How Intracellular Bacteria Survive: Surface Modifications That Promote Resistance to Host Innate Immune Responses

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Bacterial pathogens regulate the expression of virulence factors in response to environmental signals. In the case of salmonellae, many virulence factors are regulated via PhoP/PhoQ, a two-component signal transduction system that is repressed by magnesium and calcium in vitro. PhoP/PhoQ-activated genes promote intracellular survival within macrophages, whereas PhoP-repressed genes promote entrance into epithelial cells and macrophages by macropinocytosis and stimulate epithelial cell cytokine production. PhoP-activated genes include those that alter the cell envelope through structural alterations of lipopolysaccharide and lipid A, the bioactive component of lipopolysaccharide. PhoP-activated changes in the bacterial envelope likely promote intracellular survival by increasing resistance to host cationic antimicrobial peptides and decreasing host cell cytokine production.

Microbial pathogens have evolved mechanisms to survive and replicate within complex, changing animal environments. Bacteria coordinate the expression of virulence factors so that they are expressed efficiently in environments in which they provide a selective advantage [1]. Every possible animal microenvironment is a potential pathogen survival niche, and bacteria use a wide variety of extracellular and intracellular environments as specialized sites for replication.

Host defenses and bacterial pathogenic mechanisms are constantly evolving. Bacteria have pathogenicity islands, DNA elements containing many genes that can be inherited by vertical transmission [2]. Host social and physical factors such as war, poverty, and starvation, as well as the development of therapeutic agents and newly susceptible immunosuppressed populations, contribute to the diversity of the animal environments that bacteria experience. The result is "emerging" or "reemerging" pathogens and diseases.

This review will discuss mechanisms used by bacteria to survive after phagocytosis and provide specific details about some of the mechanisms that salmonellae use to combat host intracellular killing mechanisms. We will focus on survival within macrophages, since bacteria have not evolved successful mechanisms to survive neutrophil killing. In addition, bacterial-mediated endocytosis into epithelial cells will be covered, as this may be an important bacterial mechanism to avoid phagocytosis and killing by myeloid phagocytes (macrophages and neutrophils).

After phagocytosis by macrophages, bacteria are located in a membrane-bound vacuole (phagosome), but the ensuing trafficking of this vacuole and subsequent bacterial survival strategies vary considerably [3]. If the ingested bacteria have no intracellular survival mechanisms, the bacteria-containing phagosomes fuse with the lysosomal compartment, and bacteria are digested within 15–30 min. The metabolic burst in activated phagocytes results in production of nitric oxide and reactive oxygen species, such as chloramines, hydroxyl radicals, and hydrogen peroxide, which are usually converted into the potent oxidant hypochlorous acid [4]. These compounds kill bacteria very efficiently within the phagosomal environment. The phagosomes of circulating monocytes, neutrophils, and some mucosal epithelial cells also contain various cationic antimicrobial peptides, which have significant antimicrobial activity [5]. As we discuss below, resistance mechanisms to killing by antimicrobial peptides are important to the intracellular survival of salmonellae. Since the phagosome is rapidly acidified to a pH <5.0, the acidic environment itself may be toxic or limit the replication of a pathogen. Within the phagosome, pathogens also can be deprived of certain essential nutrients. The ability to induce metabolic pathways as a result of this nutrient limitation may be essential to the survival of certain bacteria, particularly salmonellae. Lysosomes also contain a variety of hydrolytic enzymes. These enzymes function to kill bacteria and/or to digest the pathogen for presentation of bacterial antigens to the immune system.

Microbial pathogens have developed diverse strategies to avoid or survive the hostile environment of the macrophage phagosome. They can prevent phagocytosis (Versinia species), alter phagocytosis to target the bacterium to a novel phagosome (Salmonella species), escape from the phagosome into the cytosol by lysing the vacuolar membrane (Listeria and Shigella species), block the fusion of phagosomes with lysosomes (Legionella species), block or attenuate acidification of phagolysosomes (Mycobacterium tuberculosis), or adapt to resist the antimicrobial activity of the fused phagolysosome (Salmonella species) [6].

Studies of the interactions of Salmonella typhimurium with cultured mammalian cells and in an inbred mouse model of
enteric (typhoid) fever indicate that the mechanism of entrance and subsequent post-phagocytosis adaptation influence intracellular survival. Additionally, mouse models of Salmonella infection using both bacteria and mice carrying defined mutations indicate that the ability to survive within phagocytic cells in vitro predicts the ability to cause in vivo disease.

The internalization of bacterial pathogens (phagocytosis) is the initial morphologic event of host-bacteria interaction. The best-defined mechanism of bacterial phagocytosis is classical receptor-mediated endocytosis [6, 7]. This mechanism involves the direct interaction between bacterial ligands and cellular receptors, which leads to the engulfment of the bacteria by the membrane and the formation of a tightly adherent phagosome. Plasma membrane receptors that mediate phagocytosis have been found on phagocytic cells and include the Fc receptor, complement receptor, carbohydrate receptors, and cell adhesion molecules. To facilitate receptor-mediated endocytosis by ligand–host cell receptor interaction, some bacteria require opsonization by serum complement factors or immunoglobulins. Bacteria also display a wide variety of surface-exposed lectins that can mediate phagocytosis through interaction with host cell glycoproteins and glycolipids.

Phagocytosis of S. typhimurium by macrophages is unconventional, both in mechanism of entry and in morphology of the phagosome formed. After contact with the host membrane, internalization of the bacteria occurs via a mechanism termed macropinocytosis. Phagocytosis occurs very rapidly, within seconds to minutes, and is characterized by localized cytoskeletal rearrangements and membrane ruffling [8, 9]. In epithelial cells, membrane ruffling is localized to an area adjacent to the bacteria, whereas in macrophages it is diffuse. Bacteria that contact the cell surface are internalized by the fusion of membrane ruffles formed from cytoskeletal rearrangements that include filamentous actin. Although both macropinocytosis and receptor-mediated endocytosis require actin polymerization, they are morphologically different and use different host cell signaling mechanisms [10]. The Salmonella-containing vacuoles are large (2–5 μm) and are termed “spacious phagosomes” [9]. Spacious phagosome formation likely promotes Salmonella survival by dilution of toxic lysosomal compounds or attenuation of antimicrobial factors, including decreased phagosomal acidification. Neutrophils rapidly kill salmonellae, with <10% of an initial inoculum surviving after phagocytosis in vitro [11]. Therefore, the ability of the Salmonella organism to induce its own uptake into epithelial cells and macrophages may be a means to avoid neutrophil-mediated killing.

Although Salmonella-induced macropinocytosis and spacious phagosome formation look very similar in myeloid and epithelial cells, different bacterial factors are required for these processes. A specialized secretion system, termed the type III secretion system (TTSS), as well as proteins secreted by this system, are encoded in Salmonella pathogenicity island 1 (SPI1). This system is required for entry into epithelial cells [2, 12]. The TTSS functions to deliver proteins from the bacterial cytoplasm to the eukaryotic cell cytoplasm on bacterial contact. The exact mechanisms of bacterial contact and of delivery of these proteins are unknown. It is proposed that a bacterial surface structure is formed that opens a pore in the eukaryotic cell membrane for transfer of molecules into the eukaryotic cell cytoplasm. TTSS are used by bacterial pathogens to inhibit their phagocytosis, induce eukaryotic cell death, and alter the host cell cytoskeleton. Salmonella species have at least one other TTSS encoded on SPI2 [2, 13] that appears to be involved in intracellular survival.

Many of the properties of Salmonella that contribute to intracellular survival and induction of macropinocytosis and spacious phagosome formation are controlled by a master regulator termed PhoP/PhoQ. PhoP and PhoQ are members of a large family of two-component regulatory systems that allow bacteria to sense and respond to environmental cues by altering gene expression [14]. PhoQ is an integral membrane protein exhibiting histidine kinase activity. PhoQ responds to signals by transferring phosphate to a conserved residue in the amino-terminus of PhoP. Phosphorylated PhoP binds to specific promoters to induce or repress expression of >40 genes, termed PhoP-activated genes and PhoP-repressed genes, respectively (figure 1). Additionally, a single point mutation in phoQ (Thr to Ile at amino acid 48) results in increased phosphorylation of PhoP and leads to increased expression of PhoP-activated genes [15].

Studies into the regulation of the PhoP-activated genes, in both S. typhimurium and Escherichia coli, demonstrated that the kinase activity of the PhoQ can be repressed by increased concentrations of the divalent cations Mg²⁺ and Ca²⁺ [16]. Recently, Garcia Vescovi et al. [17] demonstrated that distinct binding sites for both Mg²⁺ and Ca²⁺ are probably located within the periplasmic domain of PhoQ. Binding of these cations to PhoQ is hypothesized to cause conformational changes in the protein, leading to decreased kinase activity and repression of PhoP-activated gene expression. Therefore, displacement of divalent cations from PhoQ within the host intracellular environment could activate PhoP-activated gene expression and promote intracellular survival.

PhoP-activated gene products have been shown to play important roles in Salmonella pathogenesis, including survival within macrophages, induction of spacious phagosome formation, increased resistance to host defense cationic antimicrobial peptides and to low pH, and altered antigen presentation. PhoP-activated genes include >10 secreted and integral outer membrane proteins, a nonspecific acid phosphatase (encoded by phoN), high-affinity magnesium transporters (encoded by mgtA and mgtCB), and proteins involved in increased resistance to host antimicrobial peptides of the polymyxin class (pagA and pmrA/B). Certain PhoP-activated genes have been shown to play a role in the modification of outer membrane structure by creating structural changes in the lipid A portion of lipopolysaccharide (LPS). These modifications may, in part, be responsible for increased cationic antimicrobial peptide resistance and survival within macrophages.
Antimicrobial peptides are important components of the host innate immune system that can disrupt the outer membranes of gram-negative bacteria. Cationic antimicrobial peptides target the bacterial membranes through electrostatic interaction [5]. Pathogens protect themselves from attack by antimicrobial peptides primarily by modification of their cell surfaces to prevent this electrostatic interaction. LPS is a pathogenic factor of gram-negative bacteria and consists of three distinct regions: O antigen, core, and lipid A (figure 2). Both O antigen and core consist of polysaccharide chains, whereas lipid A is formed primarily of fatty acid and phosphate substitutes bonded to a central glucosamine dimer.

Recently, our laboratory demonstrated the role of PhoP in regulating the structural modifications of lipid A, the component of LPS that stimulates cytokine release in the host [18, 19]. Significant structural differences in the lipid A of wild type, PhoP\(^+\) (null mutation), and PhoP\(^-\) (PhoP-activated) isogenic strains of \(S.\ typhimurium\) were observed. Analysis by mass spectroscopy and gas chromatography demonstrated that growth in low Mg\(^{2+}\) resulted in the addition of aminoarabinose, 2-hydroxymyristate, and palmitate to lipid A [18].

These changes in lipid A were mediated by PhoP/PhoQ and they have significant functional consequences. Aminoarabinose modification of lipid A results in increased resistance to polymyxin. Further analysis of polymyxin-resistant strains of \(S.\ typhimurium\) has shown that lipid A modification requires a second two-component regulatory system, PmrA/PmrB [20]. Activation of PhoP/PhoQ or low pH stimulates increased synthesis of PmrA/PmrB. PmrA/PmrB regulates resistance to polymyxin by inducing expression of \(pmrE\), previously known as \(pagA\) or \(ugd\), which is predicted to encode a UDP-glucose dehydrogenase (UDG). UGD is thought to be involved in the production of aminoarabinose by catalyzing the formation of an intermediate in the biosynthesis of UDP-arabinose. A second PmrA/PmrB-regulated locus required for polymyxin resistance has been defined. \(pmrF\) is part of a putative operon that contains genes predicted to encode proteins with similarity to glycosyltransferases and other complex carbohydrate biosynthesis molecules [21].

Modified \(Salmonella\) lipid A has been shown to reduce LPS-mediated expression of the adhesion molecule E-selectin by human endothelial cells and tumor necrosis factor-\(\alpha\) by human
monocytes [18]. It is likely that the mechanism of reduced host cell recognition is a PhoP-mediated change in lipid A composition that involves replacement of myristate with 2-hydroxymyristate. This supposition is based on data from our laboratory indicating that the addition of palmitate or aminoarabinose to lipid A does not alter host cell recognition. In addition, myristate-deficient lipid A, such as that derived from Helicobacter pylori, Porphyromonas gingivalis, or E. coli mutants, cannot induce typical lipid A-mediated host cell responses. Given these results, we hypothesize that the replacement of myristate by 2-hydroxymyristate is responsible for the observed reduced host cell recognition. This reduced recognition by host cells is likely advantageous to the pathogen during systemic illness. This illness is characterized immunologically by a chronic rather than an acute inflammatory response, which may be in part a result of modification of lipid A and thus of host cell recognition. Reduced host cell recognition and consequently an attenuated innate immune response may result in chronic (macrophage and monocytic) rather than acute (neutrophilic) inflammatory responses.

Finally, our laboratory has identified an additional PhoP-regulated gene, pagP, which is essential for addition of palmitate to Salmonella lipid A [22]. This modification confers increased resistance to α-helical cationic peptides. PagP-mediated addition of palmitate to lipid A may have evolved as a general mechanism for gram-negative bacterial resistance to α-helical peptides. Other gram-negative bacteria, including E. coli and Pseudomonas aeruginosa, have mechanisms by which to add palmitate to lipid A [22] (unpublished data).

Evasion of intracellular killing mechanisms within the host is an important step in the pathogenesis of many bacteria. The study of the S. typhimurium PhoP regulon has generated important insights into mechanisms that allow salmonellae to adapt to complex environments and facilitate intracellular host survival. A general mechanism of intracellular survival likely involves alteration of the microbial cell surface to avoid host killing factors. In the case of salmonellae, lipid A modifications may be advantageous to bacteria by attenuating host cell innate immune responses and by promoting resistance to host antimicrobial factors. Further studies of the regulated processes involved in the modifications of bacterial outer membrane permeability may lead to better understanding of how bacteria combat the host innate immune response. Additionally, these studies may lead to the development of new peptide antibiotics, used individually or in conjunction with current antibiotic therapies, for treatment of numerous infectious diseases.
The study of *Salmonella* pathogenesis uses a number of systems that allow specific molecular details of pathogenic mechanisms to be defined. Studies indicate that the ability to alter the cell envelope is an important mechanism for intracellular survival. It remains to be determined whether the principles learned from the study of *Salmonella* virulence mechanisms are applicable to other microorganisms, including protozoa and viruses.

**References**