Evolutionary Perspectives on the Moonlighting Functions of Bacterial Factors That Support Actin-Based Motility

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ABSTRACT Various bacterial pathogens display an intracellular lifestyle and spread from cell to cell through actin-based motility (ABM). ABM requires actin polymerization at the bacterial pole and is mediated by the expression of bacterial factors that hijack the host cell actin nucleation machinery or exhibit intrinsic actin nucleation properties. It is increasingly recognized that bacterial ABM factors, in addition to having a crucial task during the intracellular phase of infection, display “moonlighting” adhesin functions, such as bacterial aggregation, biofilm formation, and host cell adhesion/invasion. Here, we review our current knowledge of ABM factors and their additional functions, and we propose that intracellular ABM functions have evolved from ancestral, extracellular adhesin functions.

KEYWORDS ActA, actin-based motility, IcsA, Listeria, Shigella, adhesion, aggregation, biofilm, extracellular pathogen, intracellular pathogen, invasion, moonlighting

The functions of genes are generally discovered through the identification of genetic alterations (genotype) that correlate with alterations in observable biological traits (phenotype). For reasons that may be related to the enlightening nature of uncovering the unknown, it is dogmatically accepted that genes display one, and only one, function, that is, the biological function under investigation at the time of gene discovery. However, it is becoming increasingly apparent that a given gene may encode a single protein that displays various functions in addition to its first-discovered “canonical” function. This ability of a protein to have more than one biological function is referred to as “moonlighting” (1). Diverse sets of proteins from all domains of life demonstrate moonlighting functions, and they include metabolic enzymes, transcription factors, chaperones, and ribosomal proteins (1, 2). For instance, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) conducts alternative tasks both in eukaryotic and prokaryotic cells. On the surfaces of macrophages, GAPDH isoforms participate in the maintenance of iron homeostasis, functioning as a receptor for iron binding proteins, such as lactoferrin (3), transferrin (4), and apotransferrin (5). In addition, when localized to the nucleus, GAPDH promotes either cell death or increased cell survival (6). In bacterial pathogens, extracellular GAPDH operates as a virulence factor, contributing to bacterial adherence to host cells (7–9), to interactions between different bacterial species that facilitate host colonization (10), and to evasion from the host immune system (7, 11, 12).

Here, we discuss the extracellular moonlighting functions of bacterial factors that support the intracellular lifestyle of cytosolic pathogens displaying actin-based motility (ABM). Various intracellular pathogens, such as Listeria monocytogenes, Shigella flexneri, Rickettsia spp., and Burkholderia spp., reside in the cytosol of infected cells, where they acquire ABM through expression of bacterial factors that hijack the host cell actin polymerization machinery or exhibit intrinsic actin nucleation capacity (13). Expression of these ABM factors in heterologous hosts is sufficient to confer actin-based motility (14, 15). Actin polymerization at the bacterial pole generates forces that propel the...
pathogen throughout the cytosol (Fig. 1A). At cell-cell contacts, ABM mediates intercellular spread through the formation of membrane protrusions that resolve into vacuoles from which the pathogen escapes, thereby gaining access to the cytosolic compartment of adjacent cells (16, 17) (Fig. 1A). It has recently emerged that bacterial
ABM factors, such as Listeria monocytogenes ActA and Shigella flexneri IcsA, perform extracellular moonlighting adhesin functions that promote self-aggregation and host cell adhesion, in addition to having a paramount role in intracellular ABM (Fig. 1A).

L. monocytogenes ABM relies on ActA (18), a bacterial factor that binds and activates the Arp2/3 complex, a critical host cell actin nucleator (19) (Fig. 1B, panel I). ActA is displayed at the bacterial pole, which is critical for actin polymerization and generation of forces that propel the pathogen throughout the cytosol (20). Seminal studies uncovered the ActA structural determinants that mediate ActA-Arp2/3 interaction. The actin nucleation activity of the Arp2/3 complex is stimulated by the N-terminal domain of ActA (21), which mimics the regulatory activity of the host cell nucleation-promoting factor neural Wiskott-Aldrich syndrome protein (N-WASP), leading to recruitment and activation of the Arp2/3 complex (22) (Fig. 1B, panel I, region from amino acids 30 to 262), as well as to the recruitment of additional host cell actin cytoskeleton regulators, such as Ena/VASP proteins (23) (Fig. 1B, panel I, PRR region). The C-terminal domain anchors ActA to the bacterial cell wall and is not known to interact with any actin cytoskeleton components (Fig. 1B, panel I).

Subsequent to the discovery of the intracellular role of ActA in ABM, various reports revealed that ActA mediates extracellular moonlighting functions, including adhesion to and invasion of host cells and host colonization. L. monocytogenes invades different host cell types, primarily through the internalin proteins, such as InlA and InlB, which bind host cell receptors (24). In addition to internalins, ActA was suggested to be required for epithelial cell invasion, potentially through adhesion to microvillus structures at the apical surfaces of epithelial cells (25) (Fig. 1B, panel I). In addition to invading host cells, ActA mediates L. monocytogenes aggregation in vitro as well as biofilm formation, through ActA-ActA self-interaction (26). Importantly, ActA-mediated aggregation was also observed in vivo in a mouse model of intestinal infection and facilitated persistent L. monocytogenes colonization. In vitro aggregation and long-term intestinal colonization require full-length ActA, and structure/function analysis revealed an aggregation-specific role for the C-terminal domain of ActA (Fig. 1B, C-terminal G394-R585 region), which is not required for ABM (26).

As with L. monocytogenes ActA, the bacterial factor supporting S. flexneri ABM, IcsA (27), exhibits extracellular moonlighting functions, including biofilm formation (28, 29) (Fig. 1A). IcsA bears the classical domain organization of type Va autotransporters, which is composed of an N-terminal signal sequence, a surface-exposed passenger domain, and the beta-barrel translocation domain (30) (Fig. 1B, panel II). As with ActA, IcsA is displayed at the bacterial pole (31, 32). Unlike ActA, which promotes the nucleation activity of the Arp2/3 complex, IcsA recruits the host cell actin nucleation-promoting factor N-WASP, which subsequently binds and activates the Arp2/3 complex (33, 34). Structure/function analyses of the IcsA passenger domain showed that the region from R103 to A433 is responsible for N-WASP binding in vivo and in vitro (35, 36) (Fig. 1B, panel II).

In addition to having a role in ABM, IcsA functions as a polar adhesin and promotes invasion upon exposure to bile salts (37). The ABM and adhesin functions of IcsA were genetically dissected. A mutant IcsA protein carrying two individual insertions with no apparent effect on ABM (36) displayed decreased adhesion and invasion upon bile salt exposure (37) (Fig. 1B, panel II, black stars). The role of bile salts was also investigated in the context of in vitro biofilm formation (28, 29). The structural determinant(s) supporting IcsA-mediated biofilm formation upon bile salt exposure remains to be determined. However, IcsA self-associates at the bacterial poles of individual bacteria, which is critical for N-WASP recruitment (38). Thus, one potential scenario in the context of biofilm formation is that IcsA interbacterial self-association may contribute to bacterial aggregation (29). Interestingly, IcsA shares structural similarities with autotransporter adhesins, such as Escherichia coli Ag43 (35), that mediate aggregation and biofilm formation through self-association (39). We note that what distinguishes IcsA from these adhesins is the requirement of bile salt exposure for robust biofilm formation (29).
In addition to *L. monocytogenes* and *S. flexneri*, *Rickettsia spp.* and *Burkholderia spp.* display ABM in infected cells. *Burkholderia* spp. ABM is supported by the polar protein BimA (40). While *Burkholderia thailandensis* BimA activates the Arp2/3 complex, *Burkholderia pseudomallei* BimA facilitates actin nucleation and elongation by mimicking the nucleation activity of host cell Ena/VASP proteins (41). *Rickettsia* spp. exhibit early and late ABM phases driven by different surface proteins (42). Early ABM of *Rickettsia* spp. requires surface protein RickA, which stimulates Arp2/3 nucleation activity (43, 44). In the late ABM phase, the autotransporter Sca2 is needed for actin tail formation, independent of the Arp2/3 complex (42, 45), through molecular mimicry of host cell formin nucleation activity (46). It is unknown whether, as with ActA and IcsA, the ABM factors BimA, RickA, and Sca2 perform moonlighting functions. Interestingly, Sca2 promotes host cell adhesion and invasion when expressed in *E. coli* (47). However, the potential moonlighting adhesin functions of Sca2 have not been tested in *Rickettsia* spp.

How bacterial pathogens have evolved the ability to display ABM is a daunting question. The discovery of their moonlighting functions as discussed in this article may, however, offer some evolutionary perspectives. The protein sequence of ActA appears unique compared to existing sequences in publicly available databases, and the exact mechanisms supporting bacterial aggregation remain to be determined. ActA is a typical example of molecular mimicry, displaying short structural motifs that resemble motifs found in eukaryotic proteins (13). Whether these motifs have been acquired through convergent evolution or have been acquired from a eukaryotic protein through gene transfer remains an open debate. In contrast to the uniqueness of ActA, IcsA belongs to a large family of autotransporters whose passenger domain adopts an L-shaped β-helical structure that mediates self-association (35, 48). Most self-associating auto-transporters are produced by extracellular pathogens, and it is thus reasonable to assume that these factors do not bear intracellular ABM functions. Consequently, we propose that ABM factors have evolved from existing adhesins that were primordially dedicated to extracellular colonization of the host.

Our evolutionary perspectives predict the feasibility of genetically uncoupling ABM and adhesion functions. This task may be complex, as self-association properties of ABM factors are important for ABM efficiency (38, 49, 50). Structure/function analyses have so far suggested that disruption of ABM functions leads to disruption of adhesin functions (26, 37). However, the corresponding structure/function analyses relied on gross molecular lesions (deletions and insertions) that may have severely affected the scaffold of the ABM factors under investigation. Thus, identifying discrete mutations that specifically abrogate ABM functions but preserve ancestral adhesin functions, in a process that we refer to as “reverse evolution,” will constitute a critical endeavor for providing experimental support to the notion that ABM factors have evolved from ancestral adhesins.

Although functional novelties may arise neutrally in preexisting scaffolds, it has been proposed that some scaffolds may offer more flexibility in the evolution of novel functions in proteins, while maintaining ancestral functions (51). These scaffolds include disordered regions and loops in proteins. Interestingly, ActA has been shown to exist as a natively unfolded protein (49). Moreover, the predicted β-helical structure of autotransporters, such as Ag43 and IcsA, displays numerous nonstranded loops that may well accommodate substitutions in residues not essential for self-association (35, 48). Gene duplication is an important aspect of evolution that creates functional redundancy and opportunities for exploring mutational space, without jeopardizing ancestral functions. Eight of the nine trimeric auto-transporters present in *B. pseudomallei* enable bacterial adhesion to mammalian cells (52). Since these proteins appeared to play redundant roles, it is conceivable that mutational space was available for one of these proteins (BimA) to evolve ABM functions.

In conclusion, we speculate that, as extracellular pathogens evolved the ability to invade host cells and gain access to the cytosolic compartment, they encountered new selective pressures. In that context, we propose that ancestral extracellular adhesins...
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