MiniReview

Secretory delivery of recombinant proteins in attenuated Salmonella strains: potential and limitations of Type I protein transporters

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Abstract

Live attenuated Salmonella strains have been extensively explored as oral delivery systems for recombinant vaccine antigens and effector proteins with immunoadjuvant and immunomodulatory potential. The feasibility of this approach was demonstrated in human vaccination trials for various antigens. However, immunization efficiencies with live vaccines are generally significantly lower compared to those monitored in parenteral immunizations with the same vaccine antigen. This is, at least partly, due to the lack of secretory expression systems, enabling large-scale extracellular delivery of vaccine and effector proteins by these strains. Because of their low complexity and the terminal location of the secretion signal in the secreted protein, Type I (ATP-binding cassette) secretion systems appear to be particularly suited for development of such recombinant extracellular expression systems. So far, the Escherichia coli hemolysin system is the only Type I secretion system, which has been adapted to recombinant protein secretion in Salmonella. However, this system has a number of disadvantages, including low secretion capacity, complex genetic regulation, and structural restriction to the secreted protein, which eventually hinder high-level in vivo delivery of recombinant vaccines and effector proteins. Thus, the development of more efficient recombinant protein secretion systems, based on Type I exporters can help to improve efficacies of live recombinant Salmonella vaccines. Type I secretion systems, mediating secretion of bacterial surface layer proteins, such as RsaA in Caulobacter crescentus, are discussed as promising candidates for improved secretory delivery systems.

Keywords: ATP-binding cassette transporter; Caulobacter crescentus; Hemolysin; Protein secretion; Salmonella; Surface layer

1. Introduction

Live attenuated Salmonella strains expressing heterologous antigens or effector proteins are promising vaccine candidates for humans and animals. They are generally immunogenic, well tolerated and considered to be safe. Due to a number of mutations in virulence-associated genes such strains experience a drastic reduction in pathogenicity, but remaining virulence is still sufficient to trigger efficiently immune responses [1–3].

Despite some major progress over the past years on the construction of improved and better tolerated Salmonella vaccine strains [4,5], Salmonella-based recombinant vaccines reveal one major drawback. Particularly in humans, immune responses triggered against the heterologous antigens expressed by the Salmonella cell are generally masked by a dominant immune response against the bacterial antigens. Therefore, immune responses against recombinantly expressed antigens in Salmonella vaccines are relatively weak compared to immune responses induced through parenteral administration of the recombinant vaccine antigen. This is probably the reason that no recombinant Salmonella vaccine has commercially outperformed its parenteral counterpart yet.

There is clear experimental evidence that the expression level and, more importantly, the cellular location of the vaccine antigen or effector protein, can strongly influence the immune responses induced by live recombinant Salmonella, stressing the need for efficient recombinant protein delivery systems [6,7]. Secretion of recombinant proteins in Salmonella like in other Gram-negative bacteria requires specific translocation of the exoprotein across the inner and outer membrane and depends on naturally occurring secretion systems, which have been adapted to recombinant protein secretion by genetic modifications.

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Taking fundamental differences in the secretion process into account, Gram-negative bacteria have acquired five different basic protein secretion mechanisms, which are classified in Type I to Type IV secretion systems and autotransporters, also referred to as Type V secretion systems [8,9]. Despite various attempts to utilize Type III and Type V secretion systems for recombinant protein secretion in Salmonella [10,11], only Type I secretion systems combine a number of striking features which make them superior to other secretion systems for recombinant protein delivery [12,13]. Among those Type I secretion systems the Escherichia coli hemolysin (Hly) system is currently the only protein exporter, which has been developed to a versatile and universally applicable secretory expression system [14].

Supported by own data, this review provides an update on extracellular delivery of foreign proteins in Salmonella via the Hly system, critically examining the advantages and limitations of the system. Apart from that, the Caulobacter crescentus S-layer protein exporter is introduced as an alternative secretion system, which, for many reasons, is superior to the Hly system and eventually can provide a platform for development of more efficient live recombinant Salmonella vaccines.

2. Proteins delivered by attenuated Salmonella

Besides vaccine antigens mucosal adjuvant proteins, such as subunits of CT, LT and the tetanus toxin fragment C, have been coexpressed in Salmonella vaccine strains and their immunoadjuvancy and immunogenicity assessed [15,16]. Furthermore, cytokines, including interleukin (IL)-4, IL-5, and IL-6, have been expressed in attenuated Salmonella strains, hypothesizing that in situ delivery of bioactive cytokines may enhance or modulate antibody responses [17-20]. Apart from that, recombinantly expressed cytokines were explored as therapeutic proteins [21,22]. Attempting to influence the intracellular location of Salmonella cells, cytolsin genes were introduced in vaccine strains [23]. More recently, attenuated Salmonella have attracted the interest for targeted delivery of tumoricidal proteins as another class of effector proteins [24].

3. Advantages of extracellular protein delivery

In case of intracellular accumulation of a recombinantly expressed vaccine antigen, the induction of the immune response depends on the portion of antigen, which becomes released during the disintegration of the bacterial carrier. Indeed, experimental evidence has been gathered, clearly supporting the immunostimulatory effect of extracellular antigen delivery in Salmonella vaccine strains (7,25,26; Li et al., unpublished results).

Extracellular protein expression appears to be even more important for effector proteins, where correct folding of the protein is crucial for maximal biological and enzymological activity, particularly when essential disulfide bond formation is involved. On the one hand, export of the effector proteins from the reducing bacterial cytosol eventually facilitates intramolecular disulfide bond formation. On the other hand, export of the effector protein prevents the formation of insoluble protein aggregates within the cells, which is generally a problem with most cytokines, thereby contributing to the solubility of the protein. Finally, as for vaccine antigens, extracellular presentation of the effector protein is required for optimal action.

To substantiate putative immunity-modulating effects of in vivo expressed cytokines, we have depicted human IL-6 (hIL-6) as model cytokine [20,27,28]. Comparative immunization data with Salmonella typhimurium strains, expressing genetic fusions of hIL-6 with E. coli α-Hly (HlyA) clearly supported that secretory expression of hIL-6 enhances systemic and mucosal antibody responses against bacterial antigens more efficiently than intracellular arresting of the same hIL-6 fusion protein [29]. These data are in contrast to previous studies with intracellularly expressed mouse IL-6 and IL-4, which failed to demonstrate any effect on the systemic humoral immunity [18,19]. On the other end, high-level intracellular expression of IL-1β and IL-2 in S. typhimurium as therapeutic proteins provoked a significant biological effect in mouse immunization models [21,22]. In contrast to IL-6 and IL-4, which tend to form insoluble protein aggregates, the latter are expressed as soluble proteins in bacterial cells, suggesting that the biological effect observed is caused by recombinant cytokine released from lysed cells in consequence of the protein overexpression.

Thus for both, protein antigens and effector proteins, extracellular delivery and bioavailability is required for maximal immunostimulatory and immunomodulatory action.

4. Secretion systems in Gram-negative bacteria

Among the different secretion systems found in Gram-negative bacteria, Type I secretion systems are found in almost any bacteria genera and are involved in the direct export of a large number of different proteins [12,13]. Type II secretion systems depend on the Sec pathway for transport of the protein into the periplasm. Export across the outer membrane requires a second specialized transport apparatus, consisting of at least 12 components, constituting a gated channel between the periplasm and the external medium [30]. Both, Type III and Type IV secretion systems are of still higher complexity, consisting of a minimum of 17 and nine proteins, respectively. Type III secretion systems are mostly responsible for delivery of cytoplasmic proteins into the host cells by different patho-
genic bacteria [31]. Type IV secretion systems are responsible for translocation of large multisubunit proteins across the outer membrane, showing a striking similarity with the Agrobacterium tumefaciens T-DNA transport machinery [9]. Additionally, Gram-negative bacteria utilize autotransporter mechanisms for protein export [8]. Autotransporter or Type V secretion systems display a common three-domain structure, consisting of an N-terminal leader peptide domain, a ‘passenger domain’ encompassing the secreted exoprotein, and a C-terminal translocator domain, forming a β-barrel-like structure in the outer membrane which functions as secretion pore [32].

Because of conserved structure and sequence features in the targeting signals of the secreted proteins only Type I and Type III exporters as well as autotransporters are potentially useful for extracellular recombinant antigen delivery. S. typhimurium Type III secretion systems enable recombinant antigen delivery right to the cytosol of host cells [10]. However, their complexity and the involvement of multiple secretion pathways for the transport of different exoproteins restrict the development of efficient and universally applicable antigen and effector protein secretion systems on the basis of the Type III secretion machinery. Autotransporters on the other hand allow cell surface display of recombinant proteins only and proteolytic release of the recombinant protein moiety relies on the Omp T protease [11,33].

5. Type I protein exporter

Type I or ABC (ATP-binding cassette) secretion systems are most suited for recombinant protein secretion in heterologous systems for a number of reasons [12,13,34]. First, the Type I secretion apparatus consists of three proteins only and, thus reveals a relative low complexity. Secondly, Type I secretion systems rely on a distinct secretion signal localized at the C-terminus of the secreted protein. Thirdly, the topology of the secretion apparatus and the export mechanism is comparably well understood.

Generally the Type I secretion apparatus consists of an ABC translocase and two auxiliary protein components, forming a transporter complex, which spans both, inner and outer membrane, and intervenic periplasmic space. The ABC translocase is integrated in the inner membrane and contains a cytoplasmic domain comprising a characteristic ATP binding cassette sequence motif [35]. Because of its topological structure the first auxiliary component belongs to the family of membrane fusion proteins (MFPs) [36,37]. The MFP is anchored via a short N-terminal hydrophobic sequence in the inner membrane. The extended hydrophilic region of the protein is protruding into the periplasm and interacting with the second auxiliary component, an outer membrane protein (OMP), which is often provided by a multifunctional OMP component of the host cell [38].

Based on the structural and functional relationship of the translocated exoproteins Type I protein exporters are divided into two families [39]. Transporters of the RTX toxin exporter family mediate the secretion of pore-forming cytolsins while lipases, proteases, and other degrading enzymes are transported by members of a different transporter family. The prototype of RTX toxin exporters and the by far best-characterized ABC transporter is the E. coli HlyA secretion system [34].

A series of biochemical and genetic evidence along with the recent elucidation of the three-dimensional crystal structure of TolC [40–42], the OMP component of the HlyA exporter, supports the following model for the Type I secretion process. Importantly, the Type I secretion apparatus appears to be a dynamic structure. Transporter components, which are not immediately engaged in protein export, reside in a disassembled state within the inner and outer membrane, respectively.

Apparently, a trimeric form of the ABC translocase is transferred into an active state by ATP hydrolysis through the cytoplasmic domain [41]. The resulting conformational change of the energized ABC translocase allows a specific interaction with the secreted protein, most probably via the C-terminal secretion signal [43]. Upon interaction with the secreted protein, the membrane-bound region of the ABC translocase assembles with the MFP and OMP components [41,44]. Presumably in a two-step process three MFP subunits are recruited by the activated ABC translocase, forming a MFP–ABC translocase complex, which is associated with the secreted protein. Putative conformational changes in the MFP subunits are then responsible for specific aggregation with the homotrimeric OMP component [42]. The OMP subunits form a continuous solvent-accessible conduit, which spans both the outer membrane and the periplasmic space, and is sealed at the periplasmic end [41]. Interactions with the MFP–ABC translocase complex finally lead to an opening of the channel by an allosteric mechanism resulting in the establishment of a continuous conduit that transiently connects the cell cytosol to the external environment, through which the unfolded polypeptide chain of the exoprotein is transported [40]. Once the transported protein is released on the outside from the secretion pore, the transporter disengages and the protein components revert to their separate inner and outer membrane resting state [41]. Thus, the energy stemming from the ATP hydrolysis and stored in an activated conformation of the translocase provides the driving force for the transport of the exoprotein.

To some extent this model is rather speculative and it may be true for HlyA and closely related RTX-type exporters only. However, it provides an explanation of how the cytosol is connected to the external environment to allow in a single energy-coupled step direct passage of an exoprotein through the inner and outer membrane and the intervening periplasmic space.
6. The HlyA secretion machinery

6.1. Genetic organization

The plasmid-encoded HlyA secretion system initially characterized by Goebel and coworkers [45] comprises four structural genes, organized in a single transcriptional unit (Fig. 1). Besides the genes for HlyA (hlyA), the ABC translocase (hlyB), and the MFP component (hlyD), the operon encodes an acetylase (hlyC) required for posttranslational activation of HlyA. The gene encoding the OMP component is not linked to the hlyCABD operon upstream of the mapped transcription start site [87], while P_p and P_om indicate the two promoters involved in the SLP secretion in S. marcescens. The horizontal arrows indicate the transcriptional direction of the genes. All three operons are flanked by predicted stem-loop structures indicating rho-independent terminator sequences. For both, the hly and rsa operon, rho-independent terminator sequences have additionally been identified in the intergenic region downstream of the gene encoding the secreted protein. The filled area at the 3’-end of hlyA and rsaA, respectively, highlights the minimal secretion signal sequences identified for both exoproteins. SstI is a predicted gene without a known function [75].

6.2. Secretion of foreign proteins

Mainly by Goebel and coworkers, the Hly secretion system has been adapted to recombinant protein secretion and utilized for secretion of a variety of protein and peptide antigens, including toxins and toxin fragments for elicitation of neutralizing antitoxins. Besides that, the Hly system has been explored as secretory expression system for biologically active effector proteins, including bacterial invasines, cytolysins, and cytokines (Table 1). Two lines of Hly-based expression systems have been developed, depending on a cis- or trans-located expression site for the secretion competent HlyA fusion protein in a single plasmid and two-plasmid expression system, respectively [14,61,62]. The single plasmid system comprises the natural hly operon, utilizing a singular NsiI restriction site to generate genetic fusions of a foreign protein to a residual portion of the hlyA gene encoding the HlyA_s secretion signal. In the two-plasmid system, hlyC and hlyA have been deleted from the operon and a copy of the truncated hlyA gene is provided on a second plasmid under tran-
Table 1
Recombinant vaccine antigens, effector proteins, and enzymes secreted via the Hly export apparatus in *E. coli* and *Salmonella* species

<table>
<thead>
<tr>
<th>Foreign protein</th>
<th>Protein type</th>
<th>Source of foreign protein</th>
<th>Host organism</th>
<th>Expression vector(s)</th>
<th>Location in host</th>
<th>Secreted protein amounts (μg ml⁻¹)</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HlyA</td>
<td>Hly</td>
<td><em>E. coli</em></td>
<td><em>E. coli</em></td>
<td>pMOhly1</td>
<td>E</td>
<td>3–3.5</td>
<td>++</td>
<td>[53]</td>
</tr>
<tr>
<td>DT</td>
<td>Toxin</td>
<td><em>Corynebacterium diphtheria</em></td>
<td><em>S. typhi</em></td>
<td>pMOhly1</td>
<td>C(100)</td>
<td>ND (WB)</td>
<td>Antitoxin</td>
<td>[54]</td>
</tr>
<tr>
<td>LLO&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cytolysin</td>
<td><em>L. monocytogenes</em></td>
<td><em>Salmonella dublin aroA</em> SL5928</td>
<td>pMOhly1</td>
<td>E(50)</td>
<td>&lt; 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>++</td>
<td>[23]</td>
</tr>
<tr>
<td>p60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Invasin</td>
<td><em>L. monocytogenes</em></td>
<td><em>S. typhimurium</em> SL7207</td>
<td>pANN202-812ΔnsrΔCluI</td>
<td>E + BS&lt;sup&gt;f&lt;/sup&gt;</td>
<td>D (WB)</td>
<td>++</td>
<td>[55]</td>
</tr>
<tr>
<td>PagC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>OMP</td>
<td><em>S. typhimurium</em></td>
<td><em>E. coli</em></td>
<td>pMOhly1</td>
<td>E(100)</td>
<td>3–3.5</td>
<td>Antigen</td>
<td>[56]</td>
</tr>
<tr>
<td>IL-6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cytokine</td>
<td>Human</td>
<td><em>S. typhimurium</em> X4064</td>
<td>pMOhly1</td>
<td>E(60) + M/BS</td>
<td>0.02–0.03</td>
<td>++</td>
<td>[20]</td>
</tr>
<tr>
<td>LLO&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Cytokine</td>
<td>Human&lt;sup&gt;g&lt;/sup&gt;</td>
<td><em>E. coli</em></td>
<td>pMOhly1</td>
<td>E(80) + M/BS</td>
<td>0.07–0.08</td>
<td>++</td>
<td>[28]</td>
</tr>
<tr>
<td>IL-6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Cytokine</td>
<td>Human&lt;sup&gt;g&lt;/sup&gt;</td>
<td><em>E. coli</em></td>
<td>pMOhly1+pYL3-1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>E(20) + M/BS + C</td>
<td>&gt; 0.25</td>
<td>n.t.</td>
<td>[29]</td>
</tr>
<tr>
<td>PiA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Pilin</td>
<td><em>P. aeruginosa</em></td>
<td><em>E. coli</em></td>
<td>pMOhly1</td>
<td>E(10) + M/C</td>
<td>&lt; 0.01</td>
<td>Antigen</td>
<td></td>
</tr>
<tr>
<td>IL-1&lt;sup&gt;λ&lt;/sup&gt;</td>
<td>Cytokine</td>
<td>Human</td>
<td><em>E. coli</em></td>
<td>n.m.</td>
<td>C(100)</td>
<td>ND</td>
<td>n.t.</td>
<td>[57]</td>
</tr>
<tr>
<td>Skc</td>
<td>Protease</td>
<td><em>Streptococcus equisimilis</em></td>
<td><em>E. coli</em> K5</td>
<td>pANN202-812</td>
<td>E(50)</td>
<td>0.5–1.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>++</td>
<td>[58]</td>
</tr>
<tr>
<td>OspA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>OMP</td>
<td><em>B. burgdorferi</em></td>
<td><em>E. coli</em></td>
<td>pLG575+pYFC99&lt;sup&gt;f&lt;/sup&gt;</td>
<td>E(50)</td>
<td>D (WB)</td>
<td>Antigen</td>
<td>[59]</td>
</tr>
<tr>
<td>p67&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Antigen</td>
<td><em>Theileria parva</em></td>
<td><em>Salmonella dublin aroA</em> SL5631</td>
<td>pMOhly1</td>
<td>E(100)</td>
<td>D (WB)</td>
<td>Antigen</td>
<td>[7]</td>
</tr>
<tr>
<td>StxB</td>
<td>Toxin</td>
<td><em>Shigella dysenteriae</em></td>
<td><em>S. typhimurium</em> SL3261</td>
<td>pLG575+pSU202&lt;sup&gt;f&lt;/sup&gt;</td>
<td>E(90)</td>
<td>D (WB)</td>
<td>Antitoxin</td>
<td>[60]</td>
</tr>
</tbody>
</table>

D (WB), detectable in concentrated culture supernatant samples by Western blot; ND, not detectable (by Western blot); n.t., not tested; n.m., not mentioned.

<sup>a</sup>DT, diphteria toxin (nontoxic mutant CRM197); LLO, listeriolysin; p60, p60 protein; PagC, OMP encoded by PhoPQ-activated gene; PiA, type pilin A protein; Skc, Streptokinase; OspA, outer surface protein A; p67, p67 sporozoite antigen; StxB, Shiga toxin B-subunit.

<sup>b</sup>C, Cytoplasm; E, extracellular; M/BS, membrane/bacterial surface; M/C, membrane/cytoplasm (estimated portion from total foreign protein amount).

<sup>c</sup>++, Same activity as native protein; +, lower activity than native protein.

<sup>d</sup>Mature protein (without N-terminal leader sequence).

<sup>e</sup>HL-6 gene adapted to *E. coli* codon bias.

<sup>f</sup>Transporter genes provided in *trans* on plasmid pMOhly1 and pLG575, respectively.

<sup*g</sup>Small amount attached to the bacterial surface.

<sup*h</sup>Estimated from published Coomassie-stained protein gel.

<sup>i</sup>Estimated from comparative zymogram analysis using commercial Skl preparation.

<sup>j</sup>Six-fold lower than wild-type protein.

<sup>k</sup>Hahn et al., unpublished results.
scriptional control of the lac promoter. Although data from comparable studies are not available there is apparently no marked difference in the secretion efficiencies of HlyA fusion proteins between the two-vector systems [14]. However, besides the construction of vector systems for stable chromosomal insertion of the hly operon and inducible gene expression [63], little progress has been made on the optimization of hly-based vectors for improved extracellular expression of recombinant proteins. This mainly stems from the poor understanding of the transcriptional regulation of the hly operon and the fact, that tightly balanced gene expression levels of HlyA and the transporter protein components are extremely crucial for maximal secretion efficiency.

6.3. Limitations of the system

6.3.1. Secretion capacity

Among various disadvantages, the most serious limitation of the Hly system is the low secretion capacity. Genttsch et al. [14,53] reported secretion efficiencies of up to 2–3 mg l⁻¹ for certain Hly fusion proteins, which are almost comparable to the efficiencies reported for the natural substrate HlyA. Furthermore, Blight and Holland [57] mentioned secretion efficiencies for heterologous fusion proteins, which even reflect an increase of 50- to 100-fold over the secretion efficiency of the wild-type system. However, from our own experience [20,28], and published data of other groups listed in Table 1, secretion protein amounts generally do not exceed 500 ng ml⁻¹ of culture supernatant, which corresponds to 30–120 ag or 5–20 secreted protein molecules per bacterial cell for a Hly fusion protein with a Mᵣ of 50 000 Da.

Qualitative analysis of the extracellular and cellular distribution of a hIL-6-HlyA fusion protein, expressed from the commonly used Hly vector pMOH1 [20; Li et al., unpublished results] clearly demonstrated that the gene expression, rather than the protein export via the Hly transporter, was the limiting step in secretion efficiency. We confirmed this data by optimization of the transcription and transcription efficiency of the hIL-6-hlyA gene. Both codon adaptation and an increase in gene copy number resulted in a three- to 10-fold enhancement of secreted fusion protein levels [28,29]. Thus, optimization of the expression level of a hlyA gene fusion may be one way to increase the secretion efficiency for a certain Hly fusion protein in Salmonella.

6.3.2. Growth-phase dependency

The activity of the Hly secretion system is tightly coupled to bacterial growth and accumulation of HlyA and Hly fusion proteins in the culture supernatant occurs only in the early and mid-exponential growth phase [20,64]. This tight regulation of the Hly secretion system most probably appears at the transcriptional level and possibly involves sequential activation of different promoters within the Pｈly complex [47]. Consequently, continuous secretion of HlyA-tagged antigens or effector proteins eventually demands permanent bacterial growth in vivo upon oral administration of live attenuated Salmonella in the course of a vaccination. It is reasonable to assume that during the initial phase of the establishment of the ‘mild infection’, when bacteria have to adapt to the in vivo environment, Hly-mediated recombinant protein secretion may be drastically reduced, if not totally impaired. Even in a later stage, when Salmonella persist within immunocompetent cells, bacteria probably do not divide at maximal rate time [65]. Therefore, Hly-mediated protein secretion by recombinant Salmonella vaccine strains may be significantly lower in vivo than in vitro.

6.3.3. Oxygen-dependent regulation

Rather unexpected we found that hIL-6-HlyAᵦ amounts in culture supernatants of recombinant E. coli and S. typhimurium depend strongly on the culture size, with protein amounts significantly declining in larger culture volumes (Fig. 2). More precise studies using standardized preculture and inoculum conditions revealed that final hIL-6-HlyAᵦ concentrations in 250-ml flask cultures were at least 10-fold lower as those reached in 5-ml tube cultures, suggesting that aeration is negatively affecting hIL-6-HlyAᵦ secretion. Comparative growth studies under anaerobic conditions confirmed the postulated oxygen-dependent regulation of the Hly secretion system (Danner et al., unpublished data). Moreover, hIL-6-HlyAᵦ secretion was no longer restricted to the early growth phase and increased levels of hIL-6-HlyAᵦ were found in exponential phase cells, indicating that the translocation has become the rate-limiting step in secretory expression of the HlyA fusion protein. This is probably due to the reduced availability of ATP within these cells. Although data are still

![Fig. 2. Effect of culture size on secretion efficiency of HlyA fusion protein. E. coli XL1-Blue/pCH1A [27] was grown aerobically in shaking cultures of 5, 50 and 250 ml to stationary phase. Proteins from cell-free culture supernatants were separated by SDS-PAGE and secreted hIL-6-HlyAᵦ fusion protein detected by hIL-6-specific Western blotting. In each lane, proteins from 200 µl supernatant were loaded. Supernatant from the strain carrying plasmid pCH2G was loaded as a control. The arrows indicate the full-length fusion protein and the main degradation product (variant I) [20].](image-url)
preliminary they confirm earlier results [66], postulating an activation of the Hly system by anaerobiosis.

Due to a reduced availability of molecular oxygen within the host upregulation of the Hly secretion system may also appear in vivo, implying higher secretion levels for vaccine antigens and effector proteins adapted to Hly-mediated export than anticipated from in vitro expression data. According to our knowledge nobody has addressed the question of the in vivo activity of the Hly secretion system yet, and there is certainly room for speculation.

6.3.4. Structural restrictions

Many HlyA, fusion proteins were secreted into the culture supernatant with varying efficiencies (Table 1) and a number of structural features within the secretion-competent proteins have been identified, which are thought to influence translocation of secretion-competent proteins. Inhibition of the secretion is observed when fusion proteins additionally carry a sec-dependent N-terminal secretion signal [58,67], most likely because of an interference of the two secretion pathways for the same substrate protein. Also the length of the Hly fusion protein is apparently affecting the secretion in that way that longer proteins are secreted at lower efficiencies [14]. Furthermore, proteins, which tend to form inclusion bodies or fold rapidly within the cytoplasm are secreted rather inefficiently [53,68]. Finally, we have found that Hly-mediated secretion is greatly impaired when secreted proteins contain extended β-sheet regions (unpublished data) which is the case for many structural proteins of bacterial surface appendages.

7. Alternative Type I secretion systems for efficient recombinant protein delivery

Presently, the Hly secretion system is the only Type I transporter, which has been successfully explored for recombinant vaccine and effector protein secretion in bacterial vaccine strains [14]. However, the various limitations of the system eventually prohibit high-level secretory delivery of recombinant proteins in Salmonella vaccine strains, which is crucial for induction of more intense immune responses and effector protein action. Although the secretion efficiency can be increased within a limiting range for certain proteins [28,29], there is probably not much room for general improvement of Hly-based secretion systems, demanding the exploration of more efficient and less restrictive secretion systems for recombinant protein export.

7.1. Type I secretion systems mediating surface (S)-layer export

Bacterial S-layers are generally composed of S-layer protein (SLP) subunits, forming a paracrystalline surface array on the outside of the bacterial cell envelope [69]. Among the large number of Type I secretion systems found in Gram-negative bacteria [12], secretion systems involved in the export of (S)-layers combine several prerequisites for development of more efficient recombinant protein exporters. Compared to Hly, which is a minor secreted extracellular protein, SLPs generally account for a large portion of the cellular protein, implementing high transporter efficiencies for the export of these proteins. Furthermore, S-layer protein synthesis, secretion, and assembly are permanently required during cell division and growth, suggesting a constitutive regulation of the transporter genes.

The majority of bacterial SLPs carries N-terminal signal sequences and is secreted via the general sec-dependent secretion pathway [12,69]. However, SLPs, which do not depend on N-terminal secretion signals and are most probably secreted via a Type I secretion mechanism involving a C-terminal secretion signal, have been identified in Serratia marcescens [70], Campylobacter fetus [71,72], Campylobacter rectus [73,74], and C. crescentus [75]. Generally, SLPs are the most abundant proteins in these bacteria, constituting as much as 10–15% of the total cell protein [76–78].

As for other ABC transporters, genes encoding SLPs and the Type I transporter components are clustered; however, genes incorporated into the S-layer operon and the complexity of the operon structure varies between the different Type I-mediated SLP secretion systems. In Campylobacter, putative genes for the ABC translocase (sapD), the MFP (sapE), and the OMP component (sapF) are located immediately downstream of a gene encoding a protein of an unknown function (sapC), most probably cotranscribed from several σ70-like promoters upstream of sapC [71]. The sapCDEF operon is located on an inversive fragment flanked by two SLP encoding-gene variants (sapA). A recA-dependent inversion of the fragment and a unique outward facing sapA promoter are responsible for the antigenic variation of SLPs in Campylobacter [79]. The S. marcescens Lip secretion system, which specifically promotes the secretion of SLP and two other unrelated proteins [70,80], reveals a similar operon architecture as the C. fetus Sap system (Fig. 1). Genes for the ABC translocase (lipB), MFP (lipC), and OMP component (lipD) are linked to a single transcription unit, which is followed by a rho-independent terminator [81]. The gene encoding the SLP (slaA) is situated immediately upstream of the lip operon in the same transcriptional orientation, while the genes encoding the two other protein substrates of the Lip exporter are not closely located to the lip gene cluster. As an unlinked monocistronic transcription unit slaA is flanked by its own promoter and terminator [70]. Because of missing sequence homologies in the secretory proteins, it remains to be determined whether the secretion signal is located at the C-terminus of the SlaA protein, as it is in many other ABC exporter substrates.
Heterologous reconstruction of SLP secretion has been demonstrated for the *C. fetus* Sap and the *S. marcescens* Lip secretion system in *E. coli* [70,71]. However, the complexity and the unknown function of certain gene components of the *sap* operon, and the comparably poor understanding of the secretion signal of the Lip exporter may hinder the development of recombinant protein secretion systems based on any of these SLP exporters [82].

### 7.2. The *C. crescentus* RsaA protein exporter

From the Type I secretion systems mentioned before, the *C. crescentus* S-layer transporter seems to be the most suitable candidate for development of more efficient recombinant antigen and effector protein expression systems. In *C. crescentus*, the regular surface array protein (RsaA) comprises 1026 amino acids [83] with an estimated 50,000 copies of the protein found per cell [77]. Compared to the amounts of Hly protein generally accumulated in the culture supernatant of *E. coli*, secreted RsaA amounts are in the range of 500 to 1000-fold larger. Thus, with respect to the secretion efficiency the RsaA secretion system is by far superior to the Hly secretion system. Whether this secretion efficiency is due to a drastically higher transporter activity or the result of a significant increase in the number of transporters per cell remains unclear. Over the past years, significant progress has been made on the understanding of the genetic organization and the regulation of the Rsa secretion system as well as the delineation of the sequences important for RsaA secretion [84-86]. Experimental evidence has been provided that, in contrast to the hly operon, the rsa operon is constitutively expressed from a single promoter located upstream of the rsaA gene and no putative activator elements have been identified yet [87] (Fig. 1). However, this promoter is not functional in *E. coli* and *Salmonella*. Interestingly in both, the hly and rsa operon, transcription of genes downstream of hlyA and rsaA, respectively, are apparently regulated by an antitermination mechanism, leading to an occasional readthrough into the adjacent translocase gene region [75].

By genetic linkage of the C-terminal secretion signal of RsaA to foreign protein sequences of various lengths, the *C. crescentus* S-layer secretion system has been repeatedly used for secreting large quantities of hybrid proteins into the medium [85]. Furthermore, plasmid-based secretion systems have been developed and commercialized, utilizing truncated rsaA genes for construction of gene fusions encoding the C-terminal secretion signal of RsaA linked to the sequences encoding the recombinant protein. Therefore, the feasibility of adapting the *C. crescentus* S-layer secretion to recombinant protein export has already been demonstrated. However, earlier attempts by our group and other researchers to express the rsa operon functionally in *E. coli* under transcriptional control of an *E. coli* promoter were not successful. This is probably due to the fact that the gene encoding the pore-forming OMP component of the S-layer secretion system in *C. crescentus* had not been identified so far.

Apparently, TolC, which provides the OMP component for the Hly transporter in *E. coli* and *Salmonella*, can not functionally complement the RsaA OMP component in *C. crescentus*. Another reason for the failure of a heterologous expression of the rsa operon may be, that in *E. coli* the termination signal downstream of the rsaA gene is not properly antiterminated, leading to a repression of the rsaD and rsaE gene expression.

Unlike other known ABC transporters mediating the secretion of S-layer proteins, but similar to the hly secretion system, in *C. crescentus* the putative omp gene is not linked to the rsa operon [75]. To provide a rational basis for heterologous reconstitution of the *C. crescentus* S-layer secretion system in *E. coli*, we have focussed on the identification of the gene for the missing OMP component. Taking advantage of the recently deciphered *C. crescentus* CB2 genome sequence [88], we have used TolC and TolC homologs from other Gram-negative bacteria to identify open reading frames for putative OMPs of Type I exporters in the *C. crescentus* genome by homology searches. Based on that approach, we have found three omp candidate genes. Because of their unknown function, predicted proteins were termed OMP58, OMP50, and OMP48, according to their calculated molecular mass (Reichelt et al., unpublished data). The three proteins display significant sequence homology among each other and to known OMPs of other ABC transporters. Previous attempts to identify the OMP required for functional S-layer transporter expression in *C. crescentus* by insertional mutagenesis of individual omp candidates and screening for an S-layer secretion negative phenotype failed ([89]; unpublished results). These data gave rise to the assumption that either none of the analyzed omp gene candidates encodes the OMP component of the *C. crescentus* S-layer secretion system or that, because of their structural relationship, the different omp gene candidates can functionally complement each other in the homologous system, thereby restoring S-layer secretion competence in the *C. crescentus* mutant strains. We have therefore started a second heterologous complementation approach, using a genetic fusion of the rsa operon to the hly operon and omp gene candidates expressed on different plasmid vectors in *E. coli*. Preliminary expression data revealed that the coexpression of one of the omp gene candidates results in specific secretion of a truncated RsaA protein genetically fused to an N-terminal Hly polypeptide sequence, indicating a functional expression and assembly of the S-layer secretion apparatus in *E. coli*. More importantly, it demonstrates the feasibility of a heterologous reconstitution of the *C. crescentus* S-layer secretion system in *E. coli* as a first step towards the development of a Rsa-based recombinant protein secretion system in *Salmonella*. 
8. Conclusions

Recombinant vaccines based on attenuated Salmonella carriers are promising vaccine candidates. However, immune responses elicited against the heterologous antigen are generally weaker than those induced by parenteral application of the same recombinant vaccine antigen. This is partly due to the fact, that the recombinantly expressed vaccine antigen is not optimally presented to the immune system and the vast majority of the immune response is directed towards the surface-exposed antigens of the carrier strain. One approach to improve immune responses against the vaccine antigen is the secretory delivery of the recombinant protein by the carrier strain. Secretory delivery is even more crucial for effector proteins, which depend on bioactivity for maximal effectiveness. Apart from that, the expressed recombinant protein amounts appear to be crucial to trigger maximal immune responses and in vivo action, respectively.

Despite various attempts to adapt different secretion systems to recombinant protein delivery in Salmonella and other vaccine strains, only Type I protein exporters have the potential to serve as high-level secretion systems for recombinant proteins with maximal biological activity. Moreover, the low complexity of the Type I export machinery makes heterologous plasmid-encoded reconstitution of such transporters in vaccine strains rather feasible.

So far, the Hly secretion system is the only Type I protein exporter, which has been explored as secretory expression system in a variety of Gram-negative bacteria. In many cases, the Hly transporter enables the secretion of detectable amounts of foreign proteins when tagged with the C-terminal HlyA secretory signal. As repeatedly documented for different proteins, the C-terminal secretion signal extension may not impair the secretion of functional fusion proteins. However, the Hly system has a number of disadvantages, which eventually affect the efficiency of secretory delivery of vaccine antigens and effector proteins by Salmonella carriers. First, depending on the nature of the protein, secreted protein levels are rather low. Secondly, the growth-dependent regulation prevents a continuous expression of foreign proteins under in vivo conditions. Thirdly, the secretability and the secretion efficiency of a recombinant protein depend on the structure of the protein. The putative oxygen regulation of the system may finally turn out to be rather an advantage than disadvantage.

From that scenario, there is need for development of more efficient recombinant protein secretion systems, which overcome one or more of the limitations of the Hly system. Among the large number of Type I secretion systems, exporters involved in the extracellular delivery of SLPs are particularly interesting. SLPs generally account for a significant portion of the total cellular protein, suggesting a high level secretory expression of these proteins. Furthermore, SLP synthesis and secretion are required throughout the cell growth, implementing a constitutive gene regulation for the S-layer secretion systems. From the three currently known Type I protein secretion systems involved in the export of S-layers, the C. crescentus Rsa transporter offers the greatest potential to serve as a high-level recombinant protein secretion system even in heterologous systems. The Rsa transporter has been genetically and biochemically characterized in greater detail. More importantly, the feasibility and versatility of heterologous protein secretion have been demonstrated for a large number of RsaA fusion proteins in C. crescentus.

Although the E. coli Hly and C. crescentus Rsa secretion system reveal similar operon architecture, heterologous expression of the transporter was so far hindered due to the unknown OMP component of the transporter. We have recently identified at least one Rsa transporter-specific OMP protein, thereby providing the basis for heterologous reconstitution of this Type I secretion system in E. coli and Salmonella. Eventually, this may ease the development of more efficient vaccine and effector protein presentation systems for Salmonella carriers.

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