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REVIEW

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Facing the challenges of multidrug-resistant *Acinetobacter baumannii*: progress and prospects in the vaccine development

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ABSTRACT

In 2017, the World Health Organization (WHO) named *A. baumannii* as one of the three antibiotic-resistant bacterial species on its list of global priority pathogens in dire need of novel and effective treatment. With only polymyxin and tigecycline antibiotics left as last-resort treatments, the need for novel alternative approaches to the control of this bacterium becomes imperative. Vaccines against numerous bacteria have had impressive records in reducing the burden of the respective diseases and addressing antimicrobial resistance; as in the case of *Haemophilus influenzae* type *b* . A similar approach could be appropriate for *A. baumannii*. Toward this end, several potentially protective antigens against *A. baumannii* were identified and evaluated as vaccine antigen candidates. A licensed vaccine for the bacteria, however, is still not in sight. Here we explore and discuss challenges in vaccine development against *A. baumannii* and the promising approaches for improving the vaccine development process.

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Introduction

Acinetobacter species are mostly free-living saprophytes with different genus inhabiting different habitats including soil, water, and sewage.[1] Among the Acinetobacters, *A. baumannii*, *A. haemolyticus*, and *A. calcoaceticus* have been reported as the species with high clinical importance.[2] *Acinetobacter baumannii* in particular, is most commonly found within the healthcare facility environments.[1] . *baumannii*, a Gram-negative coccobacillus was previously regarded as a low-grade pathogen of humans. It has in recent decades emerged as a leading cause of health-care-associated infections (HAIs).[3] The bacteria causes a wide range of infections including skin and soft-tissue infections associated with trauma, urinary tract infection, pneumonia, meningitis, and septicemia.[4,5] Early on before the 1970s, infections by *A. baumannii* were easily treatable with beta-lactams and sulfonamide antibiotics.[3] Within a few decades, however, the bacteria acquired extensive resistance to the antibiotics which led to the eventual emergence of the multidrug resistance (MDR) *A. baumannii* strains. *A. baumannii* was a major concern in conflict regions such as in Iraq and Afghanistan, where a high incidence of MDR *A. baumannii* bloodstream infections and battlefield trauma in injured military personnel were reported; which earned it the moniker "Iraqibacter".[6–8] An increasing trend in the prevalence of the bacterium in long-term care facilities and nursing homes suggests that *Acinetobacter* strains are no longer strictly a healthcare facility-associated pathogen.[9,10] Community-acquired infections caused by *A. baumannii* are increasingly being reported, especially in the tropical or subtropical regions of the world.[11,12]

Common infections with *A. baumannii* could lead to mortality if left untreated or ineffectively treated when such infections involved antibiotic-resistant (ABR) bacterial strains. The ABR as human pathogens not only poses a detrimental impact on human health but also on economic wellbeing.[13] Specifically for *A.baumannii*, infection which resulted in admission into an intensive care unit (ICU), lead to increase length of hospital stay (LOS), thus, escalating the cost of treatment (COT).[14] Economically, the LOS and mortality are proportionately related to the productivity losses for a country that directly contributing to the economic burden.[15]

Antibiotic resistance of *A. baumannii* has been linked to the "*resistome*", a well-coordinated network between antibiotic resistance genes, and other genetic elements such as those associated with critical bacterial metabolism.[16] The MDR *A. baumannii* isolates have been reported worldwide and they are resistant to at least three classes of antimicrobial agents, viz. penicillins and cephalosporins, fluoroquinolones and aminoglycosides.[17–19] Owing to its serious health concerns and global spread, the MDR *A. baumannii* was listed by the World Health Organization (WHO) as amongst the 12 bacterial families for which new drugs are urgently needed.[19] In addition, an independent Review on Antimicrobial Resistance, jointly supported by the UK Government and Wellcome Trust, issued specific recommendations to reduce dependence on antibiotics and called for the development of new vaccines, or greater use of existing vaccines against the bacterial infections.[20,21] Use of vaccines would reduce the usage of antibiotics,[21] hence, decrease the incidence of antimicrobial resistance.[22] Vaccines could also play a significant

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role as a long-term approach in preventing bacterial infections and lowering the overall disease burden.[23]

Toward vaccine development: identifying virulence factors and understanding the pathogenesis *of A. baumannii*

Over the past decades, our understanding of the pathogenesis of *A. baumannii*, including its virulence factors, antimicrobial resistance mechanisms, and quorum sensing have increased tremendously.[3,24] The numerous factors underlying the success of the bacterium in causing disease have led to the identification of candidate antigens for the development of novel vaccines.[3,25–27] Among the factors that are essential for the successful transmission of *A. baumannii* are those associated with the membrane proteins and cell surface adaptations, antibiotic tolerance, mechanism of nutrient acquisition, and bacterial community interactions.[24] Some of these factors have been demonstrated to work in concert with others.

One of the striking features of *A. baumannii* is its resistance to most antibiotics, including the last resort antimicrobials; carbapenems, colistin, and tigecycline.[28] Resistance is associated with environmental stress conditions,[29] as well as the bacterial cell surface structure which consisted of the lipopolysaccharides (LPS) as the major component of the outer leaflet of the outer membrane (OM), capsular polysaccharides (CPS), glycosylated proteins, and the peptidoglycans.[30] These bacterial components provide strong structural stabilization of the bacteria and contribute to cellular homeostasis under changing environmental conditions.[31] Additionally, the biofilm which is a complex extracellular surface structure formation is among the virulence factors of *A. baumannii* that is also associated with antimicrobial resistance.[27] The biofilm-associated proteins Bap and Blp1 have been reported to enhance attachment to host cells or abiotic surfaces.[32] Expression of other proteins within the bacterium such as autotransporter (Ata) and the extracellular poly-*N*-acetyl-β-(1-6)-glucosamine (PNAG) are shown to mediate attachment and adhesion of the cells in the biofilm.[24] The biofilm not only protects the bacterium from antibiotics but also facilitates attachment to abiotic surfaces as well as the host epithelial cells.[33] The key role of the cell surface structures, however, is in mediating immune evasion from the host, e.g. K1 CPS, which augments the bacterium's ability to survive in human serum and contribute to causing disease within the host.[34,35]

The extraordinary balance and plasticity of the *A. baumannii* genome enable the bacterium to adapt for survival, contributing to its superbug status. Further investigation into the genetics of antibiotic resistance revealed that antibiotic resistance is not dependent on an individual characteristic, but rather on a repertoire of traits including enzymatic determinants (e.g. β-lactamase), genetic manipulations (where genes are recombined, acquired, or lost), as well as efflux pumps that extrude the antibiotics.[35] *A. baumannii* possesses representatives of each of the six efflux pump families associated with resistance to various antimicrobials; major facilitator superfamily (MFS) pumps (tetracycline), multidrug and toxic compound extrusion family (MATE; aminoglycosides, quinolones, and chloramphenicol), small multidrug resistance (SMR) superfamily (erythromycin and novobiocin), resistance-nodulation-division family (RND; aminoglycosides, fluoroquinolones, tetracycline, trimethoprim, and β-lactams), ATP-binding cassette (ABC) family, and proteobacterial antimicrobial compound efflux family.[28,35] While these efflux pumps have been well-described, the full understanding of their underlying mechanisms is still limited.[28,35] A transcriptomic study of multidrug resistance in *A. baumannii* showed that the regulation of antibiotic resistance occurred at the level of operons, which involved different responses of the strains to different classes of antibiotics. The study also revealed that multiple antibiotic resistance genes were necessary to confer antibiotic resistance in MDR strains so that the broad resistance was not dependent on any single antibiotic gene.[36] It is hypothesized that the upregulation of efflux pumps responsible for antibiotic tolerance could represent a form of cellular adaptation under nutrient limiting conditions.[35]

A. baumannii can produce various low molecular weight compounds known as siderophores that have an extremely high affinity toward metals.[37,38] Metals, especially iron, are an essential nutrient for all living organisms as they are needed for various biochemical and physiological functions. They are also crucial for the survival of *A. baumanii* within the host. Regardless of the abundance of metals in the biological systems, the bioavailability of certain active metals such as ferric iron is relatively low due to sequestration by compounds like heme, lactoferrin, and transferrin.[28,39] *A. baumannii* produces three classes of siderophore; namely acinetobactin,[40] fimsbactins,[41] and baumannoferrins,[42] which are transported by their respective TonB-dependent transporters.[43] This metal acquisition system, particularly those involving iron, contributes to the ability of *A. baumannii* to survive and causes infection in humans.[44] It is suggested that, under conditions of low iron concentration, the RND efflux pump genes known as *ade*ABC, are co-transcribed as a single *ade*ABC operon leading to overexpression of the adeABC pump.[45] Additionally, the mutation of the TonB-dependent copper receptor in *A. baumannii* leads to a reduction in the biofilm formation, underlining the additional role of copper, besides iron, in the regulation of its virulence.[25] Iron limitation has also been reported to regulate the biofilm formation via the induction of N-acyl-homoserine lactone;[46] a signal that mediates the communication between neighboring bacteria (quorum sensing), thus, enables the bacteria to survive under limited micronutrients supply while forming the biofilms.[46] Considering this, it is plausible that interfering with iron or other essential metal transport mechanism could be the basis of a new strategy toward a broadly efficacious therapeutic or vaccine.

More virulence factors are now being reported and they offer great potential for discovering novel anti-virulence or vaccine targets against the bacterium. Nevertheless, the number of identified virulence factors is still scarce in comparison to factors identified for other Gram-negative pathogens. A few of the identified virulence factors of *A. baumannii*, however, are also bacterial strain-specific, rendering them less promising for the development of broadly efficacious therapeutic or vaccine.[3]

Progress in *A. baumannii* vaccine development

Vaccines are now beginning to be recognized as potentially highly effective tools to mitigate antimicrobial resistance (AMR).[22] Several studies reporting the potential of vaccines against

Table 1. List of developed *A. baumannii* vaccine evaluated in animal models.

Type of Vaccine	Virulence/Potential Target	Route of administration	Vaccination mode	Ref
Formalin-inactivated	Whole cells	Active: Intramuscular; Passive: Intravenous	Active and passive	47
	Antibiotic-exposed whole cells	Intramuscular	Active	49
	LPS-deficient IWC	Intramuscular	Active	56
Bacterial Ghosts	LPS/surface	Oral, subcutaneous, intramuscular, intraperitoneal	Active	50
Cells component	Outer membrane complexes (OMCs)	Active: Intramuscular; Passive: Intravenous	Active and passive	52
	LPS-free OMCs	Intramuscular	Active	55
	Outer membrane vesicles (OMVs)	Active: Intramuscular; Passive: Intravenous	Active and passive	53
	LPS-free OMVs	Intramuscular	Active	57
Bacterial polysaccharides	Poly-N-acetyl-β-(1,6)-glucosamine (PNAG)	Anti-sera production: Subcutaneous+Intravenous; Immunization: Intranasal	Passive	48
	K1 capsular polysaccharide (CPS)	Antisera production: Intraperitoneal; Immunization: Subcutaneous	Passive	62
Subunit vaccine/recombinant vaccine	OmpA	Active: Subcutaneous; Passive: Intraperitoneal	Active and passive	68
	OmpA	Intranasal	Active	69
	Trimeric autotransporter protein (Ata)	Antisera production: Subcutaneous; Immunization: Intravenous	Active and passive	76
	Omp22	Active: Subcutaneous; Passive: Intravenous	Active and passive	73
	BamA	Active: Intraperitoneal; Passive: Intravenous	Active and passive	74
	FilF	Subcutaneous	Active	82
	OmpK	Intramuscular	Active	86
	FK1B	Intramuscular	Active	86
	OmpP1	Intramuscular	Active	86
	Hybrid ZnuD protein	Active: Subcutaneous; Passive: Intravenous	Active and passive	80
	Blp1	Active: Intramuscular; Passive: Intraperitoneal	Active and passive	32
	smpA and/or PLD	Active: Subcutaneous; Passive: Intravenous	Active and passive	78
DNA vaccine	OmpA	Intramuscular	Active	70
	OmpA and Pal	Intramuscular	Active	71

A. baumannii have recently emerged,[47,48] and various types of vaccine for this bacterium have been evaluated (Table 1). The earlier vaccines for *A. baumannii* were based on the whole cells, either grown without antibiotic[47] or under antibiotic-exposed condition[49] followed by inactivation with formalin, known as inactivated whole-cell (IWC) vaccine. IWC-based vaccines have been demonstrated to induce specific antibody responses against the *A. baumannii* and confer protection against subsequent sepsis challenges with the live bacterium.[47] Low production cost adds to the attraction of this approach.[49] As an alternative to formalin-IWC, *A. baumannii* ghosts (ABGs) also hold promise. ABGs, like other bacterial ghosts, comprise the cellular surface antigens without the cytoplasm and its constituent.[50] In studies, where rats were vaccinated with the ABGs via oral, subcutaneous, intramuscular and intraperitoneal routes, all routes demonstrated full protection following challenge with a lethal dose of the live bacteria, except those vaccinated orally which showed 67% protection.[50] Nonetheless, safety remains a primary concern for the use of IWCs or ABGs, as the risk of incomplete inactivation/treatment could potentially result in undesirable side effects. For example, IWCs or ABGs containing pathogen-associated molecular patterns (PAMPs), which are known to be pro-inflammatory molecules, can lead to reactogenicity in hosts.[68 to 51]

Apart from the whole bacterium, the bacterial cell outer membrane complexes (OMCs)[52] and outer membrane vesicles (OMVs)[53,54] have also been explored for vaccine development. These cell components exhibited promising outcomes as they elicited antibodies that recognized antigens from diverse *A. baumannii* strains.[52] Application of these cell components as vaccines in humans, however, has been precluded owing to the possibility of contamination with pyrogenic LPS during vaccine preparation.[54,55] To overcome this concern, LPS-deficient IWC was developed by introducing mutation to the *lpxD* gene essential for lipid A biosynthesis. Without the lipid A, the bacteria become LPS-deficient. The LPS-deficient IWC was shown to evoke robust immune responses similar to that of the wild-type bacterium.[56] With the success of LPS-deficient IWC, the cell component vaccines using the LPS-deficient bacteria were developed and called LPS-free OMCs and LPS-free OMVs, respectively.[55,57] While these LPS-free candidate vaccines exhibit low levels of endotoxins activity, they are less immunogenic in comparison to the wild type OMCs and OMVs. This is shown in the findings whereby LPS-free OMCs and LPS-free OMVs vaccines were demonstrated to provide only partial protection against sepsis infection when challenged with a lethal dose of live bacteria.[55,57] Total protection against infection was achieved only in mice immunized with a high dose of LPS-free OMVs.[57] While concern over endotoxin contamination is lessened with LPS-deficient IWC, another concern arises from incomplete inactivation of the strain. Incomplete inactivation of LPS-deficient strain has the potential to result in dissemination of this strain among the vaccinee and possibly into the community. This would be a worrisome predicament as it has been demonstrated that loss of LPS in *A. baumannii* strains leads to resistance to colistin, among the last option antibiotic for the treatment of *A. baumannii* infections.[58] In this regard, it is uncertain if the absence of LPS components within LPS-free OMCs and LPS-free OMVs could potentially evoke similar negative effects as those observed in LPS-deficient IWC. It might be argued that, unlike the IWCs, the OMCs and OMVs are only sub-cellular components, thus, not associated with the same risk as LPS-

deficient IWC. This is reflected in the licensure of vaccines utilizing the *Neisseria meningitidis* OMVs, such as VA-MENGOC-BC, MenBvac, MeNZB and Bexero.[59] It is noteworthy that although some of the earlier described *A. baumannii* candidate vaccines displayed high immunoefficiency, their applications are limited by safety and potential regulatory concerns. Thus, the discovery of other antigens that can offer significant protective immune response, safety and ease in the manufacturing processes remains a priority.

Bacterial surface polysaccharides, particularly capsular polysaccharides (CPS), display immunomodulatory effects that make them a potential target for vaccine development.[60] The bacterial polysaccharides are covalently linked to carrier proteins, and these glycoconjugates are employed to overcome the limitation of unconjugated polysaccharides in failing to evoke immunological memory.[61] Several glycoconjugate vaccines have been licensed, such as those for the prevention of *Haemophilus influenza* type *b* (Hib), *Streptococcus pneumoniae* (23 serotypes) and *Neisseria meningitides,*[73] with more under development.[60] Specifically for *A. baumannii*, polyclonal K1 capsule antisera that have been developed for immunotherapy exhibited bactericidal activity against bacterial challenge in a rat soft tissue infection model. Nevertheless, the effect has only been observed within the K1-positive bacterial strains (13 out of 100 tested clinical isolates).[62] There are nearly 40 serovars identified and information on seroprevalence of clinical isolates is currently limited.[63] Since there are multiple serovars of the CPS, perhaps the utilization of K1 CPS could be applied as a component of a multivalent vaccine for active vaccination, as in the case of *Streptococcus pneumoniae*.[62] Another important common constituent of extracellular polysaccharides in *A. baumannii* reported to significantly influence the pathogenicity of the bacterium is the PNAG.[64] Inoculation with antiserum to PNAG; 9Glc-NH_2-TT, induced opsonic and protective antibodies and passive immunization with the antisera reduced the bacterial burden in pneumonia and bacteremia challenges.[48] Based on these findings, it is suggested that PNAG is a potential component vaccine for both active and passive immunization with broad serotype coverage.[48,63] Passive antibody immunotherapy could particularly be useful for the treatment of acute infections especially at the time of an outbreak.[65] Although passive immunization with antisera has potential therapeutic value, some drawbacks could render its wider application unlikely, such as (i) the high cost of producing the antisera, (ii) the lack of heterologous protection as observed for antisera against K1 CPS, (iii) the lack of protection against septic shock and (iv) immediate but short-lived protection.[65] In contrast to passive immunization, active immunization (prophylactic vaccines) offers long-lasting and possibly lifelong protection.[66]

Among other potential vaccine targets include the outer membrane proteins (OMPs) of *A. baumannii*, especially OmpA. It has been proposed as a potential vaccine candidate owing to its important role in the pathogenesis of the bacteria.[67] Efforts are underway for the vaccine development using OmpA as vaccine target via different technologies, either as a protein-based vaccine[68,69] or DNA vaccine (encoding OmpA alone[70] or combination of OmpA with other protein[71]). All these vaccines are shown to elicit antibodies and to induce promising protective immunity against the bacterium infection.[68,69,70,71] Polymorphism of OmpA that resulted in five major bacterium variants, however, may limit the effectiveness of vaccine from a single OmpA variant.[72] In addition to OmpA, other OMPs that have been evaluated for their potential as vaccine candidates are Omp22[73] and BamA.[74] These target proteins have been demonstrated as highly conserved among the *A. baumannii* strains (95–-99%) and displayed promising potential for active and passive immunization.[73,74] Study on anti-OmpA MAbs, however, showed that the binding of the antibody was prevented by CPS of *A. baumannii*, which in turn inhibited the activation of macrophages and complement-dependent bactericidal activity. This raised concern that not only OmpA, but other OMPs may not be viable vaccine targets as these epitopes are shielded by the CPS.[75] Hence, the surface-exposed proteins or surface-associated proteins that penetrate the capsule layer may offer better success as candidate vaccine targets.[32] Some of these surface-exposed proteins are involved in the formation of biofilm that contributes to the adhesion of the bacterium to the host cell. Among the evaluated proteins are the trimeric autotransporter protein (Ata)[76] and Blp1 protein.[32] Antibodies to Ata possess opsonophagocytic killing and bactericidal properties, while the antisera to Ata mediates both phagocyte-dependent and phagocyte-independent killing of *A. baumannii*.[76] However, *ata* gene is present in only 78% of the assessed *A. baumannii* strains.[77] This suggests that a single valent Ata protein may not be sufficient to protect against the strains without Ata, and a combination of proteins might be needed to obtain broader protective efficacy. On the other hand, Blp1 protein encoded in all the examined clinical *A. baumannii* strains of globally spread clonal lineages I and II (IC I and IC II) could be an alternative.[32] The vaccine incorporating Blp1 protein elicited efficient protection against lethal *A. baumannii* infection in a sepsis murine model, achieving 60% and 100% in the active and passive immunization, respectively.[32] The opsonophagocytic killing properties were observed in the antisera against Blp1-specific antigen.[32] Although the Blp1 was observed in all of the evaluated *A. baumannii* strains, differences in the characterization and function of Blp1 protein between IC strains in biofilm formation and adhesion to epithelial cells require further investigation.

Other bacterial components that have been evaluated as vaccine candidates were the outer membrane lipoprotein A, small protein A (smpA), and phospholipase D (PLD).[78] Not much is currently known on the function of smpA in *A. baumannii*, but a study on its homolog in *Pseudomonas aeruginosa* showed that the lipoprotein is important in the formation and maintenance of cell membrane structure and integrity.[78] PLD on the other hand, has been demonstrated to be involved in epithelial cell invasion and *in vivo* pathogenesis.[24,26] The smpA and PLD have been evaluated as a single and multi-component vaccine.[78] Mice immunized with these proteins as a single component exhibited lower pro-inflammatory cytokines in the bronchoalveolar lavage fluid (BALF) and serum, and experienced reduced lung infiltration and bacterial load.[78] Nevertheless, the combined components did not display any advantages in reducing the bacterial burden, while the protection conferred by passive immunization with the anti-sera provides a slight increase in the survival against bacterial challenge in a pneumonia model.[78] As different bacterial strains may elicit different immune responses, perhaps lipoprotein A, smpA, and

PLD, as well as K1 CPS could be included among the targets in multicomponent vaccines against *A. baumannii*.[78] In the search for vaccine antigens that provide broad protective coverage, ZnuD has been seen as a promising candidate in *Neisseria meningitidis*.[79] In *A. baumannii*, ZnuD is necessary for the internalization of zinc in low zinc conditions. In this regard, zinc acquisition plays a crucial role in bacterium growth in the human host.[80] Recently, a hybrid antigen displaying a surface loop of ZnuD from *A. baumannii* consisting of an individual loop (2, 5, 7, and 11) or the loops together in a single hybrid has been explored as a potential vaccine for *A. baumannii*.[80] The study included the refolded ZnuD protein as a control. It is reported that, when evaluated in an active immunization study, the hybrid ZnuD proteins provided better protection from lethal dose challenge in the sepsis model in comparison to the refolded ZnuD.[80] This suggested that the structure of ZnuD may need to be displayed in its native structure. Although the refolding process resulted in soluble protein, their native protein structure is possibly affected.

More recently, advancements in molecular technologies and high throughput full genome sequencing have contributed to the establishment of huge databases, allowing for the rational design of vaccines using a process known as "reverse vaccinology" (RV).[81,82] RV has led to the discovery of previously unknown antigens and has added greatly to our understanding of the biology of several pathogens.[83] It has also contributed to the development of universal vaccines for several pathogens, exemplified by the meningococcus B vaccine.[84] RV has already been applied to the development of vaccines for several bacterial pathogens including but not limited to *Bacillus anthracis, Streptococcus pneumoniae, Mycobacterium tuberculosis, and Cryptosporidium hominis*.[85]

In the development of *A. baumannii* vaccines, various RV approaches have been employed to identify potential novel vaccine candidates. These methodologies include direct RV,[82,86] antibiotic-resistant determinant-focused RV,[85] virulome-based RV,[87] and a combination of subtractive proteomics and RV.[88] These different RV methods were attempted to identify novel targets that have the potential to elicit broad protective immunoresponse. Although several potential candidates have been identified using these techniques, only a few have been validated in animal models; they include FilF, BamA, OmpK, FK1B and OmpP1.[74,82,86] FilF and OmpP1 have been shown to decrease the bacterial load in immunized and challenged mice in pneumonia model of *A. baumannii*, and increased their survival rate by up to 50%.[47,51] However, that level of protection is not considered potent enough against this evolving pathogen. As for OmpK and FK1B, they did not confer protection against pneumonia bacterial challenge despite eliciting a strong IgG response.[49] These observations demonstrate possible discordance between *in silico* and the *in vivo* findings, emphasizing the importance of further validation.

Prospects for *A. baumannii* vaccine development

Validation of antigenic protein

To date, no *A. baumannii* vaccine candidate has advanced to the clinical stage although the application of RV has demonstrated great potential to accelerate the identification of possible candidates. Furthermore, only a few have been experimentally evaluated and most of those selected were associated with the outer membrane of *A. baumannii* and were generally proteins related to antibiotic-resistance development, transport (efflux), or metabolism. A number of the candidates have been deprioritized due to their high molecular mass, since they may present challenges for efficient protein expression, purification and process development. More efforts to validate these antigens either *in vitro* and/or *in vivo* are needed. With the development of better *in vitro* cell-based or cell-free expression technologies, high-throughput platforms to screen and select the vaccine antigen candidates will become available, including those that screen for toxicity. This should greatly accelerate the vaccine development process.[89,90]

Alternative vaccine expression host

Many expression platforms for the production of recombinant proteins are now available, and these consist of the eukaryotic, plant or microbial expression systems. The microbial expression system remained a popular choice and widely used mainly because of the short production time, scalable, and low production cost.[91] Among this expression system, *Escherichia coli* has been dominantly used for over 30 years. This could be contributed by the rapid growth kinetics of *E. coli*, the easy transformation process, and the wide choices of vectors and other commercially available tools for DNA manipulation.[92,93] However, this expression system also has several disadvantages, particularly for the development of *A. baumannii* vaccines. Most highly antigenic *A. baumannii* proteins such as BamA and FilF are only expressed as insoluble inclusion bodies in *E. coli*.[74,82] Therefore, large-scale vaccine antigen production would require more complex purification and refolding processes, typically leading to a reduced yield of the final antigen product.[74,93] To overcome this limitation, *in silico* vaccine design strategy such as that designed for a soluble Omp34 immunogen with increased antigenicity was described.[94] In the study, based on the predicted topology of Omp34, the exposed linear B-cell epitope was predicted. A peptide loop that was considered "inappropriate" was removed and replaced with a more compatible sequence predicted by *in silico* analysis. This modification, however, has yet to be evaluated *in vitro* as well as *in vivo*. Although the modification may increase the solubility of the antigenic protein, it may also have detrimental effects on the immunogenicity,[95] such as those reported for the ZnuD mentioned earlier.[80] As an alternative to *E. coli*, expression in Gram-positive bacteria, such as *Bacillus* and *Corynebacterium* has been proposed.[96,97] Among the Gram-positive bacteria, the spore-forming Gram-positive *Bacillus* sp. i.e. *Bacillus subtilis*, has the potential to be used as an expression vehicle in bio-factories for vaccine production. The bacteria are nonpathogenic and with a safe record in human and animal use as probiotics and food additives.[98] The supplementation of *B. subtilis* as a probiotic has been demonstrated as a safe and effective way to stimulate systemic and mucosal responses.[99] This further affirms the potential of *B. subtilis* as a live bacterial vector that could both serve as an expression and delivery vehicle, which also possesses the much desired adjuvant properties.[100,101] Amenability to oral delivery in itself constitutes an additional advantage as a delivery platform for

the *A. baumannii* vaccine. The idea of formulating *A. baumannii* vaccines for mucosal delivery is appealing since the *A. baumannii* usually gain access into the body via mucosal sites to affect a wide range of tissues. Mucosal vaccines would be expected to elicit both the systemic and mucosal antibody responses.[69] Studies on mucosal delivery using *B. subtilis* spore-displaying antigenic protein have demonstrated the ability to elicit protection and immunogenicity responses.[98,102–104] With the advancement of expression systems such as *B. subtilis*, validation of potential vaccine antigen candidates for *A. baumannii* is anticipated.

Vaccine delivery

The elucidation of the immune pathways that are critical in defense against *A. baumannii* infection will hasten the discovery of a novel vaccine.[105] What is currently known is that LPS is a potent stimulator of TLR-4, which leads to activation of NF-κB. That, in turn, induces secretion of macrophage inflammatory protein 2 (MIP-2) and KC/IL-8,[105] and stimulates the tumor necrosis factor-alpha (TNF-α) release.[106] These cytokines, which have chemo-attractant properties, lead to the recruitment and activation of neutrophils and macrophages, in addition to a range of other host factors.[24,106] Apart from TLR-4, IL-17 has been shown to stimulate the production of CXC chemokines, which ultimately clears the bacteria during primary infection with Gram-negative bacteria.[105] With more evidence showing the critical role played by the Th17 cells (CD4+ IL-17 producing cells) in the host defense against many bacteria, a similar pathway could be important in protecting against *A. baumanni* infection. Most Th17 cells reside in the barrier tissues such as the intestinal and respiratory tracts, and the skin. The signature cytokines of Th17 cells (include IL-17A, IL-17F, IL-22, and IL-26) besides innate lymphoid cells are critical in regulating tissue homeostasis.[105,107] It was reported that Th17 effector cytokines IL-17 and IL-22 induced an array of antimicrobial peptides such as β-defensin-2, lipocalin 2, and the S100 family proteins from barrier tissues, such as gut, skin, and lung.[105,107] Based on the identified immune pathways against *A. baumannii*, it appears that the mucosal route for vaccination could be better for the elicitation of an effective and protective immune responses against *A. baumannii*. However, most of the earlier described *A. baumannii* vaccine candidates, focused on the parenteral route of administration as it was more challenging to formulate vaccines for mucosal delivery. Currently, the availability of mucosal vaccines in general is still limited. Most of the licensed oral vaccines are based on live attenuated platforms (bacteria or viruses). In mucosal vaccine delivery, the main challenges are to overcome the low immunogenicity and instability of antigens in the harsh mucosal environment.[108] Mucosal delivery vehicles that protect and preserve the structural integrity of the target antigen while simultaneously increasing the production of local and systemic neutralizing immune responses are highly needed. Numerous studies have shown that spores of *B. subtilis* possess these characteristics.[98,100,103,109] Other examples of mucosal delivery vehicles being studied include virus-like particles (VLP), immunostimulatory complexes (ISCOMs), polymeric and inorganic nanoparticles, emulsions, pollen grains, and live bacterial vector.[59,98]

Animal models and bacterial strains

In vaccine studies, preclinical assessment in animal models is one of the prerequisites before the clinical phase in human subjects. As most of the *A. baumannii* strains do not infect immunocompetent mice commonly used in vaccine studies, such as BALB/c or ICR, there is an urgent need to search for better mammalian models, especially animal model of sepsis and pneumonia. For the establishment of a pneumonia model, infection performed via intra-tracheal,[110] intranasal,[111] inhalation through nebulizer[112] and oral aspiration are feasible.[113] As for the sepsis model, infections through intraperitoneal[114] and intravenous via tail vein[113] have been performed. The effects of these different routes of infection on the biomarkers and the elicited host-immune responses, are yet to be fully evaluated. Improved mammalian models that serve as surrogate models for the assessment of vaccines for *A. baumannii* are urgently needed.[114]

Currently, to increase the infectivity of *A. baumannii*, mice to be infected are engineered to be immunocompromised, either by inducing diabetes or administrating the alkylating agent cyclophosphamide (CYP).[112] Alternatively, increased virulence of the bacterium is achieved by adding porcine mucin to the challenge bacteria.[115] These models nevertheless, are still limited in mimicking human infections.[116] Special mouse strains; A/J and C3HeB/FeJ are noted to be more susceptible to *A. baumannii* infection in comparison to the commonly used mice strains, hence, should be explored as a better model.[117] The susceptibility of A/J mice could be attributed to the delayed neutrophil recruitment to the lung caused by diminished CXC chemokine responses to the bacteria, while the reason for susceptibility of C3HeB/FeJ has yet to be explained.[117] In addition to mice, rats could represent a better animal model.[118] The rat pneumonia model demonstrates many characteristics that mimic human infection without altering the immune profile or requiring the use of porcine mucine.[118]

Apart from selecting the right animal model, it would be advantageous if the bacterial strains employed for the animal challenge are genotypically characterized. Small genomic changes within the bacteria could lead to significant differences in virulence that could then present a different drug resistance profile.[24] Hypervirulent *A. baumannii* strains have been demonstrated to persist in the blood at high bacterial densities for up to 24 hours and are less susceptible to complement-mediated killing and macrophage uptake.[117] Among the hypervirulent strains, HUMC-1,[112] AB5075,[110] LAC-4,[111,114] and VA-AB41[113] have been evaluated for their ability to cause lung infections and bacteremia in mice. Among these strains, only LAC-4 strain has been shown to elicit lethal and sublethal pneumonia and sepsis infections, in immunocompetent BALB/c and C57BL/6 mice without any neutropenic rendering procedure.[111,114]

Vaccine target population

The ultimate goal of vaccine development is a successful product licensed for use. Although WHO listed *A. baumannii* as among the bacteria that are in critical need of novel antimicrobial agents, the commercial interest may not match the urgency. This is partly due to the low prevalence of the

infection and the difficulty in establishing vaccine programs for target populations.[119] It is envisioned that three populations would benefit from vaccination against *A. baumannii*. The first population consists of hospitalized patients or those who will require prolonged treatment in the hospital due to health issues. For this population, vaccination could be incorporated into the pre-surgical care plan.[119] Although the prevalence of *A. baumannii* infection is relatively lower in comparison to other bacterial infections such as those caused by *Escherichia coli, Staphylococcus aureus*, and *Clostridium difficile*, the significant burden of the cost associated with the HAIs to the healthcare agency and the patient are still enormous.[14] On the other hand, vaccination of those in the ICU could be challenging as the target population may be already immunosuppressed. Because of this difficulty, passive immunization with monoclonal antibodies is perhaps a viable strategy.[119]

The second population that could benefit from *A. baumannii* vaccination is the elderly. The global population is rapidly aging. By 2050, it is estimated that the number of persons older than 60 years of age is expected to double.[120] Aging is a significant risk factor for *A. baumannii* infection and the severity of infection is higher in the elderly due to immunosenescence.[121] With the increasing prevalence of MDR-*A. baumannii* and limiting avenues for effective antimicrobial treatment, infection with the bacteria is foreseen to pose more burden clinically and economically, hence, preventive and treatment measures are desperately needed. The elderly population that would be suited as target population would be those above the ages of 50, or perhaps 65, and those with underlying diseases; this would be similar to the study group recommended for *Streptococcus pneumoniae*.[120] As immunosenescence in the elderly could increase susceptibility to infections, it could at the same time pose a challenge for vaccination as the capacity to respond toward vaccine is also compromised.[122] Several potential strategies to improve immunogenicity in the elderly have been suggested. These include the use of stronger adjuvants, higher antigen doses or more boosters, and different or novel routes of administration.[120–122]

The third population that could benefit from *A. baumannii* vaccination comprises the healthcare workers (HCWs) as they are in general at higher risk of contracting the infection through occupational transmission. A broader grouping could also include the military, volunteers, and humanitarian personnel who visit conflict-affected areas. Potential responses to infection, however, vary in this population group. Although most healthy people may not develop symptoms from *A. baumannii* infection, they could transmit the bacteria to cause serious health problems to the vulnerable population or they become pre-disposed following tissue injuries.[123,124]

Summary and prospects

The widespread presence of antibiotic-resistance *A. baumannii* strains and the lack of new antimicrobials on the horizon, limit the treatment options against the bacterium. Vaccination against the bacterium as an option to tackle the drug-resistance issue has increasingly been emphasized. Significant progress has been made toward it but to date, there is no viable candidate vaccine that has reached the clinical trial phase. The progress in *A. baumannii* vaccine development is hampered by several challenges. The complex interaction between the virulence factors and the pathogenesis of *A. baumannii* remains to be fully discovered. The adaptability and plasticity of the genome conferring the multidrug resistance posed a challenge in the selection of the vaccine targets. It may, therefore, be necessary to identify factors that affect the overall function of the bacterium, potentially one that is crucial for its metabolism. This would impact the bacterial replication within the host, allowing the host immune response to overcome the infection, thus, reducing the severity of the infections. While vaccines derived from the bacterial cells are more immunogenic in comparison to the subunit vaccine, the latter may be preferred as it would raise a lesser safety concern. With the successful licensure of menB vaccines, RV presents a highly feasible and attractive approach to identify novel antigenic proteins of *A. baumannii* for the development of a broadly efficacious vaccine. The application of the RV approach in combination with high throughput *in silico* screening is foreseen to accelerate the vaccine target selection. Currently, most of the studied *A. baumannii* vaccines are formulated and tested for administration through the parenteral route. Our understanding of the immune responses against *A. baumannii*, suggests mucosal route inoculation could provide a better protective response. However, mucosal-based vaccines have several challenges including potentially weak immunogenicity and stability of antigens in the mucosal environment. This could be overcome with a stable expression system such as the spore-based *Bacillus subtilis* delivery method. Additionally, this platform has a high safety profile, can potentially stimulate mucosal as well as systemic responses, and needle-free route of administration. Moreover, the platform would provide fewer manufacturing and delivery challenges, which could translate into wider availability of the vaccines. Although the recombinant spore platform has yet to be licensed as a candidate vaccine, its potential is worth exploring.

More reliable pneumonia and sepsis animal models are also needed for the development of the *A. baumannii* vaccine. The current widely used inbred mice model has its limitation, thus, genetic modification is performed to render the mice susceptible to the infection. However, this may alter the critical pathways in the host-pathogen interaction,[125] which may not reflect the natural immune responses conferred by the vaccine in humans. Alternatively, rats displayed better characteristics as the animal model for challenge study with live *A. baumannii*, as it mimics human infection, hence, should be rigorously explored.

Despite the significant threat posed by *A. baumannii* to global public health, vaccine development for this bacterium still lags behind other Gram-negative pathogens. Adapting the successful approach in identifying the vaccine targets of other bacterial vaccines (eg. 4CMenB vaccine for *Neisseria meningitidis*) and exploring alternative expression systems and delivery platforms, such as *B. subtilis* spore, could hasten the vaccine development that would eventually lead to approved *A. baumannii* vaccine. To ensure success, conscientious and continuous endeavor coordinated and harmonized efforts between the government agencies, academia, and industries are required to accelerate the discovery of alternative treatment to thwart the challenges posed by MDR *A. baumannii*.

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N.M.R., L.H-Y., U.S., and S.A conceived, wrote and edited the manuscript.

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