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A Novel Expression System for *Salmonella typhimurium* Allowing High Production Levels, Product Secretion and Efficient Recovery

Sissela Liljeqvist,* Diana Haddad,† Klavs Berzins,† Mathias Uhlén,* and Stefan Ståhl*,[1]

**Department of Biochemistry and Biotechnology, Royal Institute of Technology (KTH), S-100 44 Stockholm, Sweden; and †Department of Immunology, Stockholm University, S106 91 Stockholm, Sweden*

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A novel expression system for heterologous production in *Salmonella typhimurium,* taking advantage of the promoter, signal sequence and two IgG-binding domains (ZZ) from staphylococcal protein A, has been investigated. The production of two different fusions proteins, ZZ-M3 and ZZ-M5, was characterized in terms of production levels, product localization (periplasm or culture medium) and product quality after affinity purification. High expression levels and efficient product secretion were obtained, making the system attractive for vaccine development. The potential use of *S. typhimurium* as host for heterologous production in biotechnology is discussed.

Attenuated strains of *Salmonella* have been investigated for more than a decade as live bacterial vaccine vectors for delivery of recombinant antigens [1,2] and therapeutic agents [3]. Recently, *Salmonella* has also been investigated as an alternative to *E. coli* for the production of recombinant proteins. Martin-Gallardo and coworkers [4] demonstrated that the attachment glycoprotein from human respiratory syncytial virus could be produced and recovered using *S. typhimurium* as production host. Attempts to produce the same protein in *E. coli* failed to yield detectable amounts of recombinant protein [4].

When utilizing live *Salmonella* for the delivery of antigens, heterologous expression from the chromosome has been compared to plasmid expression [5]. The significantly higher expression levels obtained when using plasmid expression have been demonstrated to correlate with increased humoral and mucosal antibody levels to the delivered antigen [5]. This indicates that the initial amount of antigen delivered by the live *Salmonella* vector is an important factor for eliciting strong immune responses, while stable expression for a long time has less influence in this context [5]. In addition, an increase in the amount of foreign antigen expressed by recombinant *S. typhimurium* has shown to be important in overcoming genetic restriction [6]. Furthermore, the cellular localization of the heterologous antigen has been demonstrated to influence the immune responses [7–9], where targeting of foreign antigenic determinants to outer cellular locations, i.e. the cell surface or the periplasmic space, has been considered advantageous [7–9].

The promoter and secretion signals from the gene of staphylococcal protein A (SpA) from *Staphylococcus aureus* have earlier shown to be functional in the Gram-negative *E. coli* [10]. Two synthetic IgG-binding domains (ZZ) derived from SpA have been constructed and utilized as an affinity tail for affinity purification purposes [11]. The promoter, secretion signal and ZZ-domains from SpA have been used for the production and recovery of various fusion proteins, which have been found to be secreted to the periplasm or culture medium of *E. coli* [12]. Expression levels in the range of 500–800 mg per litre for fermentor cultivations have been reported [13,14], and pilot and large scale production schemes based on the SpA expression system have been evaluated [13–15].

Since there exists a need for high-level expression systems in *Salmonella* spp. used for vaccine development, and since *Salmonella* strains have been evaluated for heterologous production [4], we

[1] To whom correspondence should be addressed. Fax: +468245452.

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have investigated the use of the SpA expression system in *S. typhimurium.* The expression of two different fusion proteins, ZZ-M3 and ZZ-M5, consisting of the IgG-binding domains fused to the malarial immunogens M3 and M5, respectively, derived from the *Plasmodium falciparum* antigen Pf155/RESA [16], has been investigated. Expression levels, efficiency of the secretion system and product quality after purification by affinity chromatography have been characterized and compared with the expression of the same fusion proteins in *E. coli.*

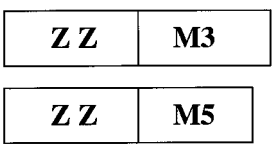
MATERIALS AND METHODS

Bacterial strains and expression vectors. Escherichia coli strain RRIδM15 [17] and *Salmonella typhimurium* aroA strain SLδ33-2 (created by Drs. Jo Ann Flynn and Maggie So, Scripps Clinic, La Jolla, CA) were used as hosts for production of the fusion proteins. The restriction negative *S. typhimurium* LB5000 [18] was used as an intermediate transformation recipient of the plasmid DNA. The construction of the plasmid vectors pEZZM3 and pEZZM5, encoding the fusion proteins ZZ-M3 and ZZ-M5, respectively, has been described elsewhere [16,19].

Protein expression, purification and characterization. The recombinant bacteria were grown in 1000 ml shake flasks at 37°C in 100 ml tryptic soy broth, 30 g/l (LabM, Lancs, UK), complemented with yeast extract, 5 g/l (Fould Springer, Maisons-Alfort, France), and ampicillin, 100 mg/l. Samples were collected every 20 minutes for optical density (OD) ($A_{595\ nm}$) measurements. At OD 1 or 4, respectively, the bacterial cells were pelleted by centrifugation at 6,000 × *g*, and periplasmic proteins were released by an osmotic shock procedure [20]. Both culture medium and periplasmic content were filtered (1.2 μm) and loaded on 5 ml columns of IgG-Sepharose [21] (Pharmacia Biotech, Uppsala, Sweden), previously equilibrated with washing buffer (50 mM tris/HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20). After loading, the columns were washed with 20 column volumes of washing buffer followed by 5 volumes of 5 mM NH_4Ac, pH 6.0, the latter to lower the buffer capacity which enables efficient elution. The proteins were eluted with 0.3 M HAc, pH 3.3, and 1 ml fractions were collected. The protein content in eluted fractions was determined by absorbance (A_{280nm}) measurements, and relevant fractions were pooled and lyophilized. The purification of ZZ-M3 and ZZ-M5 by IgG affinity chromatography was performed well below the saturation level for the IgG-Sepharose which earlier has been shown to give close to 100% recovery of ZZ-fusions [14,15]. This allowed us to use IgG affinity purified material to estimate expression levels. The affinity purified fusion proteins from culture medium or periplasm of *E. coli* and *S. typhimurium* were analyzed by gradient (8–25%) SDS-PAGE analysis under reducing conditions. The Coomassie Brilliant Blue staining correlated well with protein amounts determined by $A_{280\ nm}$ measurements.

RESULTS AND DISCUSSION

The two fusion proteins ZZ-M3 (25.2 kDa) and ZZ-M5 (22.4 kDa) (Fig. 1) were expressed in parallel in *E. coli* and *S. typhimurium.* The common ZZ portion represents an SpA-derived affinity tail enabling affinity purification on IgG-Sepharose [11]. The M3 and M5 polypeptides represent malarial immunogens which correspond to major repeat motifs from the C-terminal and central regions, respectively, of the *P. falciparum* blood stage antigen Pf155/RESA [16] (Fig. 1). The four recombinant strains, *E. coli* (ZZ-M3), *S. typhimurium* (ZZ-M3), *E. coli* (ZZ-M5) and *S. typhimurium* (ZZ-M5), were cultivated in shake flasks and harvested in early or late exponential growth phase to investigate possible differences in expression levels, product quality or product localization. Early and late exponential growth phase corresponded to optical density ($A_{595\ nm}$) values of one and four, respectively, as determined from the growth curves of the cultures (data not shown).



M3 = $(VEHDAEEN)_5(VEEN)_{10}$

M5 = $(VEEPTVADDEH)_3(VEEPTVAEEH)_2$

FIG. 1. Schematic illustration of the two produced fusion proteins, where ZZ represents the divalent IgG-binding affinity tail and M3 and M5 the two repeat immunogens derived from the *Plasmodium falciparum* malaria antigen Pf155/RESA. The amino acid sequences for M3 and M5 are given in one letter code.

Three cultivations of each of the four recombinant strains were performed. The four recombinant strains behaved similarly, but the recombinant *Salmonella* strains were found to have slightly shorter generation times (ranging from 25 to 35 minutes) as compared to the recombinant *E. coli* strains (43 to 51 minutes) (data not shown).

The gene products ZZ-M3 and ZZ-M5 were purified by IgG affinity chromatography [21], directly from the culture medium or from the periplasmic space fraction released by osmotic shock [20]. The amounts of eluted product were estimated by absorbance ($A_{280\ nm}$) measurements and also checked by Coomassie staining of SDS-PAGE gels. Total expression levels of ZZ-M3 and ZZ-M5 and product localization, at early and late exponential phase, are presented in Table 1. Neither for *E. coli* nor for *S. typhimurium* there seemed to be be any significant increase in production levels in late exponential phase. Concerning the product localization, approximately 40% of the fusion protein, being either ZZ-M3 or ZZ-M5, was found to be be targeted to the culture medium, meaning that approximately 60% was found in the periplasm of the bacteria. For ZZ-M3, there seemed to be somewhat more material released to the culture medium in late exponential phase (Table 1). The expression levels in these shake flask cultivations were high, ranging from 30 to 45 mg per litre culture. It has been demonstrated earlier that by performing the cultivations under controlled conditions in fermentors instead of in shake flasks, expression levels can be further increased at least 10-fold [14]. ZZ-M5 has indeed been produced by *E. coli* in a fermentor at an expression level of 550 mg/l [14]. In fermentor cultivations exponential growth can be maintained up to optical densities exceeding 100 [14].

To investigate possible differences in the product quality, ZZ-M3 and ZZ-M5, affinity purified from the medium and periplasmic space of both *E. coli* and *S. typhimurium*, were subjected to SDS-PAGE analysis (Fig. 2). No significant differences in the product quality when recovered from *E. coli* or *S. typhimurium* could be detected, bands corresponding to full-length ZZ-M3 and ZZ-M5 being predominat. The apparent sizes of ZZ-M3 and ZZ-M5 were approximately 31 to 29 kDa, respectively, in contrast to the predicted sizes of 25.2 and 22.4 kDa,butt such abnormal migration has been demonstrated earlier [19,21]. A slight degradation can be observed for both ZZ-M3 and ZZ-M5 but the degradation was not found to be more pronounced in *S. typhimurium.* No significant difference was seen with regard to degradation when comparing periplasmic and culture medium material, although previous studies have indicated that the culture medium material is less affected by degradation [12]. The weak bands appearing above the full-length bands (Fig. 2) represent probably fusion proteins with unprocessed signal peptide, as has been observed earlier for related secreted fusion proteins [21].

In conclusion, it has been demonstrated that the SpA-derived expression system gives high levels of expression and product secretion to the periplasm and culture medium in *S. typhimurium* as determined for the production of two different fusion proteins, ZZ-M3 and ZZ-M5. The expression

TABLE 1

Results from the Production of the Fusion Proteins ZZ-M3 and ZZ-M5 in *E. coli* and *S. typhimurium* Grown to Early or Late Exponential Growth Phase[a]

	Early exponential phase		Late exponential phase	
Bacterium/ gene product	Expression level (mg/l)	Fraction found in the culture medium (%)	Expression level (mg/l)	Fraction found in the culture medium (%)
E. coli/ZZ-M3	45	40	40	50
S. typhimurium/ZZ-M3	35	40	40	60
E. coli/ZZ-M5	30	40	40	40
S. typhimurium/ZZ-M5	30	40	30	40

[a] The values are representative results after three to five cultivations of each recombinant bacterium at two different growth phases. Early and late exponential growth phases were defined from growth curves as cultivations grown to optical density (A_{595} nm) values of one and four, respectively.

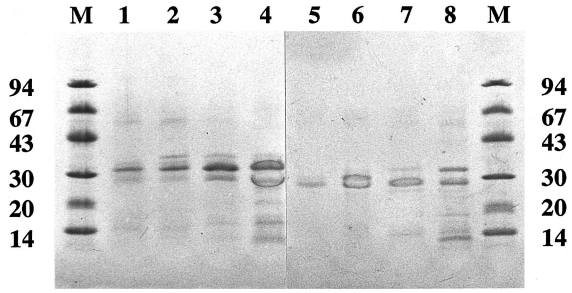


FIG. 2. SDS–PAGE analysis of the expressed and affinity-purified fusion proteins on a 8–25% polyacrylamide gel under reducing conditions. ZZ-M3 recovered from the culture medium (Lane 1) or periplasm (Lane 2) of *E. coli.* ZZ-M3 recovered from the culture medium (Lane 3) or periplasm (Lane 4) of *S. typhimurium.* ZZ-M5 recovered from the culture medium (Lane 5) or periplasm (Lane 6) of *E. coli.* ZZ-M5 recovered from the culture medium (Lane 7) or periplasm (Lane 8) of *S. typhimurium.* Lane M, marker proteins with approximate molecular masses in kDa indicated in the margins.

system could be suitable for the expression of various antigens when investigating *S. typhimurium* for live bacterial vaccine applications. In addition, various *Salmonella* subspecies could potentially be investigated as hosts for heterologous production of recombinant proteins in the field of biotechnology.

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