The *E. coli* α-hemolysin secretion system and its use in vaccine development

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Many Gram-negative bacteria use a type I secretion system to translocate proteins, including pore-forming toxins, proteases, lipases and S-layer proteins, across both the inner and outer membranes into the extracellular surroundings. The *Escherichia coli* α-hemolysin (HlyA) secretion system is the prototypical and best characterized type I secretion system. The structure and function of the components of the HlyA secretion apparatus, HlyB, HlyD and TolC, have been studied in great detail. The functional characteristics of this secretion system enable it to be used in a variety of different applications, including the presentation of heterologous antigens in live-attenuated bacterial vaccines. Such vaccines can be an effective delivery system for heterologous antigens, and the use of a type I secretion system allows the antigens to be actively exported from the cytoplasm of the bacterial carrier rather than only becoming accessible to the host immune system after bacterial disintegration.

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Four different pathways of protein secretion (types I to IV) have been described in Gram-negative bacteria [1–4]. The type I secretion systems (for reviews see [5–7]) differ from all other secretion pathways in several ways: (1) they contain only three different transport components, two in the inner membrane (IM), which are specific for the passenger protein, and one that forms a general pore in the outer membrane (OM); (2) these proteins form a 'tunnel' that links the IM and OM; (3) the passenger proteins are secreted directly into the extracellular medium without the formation of periplasmic intermediates; (4) the secretion signals recognized by these secretion systems are located at the carboxyl terminus of the secreted proteins; (5) in most cases, the secretion signals are not cleaved off during or after secretion; and (6) some type I gene clusters are located on plasmids and are therefore easily accessible for genetic manipulation. Type I secretion is exemplified by the *Escherichia coli* α-hemolysin [8,9] and *Erwinia chrysanthemi* protease [10] secretion systems.

The proteins secreted by the type I pathway can be assigned to several families: repeats in toxin (RTX) proteins [e.g. α-hemolysin (HlyA) of *E. coli*], proteases (e.g. the four different proteases of *E. chrysanthemi*), lipases (e.g. *Serratia marcescens* lipase), S-layer proteins (e.g. the SlaA protein of *S. marcescens*), hemophores (e.g. the two hemoproteins of *S. marcescens* and *Pseudomonas aeruginosa*), bacteriocins and proteins of unknown function. Interestingly, most of these proteins contain a domain consisting of tandemly arranged glycine-rich nonameric repeats (XXGGXGXDX; where X = any amino acid), which are responsible for Ca^{2+} binding [7]. Although some data indicate that the repeats themselves might be important for efficient secretion [11], their role in the secretion process is controversial.

E. coli α-hemolysin: the prototypical type I secretion system

E. coli α-hemolysin (HlyA) is the best-characterized RTX protein secreted by a type I secretion system. It is mainly produced by *E. coli* strains causing urinary tract infections (uropathogenic *E. coli*; UPEC) [12] and is an important virulence factor owing to its cytolytic and cytotoxic activity against a wide range of mammalian cell types (e.g. erythrocytes, granulocytes, monocytes and endothelial cells). The synthesis, activation and secretion of *E. coli* HlyA are determined by the *hlyCABD* operon [13]. In *E. coli*, this operon is located either on chromosome-bound pathogenicity islands or on transmissible plasmids, suggesting that the genetic determinants for type I secretion can be transmitted by horizontal gene transfer among Gram-negative bacteria [7,14]. The *hlyCABD* operon is transcribed from a promoter located upstream of *hlyC* [15], and several mechanisms are in place for its regulation. The transcription of the *hly* operon is strongly polar owing to the presence of a rho-independent terminator in the *hlyA–hlyB* intergenic region [15]. This termination is suppressed by the elongation protein RfaH and a cis-acting 5′ DNA sequence termed the JUMPstart element or ops (operon-polarity suppressor) element, which must act together to allow the efficient transcription of the entire *hly* operon [16]. The same mechanism of transcriptional elongation and anti-termination is found for the *rfa* and *tra* operons, which encode virulence and fertility factors of Gram-negative bacteria as well as their respective secretion systems [16]. In addition, the expression of the hemolysin operon is modulated by a nucleoid–protein complex that includes the proteins Hha and H-NS [17].

Pro-HlyA is activated in the cytoplasm of the bacterial cell to the hemolytically active form by HlyC, which is a fatty acid acyltransferase [18]. However, acylation is only required for hemolytic activity; the secretion of HlyA is independent of HlyC. The HlyA export machinery consists of three components: HlyB, HlyD and TolC [8,19]. Whereas

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the IM proteins HlyB and HlyD are specific components of the transport apparatus of α-hemolysin, the third component, TolC, is a multifunctional protein located in the OM of *E. coli*. The first component, HlyB, belongs to the ATP-binding cassette (ABC) superfamily of eukaryotic and prokaryotic protein transporters [6,20]. This IM protein couples ATP hydrolysis to the export of the substrate [21]. The topology of HlyB (707 amino acids) has been determined by fusion of β-lactamase [22] or of alkaline phosphatase and β-galactosidase [23] to HlyB. The data obtained by these methods suggest that HlyB is inserted in the IM by eight hydrophobic, α-helical transmembrane domains (TMDs). These TMDs extend from amino acid positions 38 to 432 of HlyB. The cytoplasmic loops between the TMDs are relatively large and carry an excess of positively charged amino acids, whereas the periplasmic loops are rather small. Interestingly, the first two TMDs can be deleted with no effect on hemolysin secretion.

The second component (HlyD) is one of the best characterized members of the membrane fusion protein (MFP) family [24]. HlyD is anchored in the cytoplasmic membrane by a single TMD and possesses a large periplasmic domain within the carboxy-terminal 100 amino acids, which are highly conserved among MFPs [25]. A recent study grouped the members of the MFP family within the superfamily of periplasmic efflux proteins (PEP) [26].

The third component (TolC) is a general outer membrane protein (OMP) [27] that is part of at least four different export systems [28]. The crystal structure of TolC shows that its trimeric state forms a trans-periplasmic channel-tunnel with an internal diameter of 35 Å and is about 140 Å in length, comprising a 40 Å-long OM β-barrel (the channel domain) anchoring a contiguous 100 Å-long α-helical barrel that projects across the periplasmic space (the tunnel domain) [29,30].

HlyA carries a carboxy-terminal secretion signal located within the last 50–60 amino acids (referred to here as HlyAs) [9,31,32]. Based on data obtained by site-directed mutagenesis, CD and NMR spectroscopy, several structural and sequence motifs in HlyAs have been proposed as being essential for its signal function, yet the precise nature of HlyAs remains basically unknown. HlyAs appears to be necessary and sufficient for the secretion of HlyA, as a peptide consisting of just the carboxy-terminal 60 amino acids of HlyA is secreted in the presence of HlyB–HlyD–TolC with the same efficiency as HlyA itself [32]. In contrast to some other type I-secreted proteins [11], the repeats within hemolysin are not required for efficient secretion [32].

Interactions between the components of the hemolysin secretion system

The first complex biochemical analysis of the interaction between the components of the HlyA secretion system was published by Thanabalu *et al.* [33]. Using a combination of co-affinity protein purification and crosslinking, these authors demonstrated that HlyB and HlyD form a stable IM complex. The formation of this complex is observed in the absence of HlyA and is independent of TolC. Binding of HlyA to the pre-formed HlyB–D complex induces contact to TolC, via a HlyD trimer, to form a trans-periplasmic export channel. However, this substrate-dependent bridge is transient and the components of the export channel revert to IM and OM states after the passage of hemolysin [33].

The secretion signal appears to be recognized initially by HlyB [34]. Mutations in HlyAs leading to secretion incompetence are partially compensated by suppressor mutations in HlyB [35]. Interestingly, HlyD can interact with HlyA even in the absence of HlyB [33]. This seems to be in contrast to results showing that HlyB alone interacts directly with the hemolysin secretion signal [23,35]. In addition, Letoffe *et al.* demonstrated that sequential assembly of the *E. chrysanthemi* metalloprotease (PrtDEF) exporter system is only induced when the protease binds to the ABC transporter [36]. A simple explanation which accomodates these data is that substrate binding by each of the two IM proteins might not be mutually exclusive, and that they bind to different sites of HlyAs, either simultaneously or sequentially. However, until now, the specific binding sites in HlyB or HlyD for the HlyA secretion signal have not been identified.

It is not yet clear whether a chaperone is involved in the transport process. Debarbieux and Wandersman recently showed that the secretion, but not the folding, of HasA, a hemoprotein of *S. marcescens* secreted via the type I pathway, is strongly dependent upon the SecB chaperone [37]. However, this is possibly a specific case, because hemolysin and protease secretion are SecB independent [38]. Other unidentified chaperones might be involved, or hemolysin secretion could occur quickly with respect to the folding process, in which case chaperones would not need be required.

Model of HlyA secretion

The secretion of HlyA has been analyzed in great detail by several groups. HlyA seems to interact with the cytoplasmic region of the pre-formed HlyB–D complex (Fig. 1). After the binding of the HlyA secretion signal by the HlyB–D complex, HlyD induces the interaction with TolC. Koronakis *et al.* [29] have produced crystal structure data for TolC that suggest a model for a possible TolC–HlyD interaction. A HlyD trimer has a mass very similar to the TolC trimer and could provide a cylinder of almost identical diameter. The periplasmic end of the TolC tunnel is sealed by sets of coiled-coils that might untwist upon contact with a region of HlyD that is also predicted to form a coiled-coil [39], thus opening the transport pore [26,29]. Furthermore, coiled-coil domains are implicated in protein dynamics and might be involved in subunit oligomerization [26,39]. The hemolysin could then move from HlyD into TolC via a continuous tunnel.

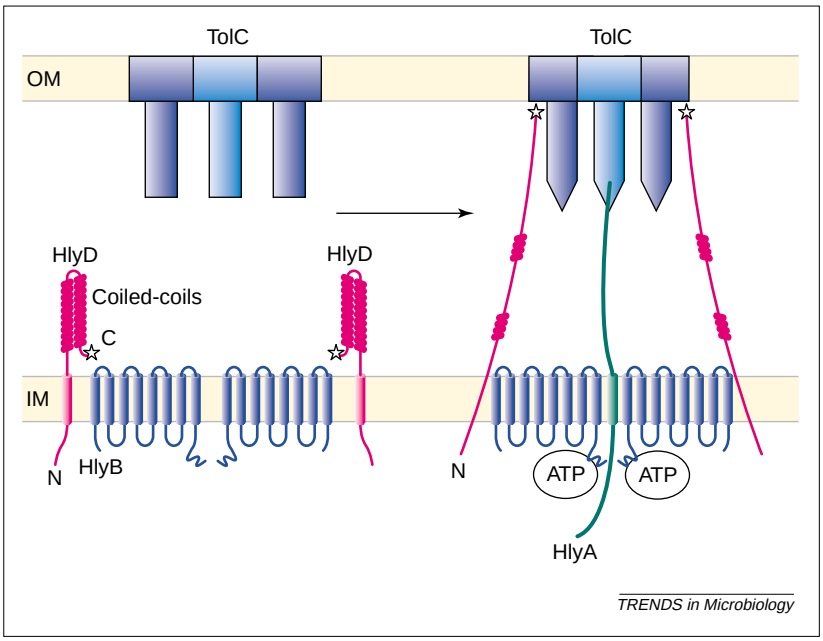

Fig. 1. Topological model of hemolysin secretion. In response to HlyA engagement, a HlyD trimer [33], which bends at the gap between the coiled-coil regions, interacts with the trimeric TolC protein to form a continuous trans-periplasmic export channel. Supported by the observation that the carboxy-terminal sequence of HlyD is in contact with periplasmic loop(s) of HlyB, Schlör *et al.* proposed HlyD has a hairpin conformation, which is essential for these interactions [63]. The HlyD coiled-coil regions are indicated and the asterisk marks the carboxy-terminal domain of HlyD. Only two HlyD molecules are shown for clarity. The circles labeled ATP denote the ATP-binding sites of HlyB. ATP hydrolysis by HlyB provides the energy for the transport process and leads to direct secretion of HlyA into the extracellular medium without formation of periplasmic intermediates. Modified from [26,30].

An important observation supporting this model is that HlyA is directly secreted into the extracellular medium without the formation of periplasmic intermediates, which is in contrast to some other secretion systems [1,9]. The energy for the transport process appears to be provided by two mechanisms: although HlyB couples the export of its substrate to the hydrolysis of ATP [21], the proton motive force could also play an important role in HlyA secretion [40].

Recently, Chang and Roth published the first high-resolution structure of a complete ABC transporter, the MsbA lipid A transporter of *E. coli* [41]. MsbA, which is closely related to HlyB, is organized as a homodimer with each subunit containing six transmembrane α-helices and a nucleotide-binding domain. The authors proposed a 'flip-flop' mechanism of substrate movement across the lipid bilayer. However, the applicability of the MsbA model to HlyB and its implications for the hemolysin transport process remain to be established.

Application of the hemolysin secretion system

Secretion of heterologous proteins

The functional characteristics of the *E. coli* α-hemolysin secretion system enable it to be used in a variety of applications. Interestingly, this system can be used to express and secrete heterologous proteins. Proteins covalently linked at their carboxyl terminus to HlyAs are recognized by the HlyB–HlyD–TolC translocator [38,42,43]. Most of these proteins (at present >400) are secreted by *E. coli* via the hemolysin secretion pathway, even though they are entirely unrelated to HlyA. The amount of fusion protein secreted under *in vitro* conditions is at least 0.5 μg per ml supernatant in cultures in late-logarithmic growth phase and is generally higher than the intracellular pool. In addition, there seem to be no limitations concerning the size or origin of the heterologous region of the HlyAs fusion proteins. The heterologous proteins can range in size from 20 amino acids to >1000 amino acids and can be derived from prokaryotes as well as eukaryotes. Another attractive feature is the apparent stabilization and enhanced solubilization of certain heterologous proteins expressed and secreted via this system [44,45].

The secretion of heterologous proteins via the hemolysin secretion system has mainly been applied to immunological and vaccine research. As HlyAs by itself is a very weak antigen for B cells and T cells, hemolysin-fusion proteins were successfully used to produce polyclonal and monoclonal antibodies [46] and to detect protective antigens of different pathogenic bacteria [47]. However, the most important application of the hemolysin secretion system is the presentation of heterologous antigens in attenuated Gram-negative bacterial live vaccines (Table 1).

Live bacterial vaccines based on the hemolysin secretion system

There is an increasing body of evidence that live-attenuated bacterial vaccine strains are an effective delivery system for recombinant antigens from a wide variety of pathogens including bacteria, viruses and parasites. However, most bacterial systems for the presentation of recombinant antigens rely on the expression of heterologous proteins in the cytoplasm of bacterial carriers; the heterologous antigens are thus only accessible to the host immune system upon disintegration of the bacteria. As an alternative to cytoplasmic antigen expression, several systems for exporting heterologous antigens from the cytoplasm of live-attenuated bacterial vaccines have been developed. These include using the MalE and OmpA export signals, or fusion to the *E. coli* heat labile toxin LT-B [48]. Alternatively, systems have been devised to insert antigenic epitopes into flagellin, fimbriae, LamB, OmpA and PhoE of salmonellae [48].
Two recently described systems for secretion of heterologous antigens by Gram-negative bacteria include the type III secretion system, which even allows for the 'injection' of the passenger antigen into the target cell [49], and the AIDA autotransporter system [50]. However, most of these systems for surface display or secretion of antigens can accommodate just an additional short peptide of the heterologous antigen.

By contrast, the hemolysin secretion system does not appear to impose any size limitations on the passenger antigens and allows the active secretion of even large heterologous antigens by attenuated bacterial carriers, which has led to the development of a wide range of recombinant live vaccines (for review,

Table 1. Examples of live vaccines based on the α-hemolysin secretion system of *E. coli*[a]

Organism	Antigen	Bacterial carrier strain	Immune response in an animal model (Model; Ab;CMR)	Protection in an animal model[b]	Refs
Bacteria					
Listeria monocytogenes	Listeriolysin	*Salmonella typhimurium aroA* (SL7207)	C57BL/6 mice;ND;+	Mice;+	[52]
L. monocytogenes	P60	*S. typhimurium aroA* (SL7207)	C57BL/6 mice;+;+	Mice;+	[52]
L. monocytogenes	Superoxide dismutase	*S. typhimurium aroA* (SL7207)	C57BL/6 mice;ND;+	Mice;+	[54]
Shigella spp.	Shiga toxin B-subunit	*S. typhimurium aroA* (SL3261)	BALB/c mice;+;ND	ND	[43,60]
		Shigella flexneri aroD (SFL124)	BALB/c mice;ND;ND	ND	
Clostridium difficile	Toxin A	*Vibrio cholerae* 0395-NT	New Zealand White rabbits;+;ND	Rabbits;+	[53]
Corynebacterium diphtheriae	Diphtheria toxin	*Salmonella typhi* CVD 908-*htrA*	BALB/c mice;+;ND	ND	[45]
Mycobacterium bovis BCG	30-kDa antigen	*S. typhimurium aroA* (SL7207)	C57BL/6 mice;ND;+	Mice;+/ –	[61]
Mycobacterium tuberculosis	ESAT-6 antigen	*S. typhimurium aroA* (SL7207)	C57BL/6 mice;ND;+	Mice; +/ –	[62]
Parasites					
Theileria parva	P67 antigen	*Salmonella dublin aroA* (SL5631)	Cattle;+;–	Cattle;+/ –	[64]
Plasmodium falciparum	Surface protein 2	*S. typhimurium aroA* (SL3261)	C57BL/6 mice;ND;+	ND	[59]
		S. typhi CVD 908-*htrA*			
Virus					
Measles virus	Nucleocapsid protein or fusion protein	*S. typhimurium aroA* (SL7207)	CH3 mice;+;+	Mice;+/ –	[65]

[a]Abbreviations: Ab, antibody response; BCG, Bacille Calmette–Guérin; CMR, cell-mediated response ($CD4^+$ and/or $CD8^+$ antigen-specific T cells, T-cell proliferation or cytokine release); ND, not determined.
[b]+,100% protection; +/–, partial protection.

see Table 1). As was shown recently, the *E. coli* Hly plasmid system replicates stably in many other Gram-negative bacteria, including different *Salmonella* serotypes, *Shigella* spp. and *Vibrio cholerae*, and the encoded HlyAs-fused antigens are likewise secreted [51]. This is caused by the presence of TolC or proteins analogous to TolC in the OM of these Gram-negative bacteria; the genes encoding HlyB and HlyD are generally provided by the plasmids used to express the fusion proteins [51]. A variety of antigens of bacterial, viral and parasitic origin have been expressed and secreted in attenuated bacterial carrier strains using the hemolysin secretion system (Table 1). The amounts of fusion protein or hemolysin secreted in this way in bacterial strains including *Salmonella typhimurium*, *Salmonella typhi*, *S. flexneri* and *V. cholerae* are comparable to or even higher than in *E. coli*.

Most heterologous proteins presented via this pathway in different vaccine carriers trigger humoral and/or cell-mediated immune responses to the donor organism. In addition, protection was observed after vaccination with recombinant *Salmonella* spp. and *V. cholerae* vaccine carriers secreting heterologous antigens [52,53]. These immune responses provide compelling evidence that there is active expression and secretion of the heterologous antigens under *in vivo* conditions. By contrast, protection was either low or absent when the corresponding antigens remained intracellularly in the vaccine strain owing to inactivation of the HlyB–HlyD–TolC translocator [52,54]. This could possibly be caused by rapid antigen degradation *in vivo*. However, as the amount of antigen in secreting and non-secreting strains was identical for the respective antigens under *in vitro* conditions [52,54], it could be caused by the fact that the heterologous antigens in the non-secreting strains are presented to the immune system only upon lysis of the bacterial carriers, and might hence be insufficient for the elicitation of B-cell and T-cell responses. These findings suggest that optimal compartmentalization of antigens within the carrier plays an important role and that antigen display via the hemolysin secretion system is superior to cytoplasmic antigen expression in recombinant live bacterial vaccines.

The application of the hemolysin secretion system allows compartmentalization of antigen expression and delivery to be finely tuned. Normally, HlyA or the HlyAs fusion proteins are completely secreted into the extracellular surroundings and only a small pool is detected inside the producing bacterial cells. However, specifically engineered plasmid variants allow the complete retention of the protein antigen within the cytoplasm of the bacteria without proteolytic degradation of the antigen *in vitro* (Fig. 2) [52]. Moreover, the compatibility of the HlyA secretion system with a variety of Gram-negative bacteria offers possibilities to modulate the immune response against a given protein antigen as desired. The bacterial carriers can colonize the cell surface (*V. cholerae*), the specialized phagosome of antigen-presenting cells (APCs) (*Salmonella*) or cytosol (*Shigella*) of infected cells and will deliver the same antigen to different compartments of APCs, hence triggering different immune responses against the same antigen (Fig. 2).

A further advantage of the hemolysin secretion system is that it is even suitable for the concomitant expression and secretion of several antigens in a single carrier bacterium, allowing the construction of multivalent vaccines [44]. Last but not least, the HlyA secretion machinery can be applied not only in the classical field of anti-infective vaccination, but also as

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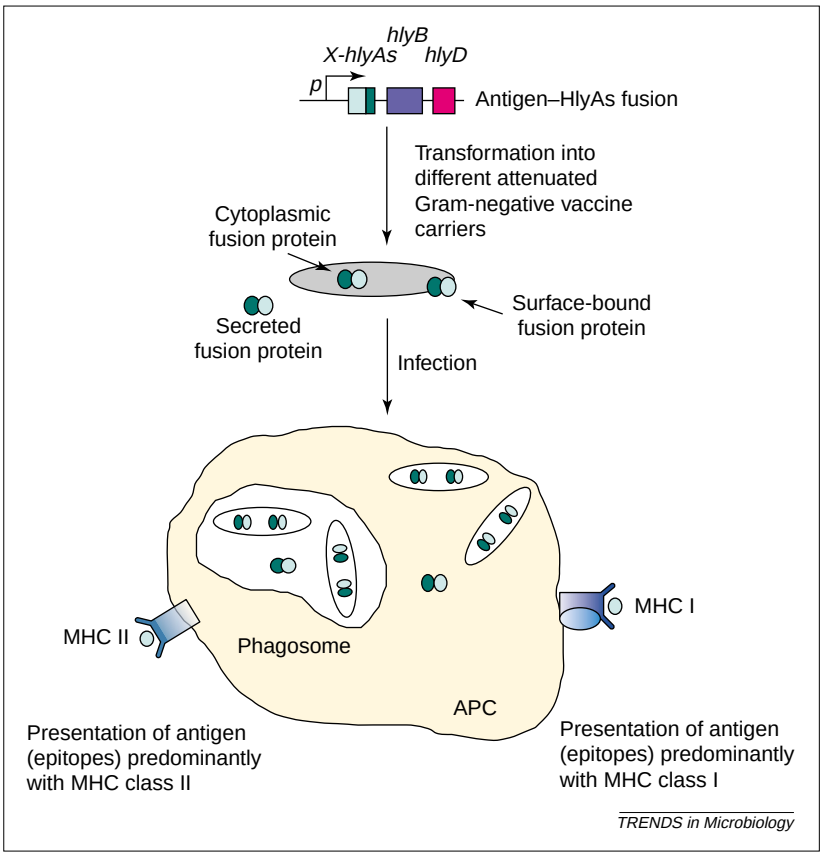

Fig. 2. Use of different attenuated Gram-negative vaccine carriers for variation of antigen presentation in antigen-presenting cells (APCs) via the hemolysin secretion machinery. Heterologous antigens can be cloned in-frame with the hemolysin secretion signal (HlyAs). Transformation into appropriate attenuated bacterial carriers leads to expression and secretion of the antigen–HlyAs fusion protein. Depending on the carrier bacterium, the antigen can either be expressed outside or in the phagosome of APCs, leading predominantly to presentation in the context of major histocompatibility complex (MHC) class II molecules, or in the cytosol of infected APCs, which results in presentation with MHC class I.

a delivery system for immunocontraceptive vaccines [55] and for co-expression and co-delivery of active cytokines [56]. Therefore, the use of the hemolysin secretion system for the expression of recombinant antigens in live-attenuated bacterial vaccines allows excellent antigen presentation and modulation of the immune response as desired.

Questions for future research

- What is the state of the transported protein (folded or unfolded)?
- Are there chaperones involved in the HlyA transport or proteins other than HlyB, HlyD and TolC involved in the structure of the translocator channel?
- What is the nature of the hemolysin secretion signal and how does it recognize the components of the secretion apparatus?
- What is the chronological order of interaction between the components of the secretion apparatus?
- Is there a direct interaction between TolC and HlyB?
- What is the stoichiometry of the components of the translocator channel for hemolysin and what is its architecture during the secretion process?
- What is the polarity of HlyA secretion?
- Can TolC as a component of different translocation channels effect the simultaneous secretion of protein toxins and toxic drugs? Is there an order of substrate priority?

Conclusions and outlook

The *E. coli* hemolysin secretion apparatus is the most thoroughly characterized type I secretion system. Its specific features enable this system to be used for the secretion of heterologous antigens in live-attenuated bacterial vaccines. Its suitability for this purpose has been demonstrated using a wide range of antigens and carrier strains as well as several animal models. Recently, similar use has been made of an alternative type I secretion system. The secretion apparatus dedicated to the secretion of the S-layer protein RsaA of *Caulobacter crescentus* has been adapted to the surface localization and antigen secretion of bacterial and viral antigens by recombinant *C. crescentus* strains, which elicited promising immune responses in mice and fish [57]. These data might indicate the general applicability of type I secretion systems for vaccine delivery. Nevertheless, at present the hemolysin secretion aparatus is the most widely used antigen-secretion system.

However, some issues with this system remain to be addressed. Little is known about the *in vivo* expression levels of heterologous antigens. Cell culture studies demonstrated antigen secretion by *Salmonella* strains in the phagosome of macrophages [52], but expression of reporter proteins such as green fluorescent protein is necessary to provide a detailed view of expression levels under *in vivo* conditions. Although the immune responses obtained clearly demonstrate *in vivo* expression under the control of P_{hly}, substitution with promoters of known *in vivo* strength or *in vivo*-inducible promoters such as P_{nirB}, P_{ompC} or P_{htrA} could enhance and control expression in vaccinated individuals. Other important issues for recombinant live bacterial vaccines are the potential instability of episomal expression systems and the reduced fitness of bacterial carriers expressing high levels of heterologous antigens [58]. Interestingly, we found *Salmonella* strains carrying *hly* antigen expression vectors stably maintained these plasmids in the absence of antibiotics over 20 generations under *in vitro* conditions, with the loss of plasmid being <1% [42], and during mouse infection studies, about 80% of all *Salmonella* bacteria recovered three weeks after oral infection still carried the plasmid vectors [52]. Additionally, there was no reduction in the persistence of the bacteria in vaccinated mice compared with control salmonellae that did not express hemolysin fusion proteins or the hemolysin secretion apparatus. These results might be owing to secretion of the heterologous antigens, which could reduce the metabolic burden for the carrier bacteria [58].

Nevertheless, chromosomal integration of the entire *hly*-determinant or incorporation of genetic systems for plasmid stabilization in the absence of antibiotic markers could enhance *in vivo* expression levels. However, the most important issue is the efficacy of the system for use in humans. The hemolysin secretion system has proven its value in a wide range of animal systems, including mice, rabbits

and even cattle. For a more thorough evaluation, however, it must be tested in humans. It has been used successfully in suitably attenuated carrier strains such as the *S. typhi* CVD908*htrA* mutant [59]. This important prerequisite should now ensure a smooth path to the application of such strains secreting passenger antigens via the hemolysin secretion system in clinical trials in the near future.

Animation

For an animated model of type I secretion please go to: http://archive.bmn.com/supp/ceb/ani1.html

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Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains

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A large, genetically related group of *Mycobacterium tuberculosis* strains, variously called W or Beijing, is distinguished by specific molecular markers and referred to as the W-Beijing family strains. Molecular epidemiological studies suggest that these strains are highly prevalent throughout Asia and the countries of the former Soviet Union and they have also been reported in several other geographical regions, including North America. Although the spread of W-Beijing family strains in diverse populations is well documented, the underlying host–pathogen factors accounting for their continued dissemination and burden of disease have yet to be determined.

Advances in our understanding of the molecular biology of *Mycobacterium tuberculosis* have proven invaluable in unraveling the epidemiology of tuberculosis (TB) and in constructing the phylogenetic structure of the species. Genomic data have revealed remarkable DNA sequence conservation between chromosomes, and the paucity of synonymous mutations led to the hypothesis that *M. tuberculosis* is a recent human pathogen dating back approximately 15 000 years [1]. Although the *M. tuberculosis* genome is highly conserved in relation to other bacterial species, there are polymorphic regions that are usually associated with insertion sequences and/or repetitive elements and it is these variable regions that form the basis of modern TB genotyping.

Several polymorphic or hypervariable genetic markers have been characterized that, together, can discriminate or sub-speciate clinical isolates of *M. tuberculosis.* The most widespread and robust genotyping tool is the insertion sequence IS*6110*, a member of the IS*3* family of transposable elements [2] that is specific for strains belonging to the *M. tuberculosis* complex [3]. Although noted 'hot spots' have been identified, IS*6110* is more or less randomly distributed around the chromosome and its copy number ranges from the rare clones that lack an insertion to strains that have 26 copies [4,5]. The standardization of a method for IS*6110* Southern blot hybridization has created 'DNA fingerprints' that can be compared between laboratories with the aid of pattern-matching software [6]. As a result, >50 000 *M. tuberculosis* isolates worldwide have been

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