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Development of a Protein Secretion System with the Application of Sec-dependent Protein Secretion Components

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Abstract In order to induce high levels of protein secretion, we have constructed a recombinant plasmid, designated pBP244, into which was incorporated key components of the type-II Sec-dependent secretion system, including LepB (signal peptidase), SecA (ATPase), and SecB (chaperone). The biological activities of the LepB, SecA, and SecB components expressed from genes harbored by pBP244 appeared to play their normal roles. In order to evaluate the protein secretion, a *pspA* (*Streptococcus pneumoniae* surface protein A) gene was cloned into pBP244, resulting in pBP438. *S. typhimurium* harboring pBP438 grown until the stationary phase, secreted a higher level of PspA into the culture supernatants than did the strain harboring pYA3494. The strain harboring pBP438 secreted a supernatant amount 1.71-fold, a periplasmic space amount 1.47-fold, and an outer membrane amount 1.49-fold higher than that of pYA3494. *S. typhimurium* χ8554 kept the Asd^+ plasmid pBP244 and pBP438 for 60 generations in LB broth harboring DAP, thereby indicating that pBP244 and pBP438 were quite stable in the *Salmonella* strain.

Keywords: pBP244, protein secretion, type II Sec-dependent secretion system, LepB, SecA, SecB

Several different protein secretion systems for bacteria have been developed thus far. Genes of interest have been constructed *via* translational fusions with genes that encode for proteins capable of secretion, and transformed into target hosts to guide expression [5, 14, 24, 32, 39]. Bacterial ghosts have also been generated for the expression of target antigens within the cytoplasmic membrane. For the membrane anchoring of target antigens or of acceptor proteins, such as streptavidin, to the cytoplasmic side of the cytoplasmic membrane, a membrane targeting system has also been developed [40, 41]. Additionally, a plasmid-based protein secretion system has been designed and constructed simply for secretion into the extracellular matrix *via* the action of signal peptides, such as pYA3493 [22].

In general, the secretion of secretory proteins is accomplished using a type II Sec-dependent secretion system, or *via* the GSP (general secretion pathway), which is exploited by many Gram-negative bacteria [11, 12, 16]. The system described herein was composed of 12–16 different GSP proteins and consisted of two distinct steps [11, 21, 35, 42]. The initial step involved translocation to the periplasmic space by the Sec-dependent system, and the other involved secretion into the extracellular matrix. The SecA protein exists in the cytosol, and recognizes the signal sequence of a translated target protein, which is then carried to the plasma membrane, in which it supports energy *via* ATP hydrolysis for the translocation of target proteins throughout the membrane [1, 19, 28, 29]. The SecB protein performs a chaperone function, and also locates itself within the cytosol and prevents against the random folding of the protein recognized by SecA [6, 7, 25, 26, 27, 36, 43]. SecYEG proteins, which locate the plasma membrane, function as a channel for the recognized protein *via* the SecA locating plasma membrane [15, 30, 34]. The signal peptide of the protein translocated to the periplasmic space is digested by LepB (signal peptidase), and then the digested protein is released freely into the periplasmic space [10, 20].

Recombinant attenuated *Salmonella* vaccine has been adapted for use as a protein antigen carrier [4, 9]. A previous study [17, 23] showed that the *Salmonella* vaccine strain secreting the antigen induced a more pronounced immune response than did the strain expressing the antigen in the cytosol. We hypothesized that the strength of the induced immune response is proportional to the level of antigen

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secretion in the *Salmonella* vaccine system. This hypothesis enforced the development of a system for augmented protein secretion.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions

The bacterial strains and plasmids employed in this study are listed in Table 1. *E. coli* and *S. typhimurium* were grown at 37°C in Luria-Bertani (LB) broth or LB agar [3]. When required, antibiotics were added to the culture media at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; tetracycline, 15 μg/ml. Diaminopimelic acid (DAP) was added at a concentration of 50 μg/ml for the growth of the *asd*⁻ (a deficient of aspartate semialdehyde dehydrogenase gene) strains. For the induction of the P_{araBAD} promoter, arabinose (0.2%) was added into the culture media.

General DNA Manipulations

DNA manipulations were conducted as described by Sambrook *et al.* [38]. Plasmids were introduced into *E. coli* or *Salmonella via* heat-shock using competent cells constructed *via* $RbCl_2$ treatment [18] or electroporation [38]. The transfer of recombinant suicide plasmids into *Salmonella* was accomplished *via* conjugation using *E. coli* χ7213 (*asd*⁻) [37] as the plasmid donor. PCR amplification was employed in order to identify the *S. typhimurium* strains, and to acquire DNA fragments for the cloning and verification of chromosomal deletion mutations. Nucleotide sequencing was conducted using an ABI 373 automatic sequencer (PE Applied Biosystems).

SDS-PAGE and Immunoblot

The bacterial culture broth was centrifuged for 10 min at 7,000 rpm in order to separate the cells from the supernatant. The collected supernatants were utilized in the analysis of secreted proteins. The precipitated cell pellet was washed twice with 0.85% NaCl, and then resuspended in 10 mM Tris HCl, at a pH of 7.5. The suspended cells were broken *via* 5 min of boiling in digestion buffer. The proteins were then separated *via* SDS-PAGE, and the separated bands were visualized by Coomassie staining [38]. For the analysis of secreted proteins, the culture supernatant was concentrated for 1 h in ice-cold TCA (trichloroacetic acid) solution. For Western blotting, proteins separated *via* SDS-PAGE were electrophoretically transferred

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

Strains or plasmids	Genotypes or phenotypes	References or sources
Bacterial strains		
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 (ϕ80lacZDM15)*	Lab stock
χ6212	F⁻ λ⁻ φ80 Δ(*lacZYA-argF*) *endA1 recA1 hadR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4*	Lab stock
χ7213	*λpir thi-1 thr-1 leuB6 supE44 towA21 lacY1 recA* RP4-2 Tc::Mu *ΔasdA4*	Lab stock
χ7122	Wild-type APEC, NalR	Lab stock
Salmonella		
S. typhimurium χ3339	Wild-type, SL1344, *hisG rpsL*	Lab stock
S. typhimurium χ8554	*Δasd*A16, a derivative of *S. typhimurium* χ3339	Lab stock
CK49	*S. typhimurium* χ8554 *ΔsecA*	This study
CK76	χ8554::*araC*::P_{araBAD}-*lepB* a derivative of *S. typhimurium* χ3339	This study
CK87	*S. typhimurium* χ8554 *ΔsecB*	This study
Plasmids		
T-vector	Cloning vector; pUC*ori* Amp^R	Promega
pMEG375	Suicide vector	Lab stock
pBP364	pMEG375 derivative containing *araC*::P_{BAD}-*lepB*	This study
pYA3342	Asd^+ vector; pBR322 *ori*	Kang *et al.* [22]
pYA3493	Derivative β-lactamase signal sequence-based periplasmic secretion plasmid	Kang *et al.* [22]
pYA3494	pYA3493 derivative containing *pspA* gene	Kang *et al.* [22]
pBP69	pYA3342 derivative containing P_{trc}-*lepB*	This study
pBP103	T-vector derivative containing P_{lac}-*secB*	This study
pBP110	T-vector derivative containing P_{lac}-*secBA*	This study
pBP158	pYA3493 derivative containing *lepB* gene	This study
pBP222	pBP158 derivative containing *pspA* gene	This study
pBP244	pYA3493 derivative containing *lepB*, *secA*, and *secB* genes	This study
pBP438	pBP244 derivative containing *pspA* gene	This study

to nitrocellulose membranes. The Western blotting was generally conducted in accordance with the protocols established by Sambrook *et al.* [38]. In order to identify the PspA protein, Xi126 monoclonal antibody was utilized [31]. The polyclonal antibodies utilized in the detection of LepB, SecA, SecB, Lon, and OmpA were prepared from rabbits *via* standard immunological procedures.

Salmonella Subcellular Fractionation

S. typhimurium χ8554 [pYA3494] or [pBP438] was cultured until the stationary phase (3.0 at OD_{600}) after inoculation in LB broth. The *Salmonella* culture broths were centrifuged for 10 min at 7,000 rpm, and the supernatant fluids were saved for the analysis of secreted proteins. The periplasmic and outer membrane fractions were then prepared *via* a modified version of the lysozyme-osmotic shock method [44]. The cell pellets were resuspended in 800 μl of 100 mM Tris-HCl buffer (pH 8.6) containing 500 mM sucrose and 0.5 mM EDTA. Hen egg-white lysozymes (40 μl of a 4 mg/ml stock solution) were added, followed immediately by the addition of 3.2 ml of 50 mM Tris-HCl buffer (pH 8.6) containing 250 mM sucrose, 0.25 mM EDTA, and 2.5 mM $MgCl_2$. After gentle agitation, the suspension was incubated for 15 min in an ice bath. The cells were removed *via* 6 min of centrifugation at 7,000 ×*g*, followed by the filtration of the supernatant through a 0.45 μm-pore-size filter. The filtered supernatant fluids were then employed as the periplasmic fraction. Cells resuspended in 4 ml of 20 mM Tris-HCl (pH 8.6) were then disrupted *via* two passages through a French pressure cell (American Instrument Company, Silver Spring, MD, U.S.A.). The cell lysates were centrifuged for 6 min at 7,000 ×*g* at 4°C in order to remove any unbroken cells. The supernatant fluid was then centrifuged for 1 h at 132,000 ×*g* at 4°C in order to separate the soluble fraction from the insoluble cell envelopes. The soluble fraction harbored the cytoplasmic proteins. In order to isolate the outer membrane fraction, total envelope pellets were suspended in 4 ml of 20 mM Tris-HCl (pH 8.6) containing 1% Sarkosyl, and incubated for 30 min on ice. The outer membrane fraction was obtained as a pellet after 1 h of centrifugation at 132,000 ×*g* at 4°C. The pellet was then resuspended in 4 ml of 20 mM Tris-HCl buffer (pH 8.6). The original culture supernatant was filtered (0.22 μm-pore-size filter), and the secreted proteins were precipitated with a final concentration of 10% trichloroacetic acid (1 h at 4°C). An appropriate volume of each of the fraction samples was separated *via* SDS-PAGE for Western blot analysis. The target proteins harbored in each fraction were separated *via* SDS-PAGE and identified *via* immunoblotting with the appropriate antibodies.

RESULTS AND DISCUSSION

Construction of a New Protein Secretion System

Bacterial proteins secreted into the extracellular matrix are generally mediated by the GSP system. Therefore, we have planned to incorporate several major components of the GSP system into a plasmid system that could be utilized in conventional protein secretion, a measure that was expected to improve protein secretion yields. Among the variety of GSP proteins, the LepB [10, 20], SecA [1, 19, 28, 29], and SecB [6, 7, 25–27, 36, 43] proteins were selected as components for the construction of a novel secretion system. These proteins play essential roles in the translocation of the target proteins from the cytosol toward the periplasm and the extracellular environment. The *lepB* gene, which encodes for signal peptidase, is harbored in an operon with the *lepA* gene in the *Salmonella* chromosome [13]. As the position of the *lepA* gene precedes the *lepB* gene, a promoter for the *lepB* gene is required for its expression when we clone the *lepB* gene. For its promoter, the P_{trc} promoter on the pYA3342 plasmid was utilized as a promoter for the *lepB* gene.

The promoterless *lepB* gene was PCR-amplified using *S. typhimurium* χ3339 chromosomal DNA as a template with the *lepB*-NcoI and *lepB*-R-XbaI oligonucleotide set (Table 2). Amplified DNA digested with NcoI and SalI was cloned into pYA3342 digested with the same restriction enzymes, thereby resulting in pBP69 (Fig. 1). A DNA segment encompassing the P_{trc}-*lepB* gene in pBP69 was re-

Table 2. Synthetic oligonucleotides used for this study.

Synthetic oligonucleotide names	Oligonucleotide sequences
lepB-NcoI	5'-GCCCATGGCGAACATGTTTGCCCTG-3'
lepB-R-XbaI	5'-GCTCTAGATAATTCCGCCAGCGTATT-3'
P_{trc}-L-XbaI	5'-TCTAGATTCTGAAATGAGCTGTTGACA-3'
lacZ-L-BglII	5'-AGATCTGCGCAACGCAATTAATGTGA-3'
lacZ-R-over	5'-GTGTTATTTTGTTCTGACATAGCTGTTTCCTGTGTG-3'
secB-L-over	5'-CACACAGGAAACAGCTATGTCAGAACAAAATAACAC-3'
secB-R-XhoI	5'-CTCGAGTCAGGCATCCTGATGTTCTTC-3'
secA-L-XhoI	5'-CTCGAGACGAAAAAAGACCCTTTTAC-3'
secA-R-BglII	5'-AGATCTTGTTGCATCCATAAAAAAGG-3'

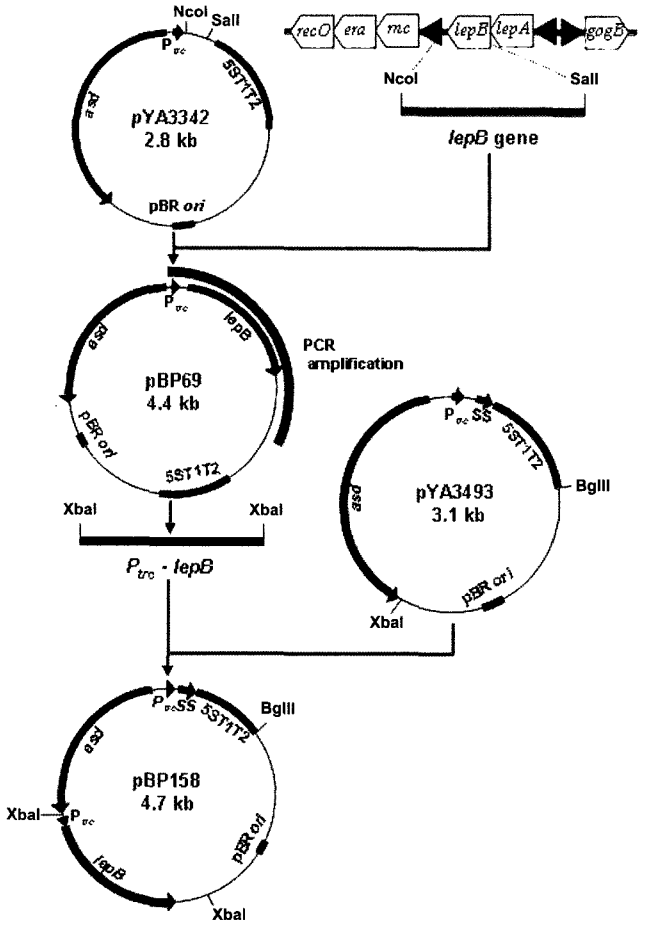

Fig. 1. Construction of an Asd$^+$ plasmid carrying the *lepB* gene. A promoterless *lepB* gene was PCR-amplified from the *S. typhimurium* chromosome and cloned into pYA3342, and designated as pBP69. The P_{trc}-*lepB* DNA amplified by PCR from pBP69 was trimmed with the XbaI enzyme and cloned into pYA3493, resulting in pBP158. The *SS* indicates the signal peptide originating from *b*-lactamase. P_{trc}, *trc* promoter; 5ST1T2, 5S rRNA terminator; *asd*, aspartate semialdehyde dehydrogenase gene.

amplified *via* PCR using the P_{trc}-L-XbaI and *lepB*-R-XbaI oligonucleotide set. The ends of the amplified DNA fragment were trimmed with the XbaI enzyme, and then cloned into pYA3493 digested with the same restriction enzymes, and designated plasmid pBP158 (Fig. 1).

The *secA* and *secB* genes are likely to consist of an operon with neighboring *yacA* and *yibN*, respectively, in the *Salmonella* chromosome. In order to obtain functional *secA* and *secB* genes, a complicated cloning strategy was required. The first required step involved the preparation of the *secB* gene under the control of a promoter, followed by the fusion of a *secA* gene to the 3'-flanking region of the *secB* gene, resulting in an artificial *secBA* operon controlled by a promoter. The P_{lac} promoter was selected as a promoter for this operon. The P_{lac} promoter and the *secB* gene were amplified from the chromosomal DNA of *E. coli* χ7122 and *S. typhimurium* χ3339, respectively.

The *secB* gene under the control of the P_{lac} promoter was constructed *via* the overlapping technique [33]. For overlapping PCR, the first left (P_{lac} promoter region) and right (*secB* gene) arm fragments were PCR amplified with the *lacZ*-L-BglII/*secB*-L-over and *lacZ*-R-over/*secB*-R-XhoI oligonucleotide sets (Table 2), respectively. *E. coli* χ7122 (wild-type) and *S. typhimurium* (wild-type) were employed as template DNAs for the amplification of the P_{lac} and *secB* genes, respectively. The amplified DNA fragments harbor the overlapped sequence (36 nucleotides) between the *lacZ*-R-over and *secB*-L-over oligonucleotides. For the joining of the P_{lac} and *secB* genes, each amplified DNA was mixed together, allowing for a partial hybridization between overlapped sequences. Then, the extensions of the overlapped segments, resulting in P_{lac}-*secB*, were conducted with the *lacZ*-L-BglII/*secB*-R-XhoI oligonucleotide set, *via* the regular PCR process. In order to fuse the *secA* gene to the 3'-region of the *secB* gene, the following *secA* gene was PCR-amplified from *S. typhimurium* χ3339 chromosomal DNA with the *secA*-L-XhoI and *secA*-R-BglII oligonucleotide set (Table 2), and then ligated with P_{lac}-*secB*. The operon DNA fragment acquired *via* the digestion of the BglII enzyme was cloned into pBP158 digested with the BglII enzyme, resulting in the formation of pBP244 (Fig. 2). This construct was verified using restriction enzyme analyses and nucleotide sequencing (data not shown).

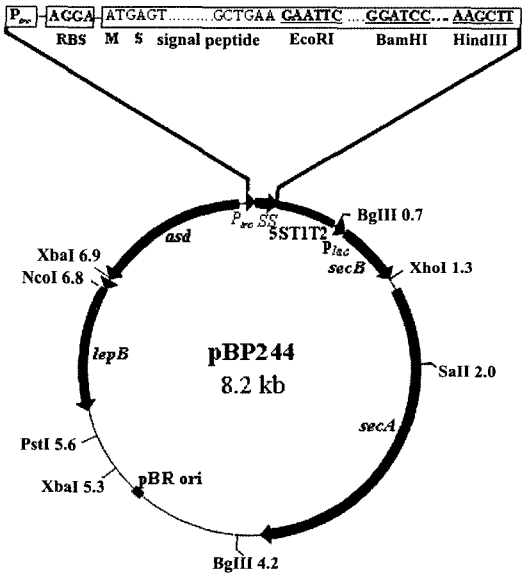

Fig. 2. Genetic map for pBP244.
The physical map of pBP244 is indicated with its representative restriction enzyme sites. The P_{trc} promoter region, ribosome binding site (RBS), β-lactamase signal sequence, and multicloning sites (MCS) on the upper panel are enlarged. Unique enzyme sites in MCS are designated by underlined bold letters. P_{trc}, *trc* promoter; P_{lac}, *lac* promoter; *SS*, β-lactamase signal sequence; 5ST1T2, 5S rRNA terminator; *secB*, *secB* gene; *secA*, *secA* gene; *lepB*, *lepB* gene; *asd*, aspartate semialdehyde dehydrogenase gene.

Identification of Activity and Expression of Each Factor Cloned in the Plasmid System

The pBP244 plasmid harbors two artificially constructed genetic elements, the P_{trc}-*lepB* gene and P_{lac}-*secBA* operon (Fig. 2). Although these genetic elements were physically integrated into the plasmid, evidence was required with regard to the biological activity of the products of the integrated genetic elements. The deletion of the *lepB* gene from *Salmonella* has been reported to be fatal [20]. Several reports [2, 10] have suggested that a conditional *lepB* mutant in the *S. typhimurium* chromosome can be used to verify the functional activity of the LepB supplied *in trans*. As an initial step in the generation of a *S. typhimurium* mutant conditionally expressing LepB, a recombinant suicide pBP364 was constructed (Table 1). The pBP364 carries a recombinant DNA fragment harboring *araC*::P_{araBAD}-*lepB*. A conditional *lepB* mutant was generated *via* the integration of pBP364 into the chromosome of *S. typhimurium* χ8554 *via* a crossover between the *lepB* DNA located in both pBP364 and the chromosome, resulting in the formation of *S. typhimurium* CK76 (Table 1). *S. typhimurium* CK76 maintained its viability in the presence of arabinose, but did not retain its viability in the absence of arabinose (Table 3). As had been expected, the CK76 harboring pBP158 or pBP244 evidenced growth, although arabinose was absent from the culture medium (Table 3). These results show that the LepB protein expressed by the gene cloned in the plasmids functions in the manner of a normal signal peptidase.

In order to determine the expression of each component cloned within the plasmid, Western blot analyses were conducted with specific antibodies against each of the components (Fig. 3). Endogenous LepB protein was detected in *S. typhimurim* CK76 [pYA3493 (vector control)] grown in the presence of arabinose. The CK76 harboring pBP158 or pBP244 expressed LepB protein in the absence of arabinose. These results were consistent with the results obtained in the genetic complementation examination. In order to assess both SecA and SecB factors, *S. typhimurium* CK49 (*ΔsecA*) and CK87 (*ΔsecB*) were utilized, respectively.

Table 3. Arabinose-dependent viability of *S. typhimurium* CK76.

Salmonella strain[a]	Growth[b]	
	with arabinose[c]	without arabinose
χ3339	+	+
χ8554	+	+
CK76	+	-
CK76 [pYA3493]	+	-
CK76 [pBP158]	+	+
CK76 [pBP244]	+	+

[a]*Salmonella* cells were inoculated onto MacConkey agar.
[b]+, growth; -, no growth.
[c]Arabinose (0.2%) was supplemented into MacConkey agar.

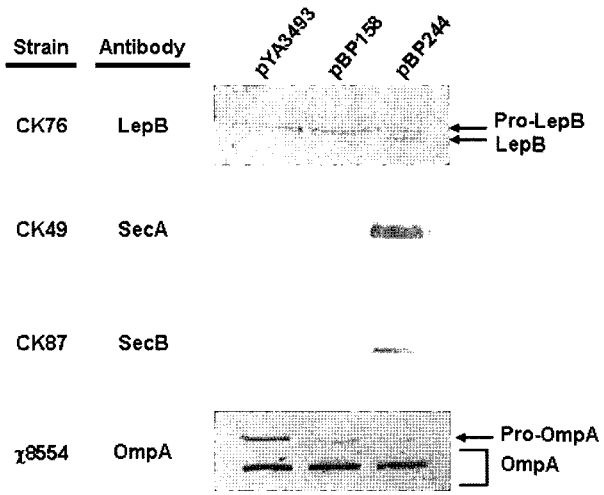

Fig. 3. Protein expression from genes cloned in the plasmid.
S. typhimurium strains harboring pYA3493, pBP158, or pBP244 were grown in LB broth, except for CK76. The CK76 strain was grown in LB broth supplemented with 0.2% arabinose. Equal amounts of cell lysates were subjected to SDS-PAGE followed by immunoblot analyses with specific antibodies for each component. Polyclonal antibodies used to detect the LepB, SecA, SecB, and OmpA were prepared from rabbits *via* standard immunological procedures. *S. typhimurium* strains; CK76 (*Δasd*, *araC*::P_{BAD}-*lepB*), CK49 (*Δasd*, *ΔsecA*), CK87 (*Δasd*, *ΔsecB*), χ8554 (*Δasd*).

SecA-specific antibody was used to detect SecA protein expression in CK49 [pBP244], thereby indicating the production of SecA from the cloned gene. In the same manner, CK87 [pBP244] expressed the SecB protein. OmpA was expressed at levels consistent with those observed in all of the hosts, and it exists as a partial precursor in pYA3493; however, those in pBP158 and pB244 uniformly exhibit a change to the mature form. These results show that LepB in the delivery system functions as a normal signal peptidase, and that SecA and SecB maintain high expression levels. The pBP244 in the *Salmonella* host was observed to retain stability through more than 60 generations of continuous subculture. Although the expression of LepB, SecA, and SecB from a multicopy gene can induce cytotoxicity in cells, we were unable to detect any cytotoxicity in the *Salmonella* harboring pBP244.

Improved Secretion of the Protein Through a New Plasmid System

In order to investigate the secretion of proteins by the new system, we employed PspA as a target protein. Among the 588 amino acid residues of the PspA protein, 3–257 of the amino acid residues used in this study corresponded to the immunodominant region of PspA. The modified *pspA* gene was cloned into pBP158 and pBP244, and the clones were designated as pBP222 and pBP438, respectively. *Salmonella* samples harboring various plasmids were cultured in LB broth. The secreted proteins were obtained

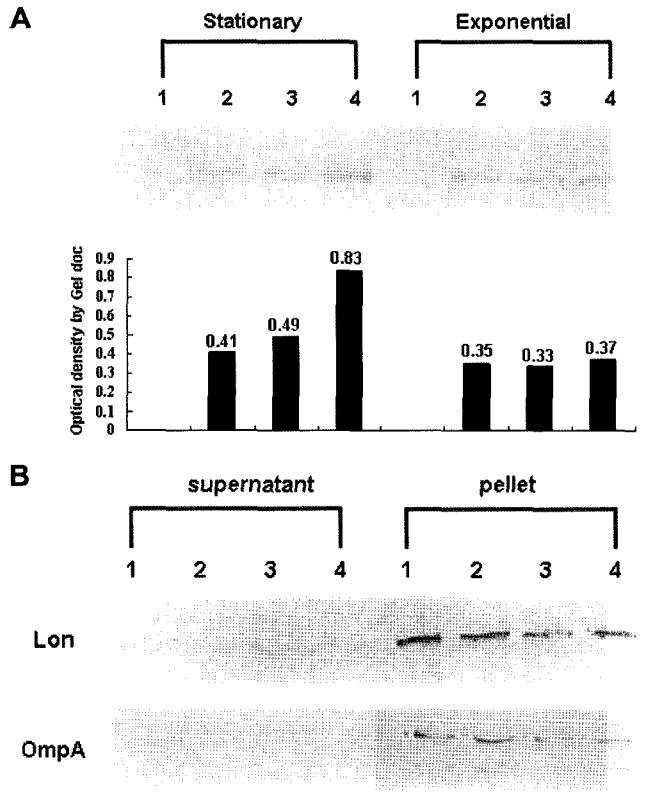

Fig. 4. Analyses of PspA secretion.
A. *Comparisons of secretion yields at different growth stages. S. typhimurium* χ8554 harboring plasmids was cultured in LB broth until either the exponential (0.8 at OD_{600}) or stationary (3.0 at OD_{600}) phase. A volume corresponding to 200 ml of cell-free culture supernatant was subjected to immunoblot analysis. Relative immuno-reactive intensity, as analyzed by Gel doc MyImager (SLB), is shown in the bottom panel. The PspA protein was detected by the PspA-specific monoclonal antibody Xi126 [31]. Lanes: 1, pYA3493 (vector control); 2, pYA3494; 3, pBP222; 4, pBP438. **B.** Analyses for absence of cell-lysis-mediated cell-associated proteins in the supernatant. To examine cell lysis or leaks, the culture supernatants at stationary phase (3.0 at OD_{600}) corresponding to 200 μl volumes or cells (10 μl culture) were subjected to immunoblotting. Lon protease (cytosolic) and OmpA (outer membrane) were utilized as indicator proteins for cell-associated proteins. Polyclonal antibodies specific for Lon or OmpA were employed in the detection of Lon or OmpA. Lanes are the same as in panel A.

from the cell-free culture supernatants of the TCA-precipitation process. The secreted PspA protein was then detected *via* immunoblotting with the PspA-specific monoclonal antibody, Xi126 [31]. The level of PspA secretion in the culture at exponential phase was similar to that of all of the tested samples (Fig. 4A). However, when the *Salmonella* harboring pBP438 was cultured to stationary phase, it exhibited elevated PspA secretion as compared with what was observed with pYA3494 (Fig. 4A). Interestingly, the *Salmonella* [pBP222] harboring the *lepB* and *pspA* genes evidenced no improved secretion, thereby indicating that LepB plays only a minor role in the improvement of secretion. The Lon protease (cytosolic protein) and OmpA (outer membrane protein) were employed as indicators for the examinations of cell integrity. These proteins were not determined to exist within the extracellular matrix (Fig. 4B), which indicates that the PspA detected in this experiment was not released as the result of cell lysis, but was translocated to the extracellular matrix *via* normal secretion with the aid of components cloned in pBP244.

Accumulation of PspA Protein in Periplasmic Space, Outer Membrane, and Supernatant

The components cloned in pBP244 were involved in the sending out of the target proteins to the extracellular component or envelope including the periplasmic space and the outer membrane. Although we detected the secreted PspA, it was first required to assess the distribution of the expressed PspA to the subcellular fractions. In order to characterize PspA localization, we conducted subcellular fractionations for strains harboring pYA3494 or pBP438. The strain harboring pBP438 evidenced a supernatant amount 1.71-fold, a periplasmic space amount 1.47-fold, and an outer membrane amount 1.49-fold of that of pYA3494 (Fig. 5). A Lon protease was employed as a cytosolic indicator in the cell fraction analysis. The Lon was detected only in the cytoplasmic fraction, thereby indicating a lack

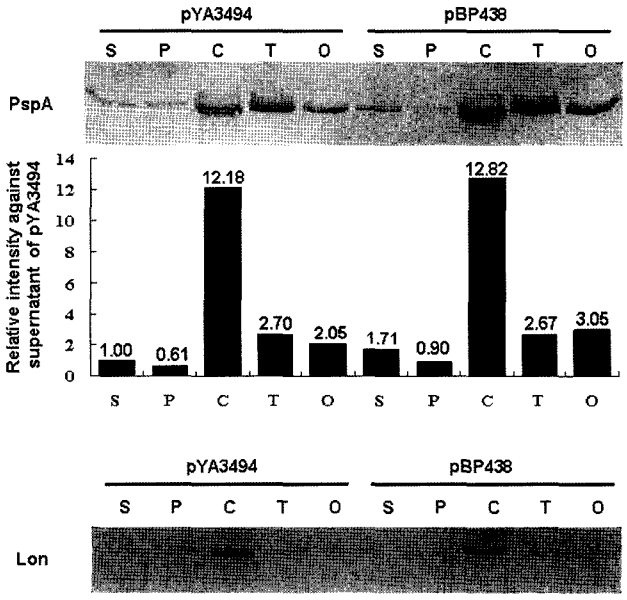

Fig. 5. Subcellular localization of the expressed PspA.
The χ8554 [pYA3494] or [pBP438] was grown to stationary phase, and then fractionated into various subcellular compartments. Fractions equivalent to 250 μl of volume of the culture at an OD_{600} of 3.0 were analyzed *via* SDS-PAGE, and the PspA was detected *via* immunoblotting with the PspA-specific monoclonal antibody, Xi126 [31]. The relative intensity against PspA in the supernatant of χ8554 [pYA3494] was analyzed using a Gel doc MyImager system (SLB) and is presented as a bar chart in the middle panel. Lon was used as a fraction control for the cytoplasmic fraction. Lanes: S, supernatant; P, periplasmic space; C, cytosol; T, total envelope; O, outer membrane.

of cell lysis and/or leaks. It has been reported that the PspA protein was not detected in the outer membrane of *Salmonella* harboring pYA3494 [22]. However, the protein was found in this study to be localized in the outer membrane. Sampling time points at different growth phases may induce variations in the cellular localization of PspA. Cells at stationary phase were analyzed in this study, unlike in a previous study, in which cells at log phase were analyzed. *Salmonella* [pYA3494] or [pBP438] grew slowly on M9 [38] or Curtiss minimal media [8], although *Salmonella* harboring the control plasmids, pYA3493 or pBP244, grew well on these media (data not shown). The highly delayed growth of *Salmonella* harboring pBP3494 or pBP438 showed that the accumulation of PspA into the periplasmic space and the outer membrane may induce a retardation of growth.

In summary, we have constructed a new novel protein secretion vector system, which was shown to improve the secretion of the target protein, PspA, up to a supernatant amount 1.71-fold, a periplasmic space amount 1.47-fold, and an outer membrane amount 1.49-fold higher than that of pYA3494. Therefore, the pBP244 plasmid may be useful in the development of a recombinant attenuated-*Salmonella* vaccine, specifically with regard to the mediation of antigen delivery.

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