37 kDa protein. Interestingly, a thick 37 kDa protein band was observed in the secreted fraction of *S. typhimuium* χ8554 carrying pMMP67. Densitometer analysis of the secreted PspA in each sample shows that the secreted PspA in *Salmonella* carrying pMM67 was 1.8 fold amount higher than that in *Salmonella* carrying pMM70 which secrets the protein second level. The other three samples, *Salmonella* carrying pYA3494, pMM66 and pMM68 secrets the PspA with similar levels. Although the PspA level in total cell lysate was similar for pYA3494, pMMP67 and pMMP70 (Figure 3), the highest amount of secreted PspA was detected in the supernatant of *Salmonella* carrying pMM67. This discrepancy is not solely because of overexpression of the protein, but it might be due to the Agf SS. Thus, we speculate that the AgfA signal peptide mediates PspA secretion more efficiently than any other signal peptides used in this study. Taken all together, since signal sequences play roles as a leader sequence and a signal sequence, their existences raise individual expression and secretion of protein.

The efficient protein secretion mediated by AgfA signal sequence can extend its utility in various fields. Especially, it can be applicable in the antigen delivery of recombinant attenuated *Salmonella* vaccines (Kim et al., 2009). A previous study suggested that *Salmonella* vaccine secreting foreign antigen induces better immune responses than the vaccine localizing the antigen in cytoplasm (Kang et al., 2002). Therefore, more secretion of the foreign antigen may induce more immune responses in *Salmonella* antigen delivery system.

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Abbreviations:

SS, Signal sequencel; **Bla,** -lactamase; **AgfA,** thin aggregative fimbriae A; **StfA,** *Salmonella typhimurium* fimbriae A; **OmpW,** outer membrane protein W; **GSP,** general secretion pathway.

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