REVIEW PAPERS

Enigma still unraveled: intracellular life of *Acinetobacter baumannii* within host cells

Enigmatyczne wewnątrzkomórkowe życie Acinetobacter baumannii

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KEYWORDS:

- intracellular
- virulent
- vacuole survival
- immunoevasion
- multi-drug resistant

ABSTRACT

Acinetobacter baumannii is a common opportunistic pathogen known for its ability to cause severe infections, particularly in immunocompromised individuals. The prevalence of multidrug-resistant *A. baumannii* infections is surging at concerning rates. While traditionally considered an extracellular pathogen, emerging findings suggest that *A. baumannii* can survive and replicate within host cells, thereby evading immune responses and antimicrobial treatments. This review article summarizes recent reports of intracellular persistence and explores the survival strategies employed by *A. baumannii*, including its interactions with host cell machinery and evasion of host defenses. Understanding these mechanisms is crucial for developing targeted therapeutics to combat *A. baumannii* infections.

SŁOWA KLUCZOWE:

- wewnątrzkomórkowy
- wirulentny
- przeżywanie w wakuoli
- ucieczka od odpowiedzi komórkowej
- wielolekooporny

STRESZCZENIE

Acinetobacter baumannii to powszechny patogen oportunistyczny, znany ze swojej zdolności do wywoływania ciężkich infekcji, szczególnie u osób z obniżoną odpornością. Częstość występowania zakażeń wielolekoopornych *A. baumannii* rośnie w zastraszającym tempie. Chociaż tradycyjnie uważa się go za patogen zewnątrzkomórkowy, nowe odkrycia sugerują, że *A. baumannii* może przetrwać i replikować się w komórkach gospodarza, unikając w ten sposób odpowiedzi immunologicznej i leczenia przeciwdrobnoustrojowego. W tym artykule dokonano przeglądu ostatnich przypadków przetrwania wewnątrzkomórkowego i zbadano strategie przetrwania stosowane przez *A. baumannii*, w tym jego interakcje z maszynerią komórkową gospodarza i unikanie mechanizmów obronnych gospodarza. Zrozumienie tych mechanizmów ma kluczowe znaczenie dla opracowania leków celowanych, zwalczających zakażenia *A. baumannii*.

Introduction

Acinetobacter baumannii is a Gram-negative bacterium that can cause opportunistic infections in humans, despite its natural habitat being water and soil (1). This pathogen is a common nosocomial agent in the intensive care units (ICUs) among critically ill patients who require a large number of invasive procedures (2). *A. baumannii* is a frequent etiologic factor of ventilator-associated pneumonia (VAP), bloodstream infections often resulting in sepsis, hospital-acquired meningitis, urinary tract infection, as well as traumatic skin, soft tissue, and bone infections (3). Infections caused by multidrug-resistant (MDR) *A. baumannii* strains are increasing at alarming rates, as this pathogen develops resistance to many antibiotics, including carbapenems. MDR *A. baumannii* infections are associated with increasing morbidity and mortality rates (4, 5). Furthermore, *A. baumannii* is constantly spreading at a faster pace, and its MDR, extensively drug resistant (XDR), and even pandrug-resistant (PDR) traits make it a high-priority pathogen (6). Moreover, *A. baumannii* has been classified as a member of the ESKAPE group, comprising six highly virulent and antibiotic-resistant bacterial pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*), as specified by The Infectious Diseases Society of America (7).

A. baumannii is a significant public health concern due to its ability to develop biofilms, enabling it to survive on various

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environmental surfaces and spread within healthcare facilities (1). This bacterium has demonstrated the capacity to undergo morphological adaptations, such as thickening of cell walls, in response to dry environments (8). Additionally, its proficiency in adhering to human cells, interacting with the innate immune system cells, and exhibiting substantial genetic variation among strains are crucial factors contributing to *A. baumannii*'s virulence (9-11). Traditionally, *A. baumannii* has been characterized as an extracellular pathogen with limited intracellular survival capabilities. However, recent studies have revealed emerging evidence that these bacteria can persist and even replicate within various cell types, including endothelial and epithelial cells, macrophages, neutrophils, and amoebae (12-21). The intracellular persistence and replication of *A. baumannii* have significant clinical implications, as they can serve as mechanism of immune evasion and resistance against antimicrobials that do not effectively penetrate the cytoplasm.

This paper aims to elucidate the role of *A. baumannii's* intracellular survival in evading the host's immune response. Comprehensive understanding of the pathobiology, including pathogenicity and the identification of the replicative niche, is crucial for development of effective therapeutic strategies against *A. baumannii*.

Virulence factors

A. baumannii is a pathogen known for its ability to cause severe infections, though it is challenging to pinpoint a specific virulence factor responsible for its pathogenicity. It is assumed that *A. baumannii* employs a complex and polygenic approach to its pathogenesis.

Unlike some other Gram-negative bacteria, such as *E. coli* or *Salmonella* species, *A. baumannii* does not harbor many classical pathogenesis determinants like toxins. Instead, this pathogen is renowned for its capacity to cause infections through various mechanisms, including biofilm formation, enzyme secretion, and resistance to host immune responses.

Outer membrane proteins (OMPs) are one of the well-studied virulence factors of *A. baumannii*. These OMPs are involved in adhesion, internalization, and the induction of host cell death (22). Biofilm formation, a crucial mechanism for bacterial adhesion and internalization, requires a complex of virulence factors, including certain OMPs, secretion systems, pili, and biofilm-associated proteins (Bap). Additionally, *A. baumannii* utilizes an elaborated iron uptake mechanism using siderophores, FecA and FecI receptors, which are also implicated in biofilm formation and resistance to oxidative stress (9).

Although there are not many studies confirming the presence of *A. baumannii* within the eukaryotic cells, several factors responsible for their intracellular persistence have been identified. Pili, for instance, are essential for bacterial adherence to the host-cell surface, leading to successful beginning of infection (16). Phospholipase D, in turn, has been found to be crucial for both invasion and serum resistance (23).

The detailed information on the virulence factors of *A. baumannii* was presented in our previous review (24). Table 1 below summarizes the main virulence factors of *A. baumannii* and their function in pathogenesis.

Table. 1 Virulences factors of *Acinetobacter baumannii* and their significance in pathogenesis.

Virulence factors	Functions	References
Acinetobactin	Iron acquisition	(25)
Biofilm associated proteins and quorum sensing	Promotion of adherence, formation of biofilm	(26)
Capsular polysaccharides (CPS)	Immune evasion, antimicrobial and stress resistance	(22)
CarO	Carbapenem resistance, adhesion,	(22)
Efflux pumps	Antibiotic's extrusion	(22)
Gig (Growth in Galleria) genes	Promotion of transcription of stress response genes	(22)
Lipopolysaccharides (LPS)	Stimulates pro-inflammatory cytokines release	(27)
Omp33-36	Carbapenem resistance, autophagy modulation, apoptosis induction	(28)
OmpA	Adhesion, apoptosis induction, antimicrobial resistance, formation of biofilm	(29, 30)
OmpW	Iron uptake, adhesion, invasion, biofilm formation	(31, 32)
OMVs	Contain antibiotic resistance genes and virulence markers	(33)
Phospholipases	Invasion, iron uptake	(34)
Pili	Motility, adherence, formation of biofilm	(35)
Type I secretion system	Intracellular persistence, formation of biofilm,	(36)
Type II secretion system	Serum resistance, adhesion, intracellular persistence	(37)
Type V secretion system	Promote adherence, apoptosis induction	(37, 38)

Phase variation as a novel virulence factor

Bacteria often exhibit genetic and phenotypic heterogeneity within a population. Recent studies have reported that *A. baumannii* displays phenotypic heterogeneity within a genetically homogenous population through rapid interconversion between cells capable of forming opaque or translucent colonies **(Fig. 1)**. Chin et al. have linked the virulence of *A. baumannii*, its resistance to diverse innate immune antimicrobials (CRAMP, lysozyme and H_2O_2), and its environmental persistence (resistance to hospital disinfectants and desiccation) to the high-frequency phenotypic switching (39). The virulent subpopulation (VIR-O, virulent, opaque) of *A. baumannii* possesses a thicker capsule and is resistant to host antimicrobials, unlike the translucent colonies (AV-T cells, avirulent, translucent). Furthermore, it was observed that the VIR-O subpopulation predominates during in vivo infection, while the AV-T subpopulation lacks the capability to induce acute disease, underscoring the diverse pathogenic potential of these subpopulations during infection scenarios. The phenotypic switching between the VIR-O and AV-T populations was found to be related to the TetR-type transcriptional regulator, ABUW_1645, whose overexpression in the VIR-O background drives conversion to the AV-T state and diminished pathogenicity (39). However, the later study indicated that ABUW_1132 (1132), a highly conserved gene predicted to encode a LysR-type transcriptional regulator, turned out to be a global and critical regulator of virulence and traits commonly linked to persistence in the hospital environment (40). Notably, bacteria isolated from the bloodstream of human patients exclusively align with VIR-O subpopulation (39). Variants of the opaque and translucent colonies of *A. baumannii* exhibit several phenotypic variations. During the early stationary phase, the translucent bacterial type displays a highly elongated morphology compared to the opaque form. The opaque colonies demonstrate an increased rate of surface motility, which is an additional death factor in *Galleria mellonella* waxworm model. Conversely, the transparent varieties exhibit a greater capacity for biofilm formation. Interestingly, the rates of phase variation increase at high cell density and are regulated by the accumulation of an extracellular signaling molecule, a process known as quorum sensing (40-42), which can also be considered a virulence mechanism (43).

Host immune response to *A. baumannii*

Innate immune cells play crucial roles in the host's defense against *A. baumannii* infection. Monocytes, macrophages, dendritic cells (DCs), and natural killer cells (NKs) have been identified as important effectors in the defense mechanism (44). Neutrophils emerge as the key cells essential for controlling different types of *A. baumannii* infection (1). While the detailed mechanism of that process is not entirely clear, neutrophils regulate and eradicate *A. baumannii* via phagocytosis, the production of reactive oxygen species (ROS), neutrophil extracellular traps (NETs) formation, defensins, and lysosomes (22).

Among the first immune cells to respond to *A. baumannii* are macrophages. They serve as a protective barrier in the early stages of infection, as they are able to phagocyte bacteria and release chemokines and proinflammatory cytokines to recruit neutrophils to the infection site (45). Autophagy is a regular cellular mechanism employed to neutralize invasive pathogens (46). After phagocytosis, the phagosome fuses with lysosome in a process called "maturation"

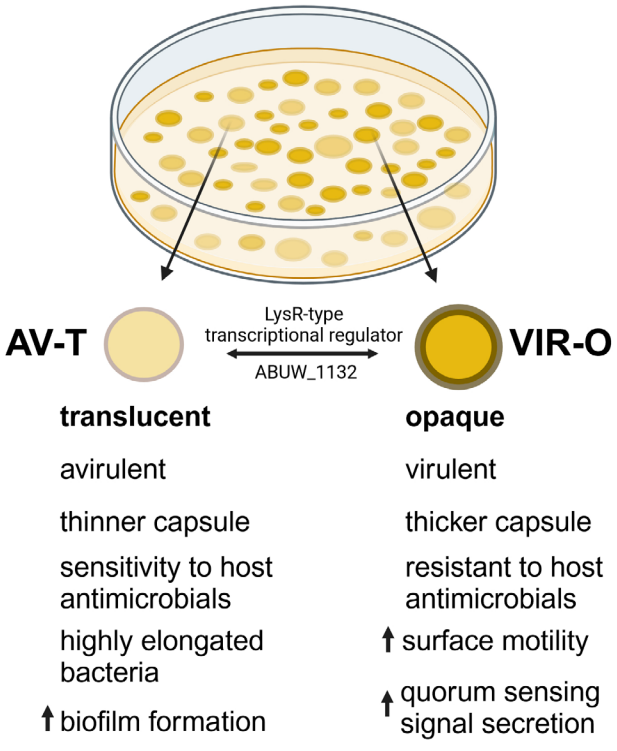

Figure. 1 Phenotypic changes resulting from a high-frequency phenotypic switch of *A. baumannii* (created with BioRender.com).

to destroy its content. The primary features of phagolysosomes are the presence of hydrolytic enzymes and an acidic pH (47). Phagocytosis stimulates the production of strong neutrophil chemoattractants such as IL-6, TNF, then IL-1β, IL-10 in later stages of infection (45).

The contribution of other innate immune cells, including NKs and DCs, remains rather ambiguous. In murine models, NKs cells utilize an indirect mechanism to attract neutrophils by releasing chemoattractants. DCs cells, on the other hand, are activated by LPS or OmpA, which triggers DCs signaling through nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs), leading to antigen presentation and IL-2 production (44).

The infection process begins when pattern recognition receptors (PPR) located on the surface of innate immune cells detect specific pathogen-associated molecular patterns (PAMPs) of *A. baumannii*, leading to the expression of pro-inflammatory cytokines and chemokines (44). The most significant surface-bound PRRs are Toll-Like receptors (TLRs), which bind to molecules such as LPS, porins, and peptidoglycan. *A. baumannii* is primarily recognized via TLR2, TLR4, and TLR9. Activation of these receptors' triggers NF-κB signaling, leading to the expression of pro-inflammatory cytokines (22). Interestingly, a recent study has demonstrated that *A. baumannii* can also induce a type I IFN response, which is dependent on the TLR4-TRIF-IRF3 pathway and phagocytosis. This has been observed *in vitro* in both VIR-O and AV-T variants, however, phase variants with reduced envelope lead to increased TLR4-dependent type I IFN induction. Enhanced signaling may depend on increased phagocytosis in the case of AV-T, which also leads to increased host cell-mediated killing (48).

The increasing number of studies confirming the presence of *A. baumannii* inside the cell has drawn attention to the role of intracellular PRRs, particularly the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (49, 50). Activation of the NOD-like receptors triggers the assembly of the cytosolic multiprotein complexes termed inflammasomes (51). During *A. baumannii* murine infection, the release of the pro-inflammatory cytokines IL-1β and IL-18 was found to be entirely dependent on the activation of the NLRP3 inflammasome. Furthermore, NLRP3 activation can induce a form of macrophage cells death known as proptosis (52).

The detailed mechanism by which *A. baumannii* modulates host cell pathways are not yet fully understood. In addition to inducing a host response signal through the activation of TLR2, TLR4, and TLR9, *A. baumannii* may employ other pathways to modulate the immune response, mediated by known virulence factors in *A. baumannii* and other Gram-negative species. Considering the critical function of the Type VI Secretion System (T6SS), which *A. baumannii* utilizes to deliver effector proteins directly into host cells, it is highly probable that the *A. baumannii* T6SS is capable of neutralizing the host's defensive strategies. These effectors can disrupt the cytoskeleton and inhibit phagocytosis. A recent study has indicated that another Gram-negative bacterium, *Edwardsiella tarda*, is able to inhibit the NLRP3 inflammasome in a manner dependent on the "T6SS effector *E. tarda* virulent protein P (EvpP)" (53) *A. baumannii* releases outer membrane vehicles (OMVs), which are spherical blebs derived from the outer membrane. These vesicles carry various virulence factors, including enzymes, toxins, that can alter host cell processes. OMVs fuse with host cell membranes, delivering their cargo into the host cytoplasm. Moreover, OMVs can deliver small RNAs that interfere with host gene expression and may lead to immune evasion (54).

Additionally, *A. baumannii* produces enzymes that can degrade host defense molecules, aiding in immune evasion and tissue invasion. *A. baumannii* inhibits the formation of neutrophil extracellular traps (NET), structures formed by neutrophils to trap and kill bacteria, by suppressing neutrophil adhesion (55). The *A. baumannii* factor responsible for this remains unknown. Nonetheless, several factors that hinder or disrupt NET formation have been discovered in other bacterial species, particularly those in the ESKAPE group. For instance, LPS is known to play this role in *K. pneumoniae*, while flagella have been implicated in *P. aeruginosa* (56).

Intracellular life of *A. baumannii* in macrophages

Until recently, *A. baumannii* was considered an extracellular pathogen with limited survival within host cells. However, recent studies have emphasized the ability of various *A. baumannii* strains to be internalized or even actively invade host cells. Studies on *A. baumannii* have predominantly focused on well-known laboratory strains, such as ATCC 19606, which were isolated many years ago (13, 21, 29, 30, 50, 57-59). Macrophages can efficiently control the replication of *A. baumannii* and eliminate the most common laboratory strains, including ATCC 17978 and ATCC 19606 (20, 45, 60). However, there is a paucity of reports utilizing current clinical strains, which have been shown to differ from the laboratory strains. In contrast to the reference strains, modern isolates have been reported to be capable of intracellular growth and replication (16-21). Novel clinical isolates have demonstrated an aptitude to overcome host cell defense mechanisms *in vitro* (16-21). Unlike the ATCC 19606, modern acquired clinical isolates of *A. baumannii* have shown the ability to persist inside murine J774A.1 cell line (16, 19), murine BMDM (16), human THP-1 macrophage-like cell line (16), human monocyte-derived macrophages (19), as well as in an *in vivo* murine pneumonia model (20). In response to intracellular multidrug-resistant *A. baumannii* (MDRAB), murine macrophages of the J774A.1 cell line produces reactive oxygen species (ROS) (19), which serve as a rapid killing mechanism against phagocytosed *A. baumannii* (61). However, MDRAB clinical isolates have been observed to exhibit resistance to the toxicity caused by hydrogen peroxide. This feature is attributed to the presence of enhanced catalase activity, which is responsible for the bacteria's defense against ROS, distinguishing these strains from ATCC 19606 reference strain (19). The elevated catalase production by *A. baumannii* could hinder the intracellular elimination by macrophages, thereby facilitating the dissemination of *A. baumannii* infections.

In contrast to the ATCC 19606 reference strain, some modern clinical isolates of *A. baumannii* have been reported to persist and replicate within spacious intracellular vacuoles, termed *A. baumannii*-Containing Vacuoles (ACVs), within J774.4, THP-1, BMDM macrophages in vitro, as well as in alveolar macrophages in a murine pneumonia model *in vivo* (16, 20). Initially, both the reference and clinical strains interact with the macrophage endocytic pathway, as evidenced by the presence of the early EEA1 (Early Endosomal Antigen 1) and the late endosomal marker LAMP1 (Lysosomal-associated membrane protein 1). However, at a later stage, while the ATCC 19606 reference strain is eliminated through the autophagy pathway, the clinical strain replicates

within the ACV and is not degraded, suggesting that replicative ACVs follow a non-canonical intracellular pathway (16, 20). Especially, since the ACVs of the clinical strains were canonically negative for the presence of the autophagy marker LC3 (microtubule-associated protein 1, light chain 3) (16, 20). Moreover, clinical strains possess the ability to escape from the macrophages through a lytic process (16, 20). ACV seems to be a non-degradative phagolysosomal compartment after the fusion with lysosomes, which is the final step of phagosomal maturation (20). *A. baumannii* utilizes a remarkable mechanism for intracellular survival that involves manipulation of pH level inside ACVs (20). Specifically, the tolerance to acidic pH and the subsequent production and secretion of ammonia to neutralize the intravacuolar pH are the key mechanisms enabling the replication of *A. baumannii* within the ACV (20). Intracellular replication of the clinical strain of *A. baumannii* strain appears to rely on the type 1 secretion system (T1SS), as well as on pAB5 plasmid, which controls the expression of several chromosomally-encoded genes (16). These observations support the conclusion that a subset of clinical strains capable of autophagy evasion is able to persist and multiply in macrophages, thereby avoiding the lytic process. A putative pathway for *A. baumannii* infection and intracellular survival in macrophages is shown in Figure 2 (**Fig. 2**).

Intracellular life of *A. baumannii* in epithelial cells

It is noteworthy that macrophages are not the sole cell type in which A baumannii can survive and replicate (17). A subset of clinical isolates of *A. baumannii* extensively multiply within non-phagocytic human lung cancer A549 epithelial cells, in primary human keratinocytes and human endothelial EA.hy 926 cells (17), and epithelial HeLA cells, although at a limited rate (18). The non-acidic ACVs positive for LAMP1 within infected cells suggest the inhibitory properties of *A. baumannii* for acidification or lysosomal fusion, and its to divert its trafficking away from the lysosomal degradative pathway (17). Rubio and colleagues hypothesized that clinical isolates of *A. baumannii* have demonstrated the ability to create a conducive environment for intracellular replication by inhibiting fusion with degradative lysosomes and impeding their acidification process. Similar to macrophages, the ACVs found within A549 cells infected with clinical strains did not originate from autophagy, which sets them apart from the ATCC 17978 strain, known for its typical autophagic pathway (17). The ATCC 17978 strain activates transcription factor EB (TFEB) to induce upregulation of the genes related to the autophagic pathway, including the gene coding for the LC3 (58), which distinguishes it from the clinical strain LC3-negative (17). Modern clinical strains are able to of altering the host response, specifically, the downregulation of COL5A1 (collagen type V alpha 1 chain) and IGF2R (insulin-like growth factor II receptor) gene expression. The declining levels of IGF2R are intriguing since this receptor is involved in the transport of lysosomal enzyme within cells. Therefore, a lower level of the IGF2 receptor suggests a compromised lysosomal function, potentially facilitating *A. baumannii* survival within cells. Furthermore, the downregulation of the collagen-producing COL5A1 may indicate a reduction in the tensile strength of the tissue (18).

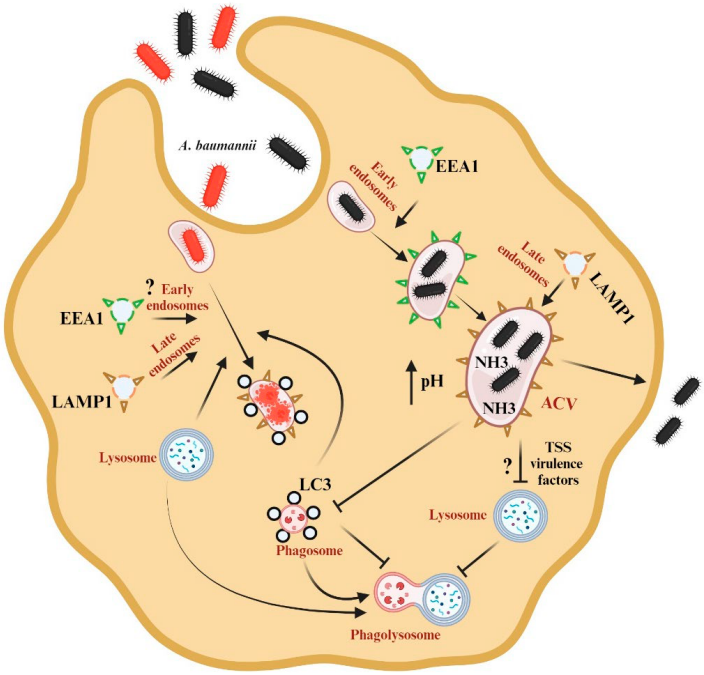

Figure 2. Proposed model of the intracellular survival and life of *A. baumannii* in human macrophages. Replicative strains of *A. baumannii* (black) sequentially acquire EEA1 and LAMP1 markers within the ACV through early and late endocytosis, respectively. However, as the ACV matures, it strategically evades interaction with the host cell's phagosomes and lysosomes. Additionally, these intracellular bacteria produce ammonia during replication in ACV, neutralizing the ACV's internal environment. Replicated bacteria that escape degradation are released outside the cell. In contrast, non-replicative strains of *A. baumannii* (red) are engulfed by macrophages and occupy a nascent phagosome. This compartment matures into a late phagosome, similar to the replicative ACV, through interactions with the endocytic pathway. Ultimately, non-replicative strains of *A. baumannii* are degraded within the phagolysosome (created with BioRender.com).

Ambrosi C. et al. demonstrated an interesting new evolutionary mechanism in which clinical *A. baumannii* strain can utilize cell adhesion molecules to facilitate invasion (21). Specifically, this pathogen exploits carcinoembryonic antigen-related cell adhesion molecule receptors CEACAM1, CEACAM5, and CEACAM6 on the A549 cell line, enabling rapid internalization within Rab5-decorated early endocytic compartments and Rab7-decorated late endocytic compartments, as well as the autophagosome marker LC3.

Despite the transient survival within host cells, *A. baumannii* ultimately succumbs to the harsh environment of the acidified vacuole (21). The mechanism underlying *A. baumannii's* survival in the cytoplasm are not yet fully understood, but potential mechanisms, such as the impaired non-canonical caspase-4 response against other classically Gram-negative extracellular pathogens, warrant further investigation. Caspase-4 is known to be involved in limiting the intracellular colonization of bacteria like *P. aeruginosa* and *Shigella flexneri*, which can be inhibited by the T3SS effector ExoS and OspC3 effector, respectively (62, 63).

Conclusions

The antibiotic-resistant *A. baumannii* is a high-priority pathogen with remarkable ability to with stand various environmental stress conditions. Here, we summarized the existing literature that reports the intracellular presence of *A. baumannii* and its capacity to persist and multiply inside eukaryotic cells. The ability to survive within the cell can partially explain the difficulty of antibiotic treatment in the hospital environment. Certain *A. baumannii* strains were found to evade the cell's typical degradative pathway, providing protection against host immune responses and potentially impeding drug effectiveness. This evasion tactic could play a role in the persistence and recurrence of *A. baumannii* infections, as well as increased mortality rates among patients. Importantly, studies on the pathogenesis of *A. baumannii* should involve modern clinical isolates and not be limited to historical strains. Identifying how this pathogen evades host response could serve as valuable guidance for the future development of antimicrobial treatments and diagnostic approaches.

Abbreviations:

MDR – multidrug-resistant
ACVs – *A. baumannii*-Containing Vacuoles
OMPs – outer membrane proteins
PPPs – pattern recognition receptors
LAMP1 – lysosomal-associated membrane protein 1
LC3 – microtubule-associated protein 1, light chain 3

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