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Bacterial differentiation, development and disease: mechanisms for survival

Sheryl S. Justice[1,2], **Alistair Harrison**[1], **Brian Becknell**[3], and **Kevin M. Mason**[1,2]

[1]Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio

[2]The Ohio State University School of Medicine, Columbus, Ohio

[3]Section of Nephrology, Nationwide Children's Hospital

Abstract

Bacteria have the exquisite ability to maintain a precise diameter, cell length and shape. The dimensions of bacteria size and shape are a classical metric in the distinction of bacterial species. Much of what we know about the particular morphology of any given species is the result of investigations of planktonic cultures. As we explore deeper into the natural habitats of bacteria, it is increasingly clear that bacteria can alter their morphology in response to the environment in which they reside. Specific morphologies are also becoming recognized as advantageous for survival in hostile environments. This is of particular importance in the context of both colonization and infection in the host. There are multiple examples of bacterial pathogens that use morphological changes as a mechanism for evasion of host immune responses and continued persistence. This review will focus on two systems where specific morphological changes are essential for persistence in animal models of human disease. We will also offer insight into the mechanism underlying the morphological changes and how these morphotypes aid in persistence. Additional examples of morphological changes associated with survival will be presented.

Keywords

Urinary Tract Infection; Otitis Media; Morphology; Persistence

Introduction

The 7th edition of "Bergey's Manual of Determinative Bacteriology" states, "no organism can be classified before its morphological … characters have been determined through a detailed study". Bergey then states that *E. coli* can be recognized as rods up to 3μm in length that exist in a coccoid form that occurs singly, in pairs or short chains. Similarly, *Haemophilus* is described as very small rods up to 2μm in length that occur singly, in pairs, short chains or long threads. Thus, in 1957, it was apparent that although bacteria could be identified primarily through a single morphological determinant, deviations from the classic

Corresponding Author: Sheryl S. Justice, sheryl.justice@nationwidechildrens.org.

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description of a bacterium's appearance occurred. Why bacteria exhibit these different morphologies was not apparent at that time. Only now are we beginning to understand why a single strain of bacteria can exhibit multiple morphotypes. Critically, many species of bacteria use changes in morphology to aid in survival in hostile environments. These mechanisms can be of particular importance as certain morphotypes are optimized for survival of innate immune effectors as well as invasion into epithelial cells. Such invasion events could provide bacteria with an environment rich in nutrients and a refuge from immune pressures and so provide temporary or long-term respite from nutrient limitation and innate immune components. Further, it is becoming clear that differentiation as a method of survival, both in the environment and in the host as part of disease progression, is an increasingly common approach. It is thus imperative that the mechanisms behind protective differentiation be fully understood.

Intracellular bacterial communities: the paradigm of uropathogenic *Escherichia coli*

The initial observations that allude to an intracellular developmental pathway for uropathogenic *Escherichia coli* (UPEC) are revealed from electron microscopic images that demonstrated bacteria within bladder umbrella cells of infected mouse bladders (Mulvey *et al.*, 1998). UPEC growth within the umbrella cells creates a bulge, or pod, on the luminal surface (Anderson *et al.*, 2003). Pods are not evident in bladders inoculated with non-uropathogenic *E. coli* strains, suggesting the formation of pods relates to infectious lifestyles. Within the urinary tract, the intracellular niche is advantageous for UPEC. Urine is nutrient poor, and the absence of urease activity reduces the access to carbon sources in the urine. In contrast, the cytoplasm of the bladder umbrella cell is a rich nutritional environment. The epithelial membrane also provides a physical barrier against the activity of phagocytes and antibody-mediated clearance mechanisms. Within the epithelium, antimicrobial agents can damage UPEC, but UPEC survives through the induction of stress responses such as the DNA damage repair response (Justice *et al.*, 2006, Li *et al.*, 2010, Gawel & Seed, 2011).

Time-lapse fluorescence video microscopy reveals the growth of UPEC intracellular bacterial communities (IBCs) within live bladder explants (Justice *et al.*, 2004). This novel approach permits dissection of discrete developmental intermediates of the UPEC intracellular life cycle. In the first branch of the developmental and differentiation pathway, internalized UPEC are non-motile, bacillary in morphology and form loosely organized colonies in the bladder umbrella cell cytoplasm (early IBC)(Figure 1). This mechanism of growth as a community is in marked contrast to the lifestyles of other previously described cytoplasmic pathogens (e.g. *Listeria, Shigella*) that harness the host actin to grow as single motile bacteria. The majority of the intracellular UPEC within each pod transition into a developmental phase characterized by a reduction in the rate of growth and an increase in organization into a biofilm-like structure with coccoid bacteria filling the host cytoplasm (mid-IBC). During the mid-IBC phase, a minority of the bacteria within each IBC proceeds through a second developmental branch, involving differentiation into a filamentous morphology (Figure 1), described in greater detail below. In addition, each IBC arises from

a single invasion event (Schwartz *et al.*, 2011), suggesting morphological changes represent a different phase of the developmental program. Therefore, the coccoid morphology is not the result of overgrowth from parallel invasion events of UPEC that exhibit different morphologies. In the third phase, peripheral bacteria of the IBC become motile and rod-like, dissociate from the community, and egress from the host cell with motility characteristic of flagellar movement. The released bacteria may attach to neighboring umbrella cells and initiate a new round of invasion (second-generation IBC). Alternatively, if urothelium is denuded in areas of umbrella cell exfoliation, the newly released UPEC may invade underlying transitional urothelial cells and establish quiescent intracellular reservoirs (Justice *et al.*, 2004, Mysorekar & Hultgren, 2006). These reservoirs consist of 1–4 bacteria in membrane-bound compartments that contain the lysosomal protein, Lamp-1, and are considered resistant to host immunity, antibiotic treatment and serve as the reservoir for recurrent infection (Mulvey *et al.*, 2001, Schilling *et al.*, 2002, Justice *et al.*, 2004, Mysorekar & Hultgren, 2006).

The majority of human clinical UPEC isolates from women with urinary tract infection exhibit IBC formation following transurethral inoculation into mice bladders (Garofalo *et al.*, 2007), suggesting the intracellular developmental lifestyle is not restricted to a single UPEC strain. Further, prototypic cystitis or pyelonephritis UPEC isolates support IBC formation in five genetically distinct inbred mouse strains (Garofalo *et al.*, 2007). IBC formation is observed to similar degrees in immunocompetent and immunocompromised hosts (Justice *et al.*, 2004, Garofalo *et al.*, 2007), suggesting that host immune pressure does not drive the developmental process with regards to the transient conversion through a coccoid morphology. Moreover, IBC-like structures are observed in the urine of women and children that present with symptoms of urinary tract infection (0 *et al.*, 2007, Robino *et al.*, 2013). Taken together, the intracellular lifestyle of UPEC occurs in multiple isolates and multiple mouse strains, suggesting that the developmental and differentiation pathway is a conserved program.

Evidence for an IBC pathway of other uropathogens as well as in human urine samples is accumulating (Table 1). *Klebsiella pneumonia*, another causative agent of urinary tract infection, the IBC pathway is present in both human urine samples as well as in the mouse model of urinary tract infection (Rosen *et al.*, 2007, Rosen *et al.*, 2008, Rosen *et al.*, 2008). Human urine samples from patients suffering UTI with *Enterococcus faecalis* also contain IBC-like structures (Horsley *et al.*, 2013). The IBC lifestyle appears to be a common lifestyle of both Gram-negative and Gram-positive urease-negative bacterial pathogens in the urinary tract.

IBCs formed by *E. coli* in mammary epithelium

The bladder epithelium is not the only tissue that supports IBC formation by *E. coli*. Similar to the contamination of the urinary tract by fecal matter in humans initiates infection, the mammary glands of bovines can be contaminated from fecal sources of *E. coli*. Intramammary introduction of *E. coli* leads to invasion and IBC formation within alveolar epithelial cells in a mouse model (Gonen *et al.*, 2007, Mintz *et al.*, 2013). In this model system, IBCs commonly develop in an immunocompromised host, suggesting bacterial

clearance by a functional immune response occurs at this privileged site. It is therefore plausible that physiological stress dampens host immune responses and promotes susceptibility to IBC formation and persistence of *E. coli* in mammary tissues.

IBCs formed by nontypeable *Haemophilus influenzae* in the middle ear epithelium

Since the initial description of IBCs formed by UPEC, evidence is accumulating for IBCs in other infectious systems (Table 1). Although the nutritional status of the pathogen is not a key mediator in the establishment of IBCs in the systems presented thus far, nutritional status contributes significantly to IBC formation and morphological changes in some organisms (Table 1). Within the host, availability of essential nutrients (i.e. iron, zinc, manganese) is sequestered to prevent microbial outgrowth. This nutritional immunity is due to the near universal requirement for specific host-derived nutrients for bacterial growth (Hood & Skaar, 2012, Cassat & Skaar, 2013). Nontypeable *Haemophilus influenzae* (NTHI), a causative agent of upper and lower respiratory infections, must obtain heme-iron from the environment. Although classically considered an extracellular, opportunistic pathogen, there is increasing evidence for *intra*cellular niches for NTHI *in vitro* (Clementi & Murphy, 2011). Further, the presence of NTHI within adenoids and bronchial epithelium suggests that an invasive phenotype may coincide with the chronic nature associated with NTHI-mediated diseases. The chronicity of NTHI infections including recalcitrance to antibiotic therapy, persistence in the presence of bactericidal antibodies and culture-negative clinical analysis are suggestive of the development of intracellular bacterial reservoirs within host cells (Hall-Stoodley & Stoodley, 2009, Clementi & Murphy, 2011). Loss of the SapA periplasmic binding protein, responsible for uptake of essential heme-iron, produces distinct differences in the capacity to invade polarized normal human bronchial cell cultures (Mason *et al.*, 2011). In contrast to the parental strain where the internalized bacteria appear to proceed through the lysosomal pathway, the *sapA* mutant is not contained within a membranous compartment but colonizes the host cytoplasm reminiscent of the cytoplasmic location of UPEC IBCs, and in most cases retain bacterial density and membrane integrity (Raffel *et al.*, 2013). These data suggest that physiological status, in response to fluctuations in the availability of the essential nutrient heme, may promote intracellular survival of NTHI.

Essential nutrients at privileged sites are normally sequestered. However, the production of inflammatory products (e.g. edema and fluid) provides sources of essential nutrients. Thus, bacteria experience fluctuations in availability of micronutrients early during infection. The ramifications of the fluctuations in the availability of heme-iron on NTHI pathogenesis are now emerging. These fluctuations in heme-iron availability can be mimicked by growth within a defined medium in the absence or presence of heme-iron to generate heme-iron ‘restricted’ and ‘replete’ NTHI, respectively (Szelestey *et al.*, 2013). Co-culture of restricted or replete NTHI with monolayers of primary middle ear epithelial cells, in the presence of heme-iron, demonstrates that NTHI transiently restricted of heme-iron are better adapted to survive inside epithelial cells. Moreover, heme-iron restricted NTHI are more persistent in the chinchilla model for human otitis media when compared to NTHI replete for heme-iron

(Szelestey *et al.*, 2013). Transiently restricted NTHI IBCs fill the entire volume of primary cultured middle ear epithelial cells, which bulge the cells and resemble UPEC pods (Szelestey *et al.*, 2013). These data suggest that exposure of NTHI to host immune pressures, which include host sequestration of essential nutrients, promote epithelial cell invasion, intracellular development and survival.

Advantages for differentiation from bacillary to coccoid morphology

There are both theoretical and evidence-based observations that a coccoid morphology has significant benefits for survival. The small and symmetric size allows for better packing of viable bacterial cells within the restricted host cell volume. A single UPEC that invades a bladder epithelial cell can multiply to 10^5 viable organisms. Further, the decrease in bacterial size provides the greatest surface area for nutrient uptake (Baker *et al.*, 1983). Although little is known regarding the signals and mechanisms for differentiation from a bacillary to a coccoid morphology within UPEC IBCs, in other systems, environmental stress is a key stimulator for differentiation into coccoid morphotypes. *Vibrio cholerae* coccoid morphotypes arise from starvation and are as infectious as the bacillary morphotype (Krebs & Taylor, 2011). Coccoid forms of *Helicobacter* result from antibiotics or acid stress and remain viable for up to 1 year in seawater while the bacillary form survives for less than 2 weeks under the same conditions (Shahamat *et al.*, 1989). Moreover, in the marine environment, transition to a coccoid morphology protects bacteria from predation by unicellular protists (Pernthaler, 2005). The similarities in the phagocytosis mechanisms of protists and professional innate immune phagocytes suggest that coccoid morphotypes may be protected from phagocytosis during disease. Taken together, the coccoid transition can provide multiple advantages to the bacterium, for example continued growth in nutrient limited environments, mediating exposure to stress, stability in nutrient deplete conditions as well as a means to subvert killing by predation and complement (Dalia & Weiser, 2011).

Second branch of differentiation within UPEC IBCs: transition from bacillary to a filamentous morphology

Bacterial communities are composed of individual bacteria that although coordinated, contain subpopulations that may serve vitally different roles in the development and persistence of the community as a whole. UPEC IBCs are a striking example of this phenomenon. During experimental urinary tract infection, a subpopulation of UPEC proceed through a developmental pathway that involves the inhibition of septation and the production of filamentous morphotypes as much as 70 μm in length (Mulvey *et al.*, 1998, Justice *et al.*, 2004) (Figure 1). Filamentous morphotypes of UPEC, *K. pneumoniae*, *Enterobacter aerogenes*, and *Proteus mirabilis* are readily observed in human urine samples of patients with UTIs (Rosen *et al.*, 2007). Therefore, differentiation into a filamentous morphology is common to multiple pathogens that cause urinary tract infections (Table 1).

Filamentation is a direct consequence of host immunity and is essential for persistence during infection

The appearance of significant populations of filamentous UPEC during experimental UTI provoked a line of investigation into the regulation of UPEC filamentation during UTI (Justice *et al.*, 2006). Deletion of SulA, a cell division inhibitor associated with the DNA damage repair response (SOS response)(Kelley, 2006), results in the absence of filamentous morphotypes, and the mutant strain is sharply attenuated in the formation of secondary IBCs and quiescent intracellular reservoirs (Justice *et al.*, 2006). Filamentous bacteria are observed in the bladders of mice with intact Toll-like receptor 4 signaling (TLR4), but are not detectable in the bladders of mice with hypomorphic *tlr4* alleles (Justice *et al.*, 2004, Justice *et al.*, 2006). As such, SulA is dispensable in hosts defective in TLR4-signal transduction (Justice *et al.*, 2006), which indicates that filamentation is required for persistence during urinary tract infections.

Fluctuations in heme-iron availability both promote NTHI intracellular survival and dramatically influence the architecture of bacterial biofilms grown on biotic and abiotic surfaces. In contrast to the mat-like extracellular biofilms that form with continuous exposure to heme-iron, extracellular biofilms that form following transient heme-iron restriction form an open, mesh-like architecture that consists of multinucleate filamentous morphotypes (Vogel *et al.*, 2012, Szelestey *et al.*, 2013). The architecture of biofilms following transient heme-iron restriction requires the activity of a SulA ortholog, suggesting that filamentation is an important component of the architectural attributes associated with fluctuations in heme-iron availability (Szelestey *et al.*, 2013). Consistent with the role of SulA in the persistence of UPEC in the urinary tract, SulA is also essential for persistence of NTHI in the chinchilla model of otitis media (Szelestey *et al.*, 2013). Although the mechanism of SulA activation in this system remains unclear, filamentation appears to be a response to the restoration of essential nutrients following transient restriction. Therefore, bacterial filamentation is a common trait of the pathogenic lifestyle and is essential for at least two independent infections caused by two different organisms.

Advantages for differentiation from bacillary to filamentous morphology

Filamentation is observed for a number of different pathogens (Table 1) and offers multiple strategies for survival. First, antimicrobial agents that target the septation are ineffective against the filamentous morphotype. In addition, recent evidence indicates that filamentous morphotypes evade killing by host phagocytes. This strategy is suggested by multiple observations. Filamentous UPEC are the predominant extracellular morphotype between rounds of IBC formation (Justice *et al.*, 2004, Justice *et al.*, 2006). Intact filamentous UPEC in immediate proximity of phagocytes engorged with UPEC rods are viable (Justice *et al.*, 2004). Following phagocytosis of populations of mixed morphologies, there is a significant decrease in the bacillary population with a minimal decrease in the filamentous morphotypes (Horvath *et al.*, 2011). This results in an enrichment of the filamentous forms when these mixed populations are exposed to both cultured and primary phagocytes (Horvath *et al.*, 2011), providing quantitative evidence that the filamentous form is resistant to phagocytosis.

The role of shape is important in the resistance to phagocytosis of plastic polymer particles, marine bacteria and yeast (Cannon & Swanson, 1992, Pernthaler, 2005, Champion & Mitragotri, 2006, Justice *et al.*, 2008). The mechanism by which filamentous shapes are not phagocytosed is unclear, but has been the subject of recent investigations. The uptake of bacillary shaped *E. coli* is independent of the orientation of initial interaction with macrophages (Moller *et al.*, 2012). In contrast, phagocytosis of filamentous bacteria only occurs when macrophages can gain access to the pole (Champion & Mitragotri, 2006, Horvath *et al.*, 2011, Moller *et al.*, 2012, Prashar *et al.*, 2013). If the pole of filamentous *E. coli* is engaged, there is no difference in the velocity of phagocytosis between filamentous *E. coli* and bacillary *E. coli* (Moller *et al.*, 2012). Formation of the actin cup, essential for initiation of phagocytosis, is only observed when macrophages engage *E. coli* filaments at the pole, and not when macrophages engage the long axis (Moller *et al.*, 2012, Champion & Mitragotri, 2006, Moller *et al.*, 2012). Engagement of macrophages with the pole of filamentous *Legionella pneumophilia* results in a remodeling of the actin cup into a tubular structure (Prashar *et al.*, 2013). The tubular cup appears as a long membranous invagination that extends around the circumference of the macrophage and requires actin treadmilling (Prashar *et al.*, 2013). The tubular structures retain the ability to fuse with lysosomes, but the compartment does not become acidified due to leaking of hydrolases into the medium (Prashar *et al.*, 2013). Filamentous morphotypes of *L. pneumophilia* are trafficked to the replicative supportive vacuole in a type IV secretion-dependent manner (Prashar *et al.*, 2013). These filamentous forms initially continue to grow as filaments, but eventually exhibit septation to produce bacillary shaped bacteria that can escape the macrophage (Prashar *et al.*, 2013). Filamentous *L. pneumophilia* also invade human lung epithelial cells using a unique “hook”, “membrane wrap” and “zipper” mechanism (Prashar *et al.*, 2012). The filamentous *L. pneumophilia* invade into the epithelial cells, septate and continue to grow within the intracellular compartment (Prashar *et al.*, 2012). Taken together, filamentous morphology provides a survival advantage in the prevention of phagocytosis and killing by macrophages.

Concluding Statements

Morphological differentiation is critical for survival of bacteria, both in the environment and in the host as part of the disease process. The role of differentiation in bacterial survival is exemplified by UPEC. Uropathogenic strains of *E. coli* form IBCs protected from the host’s immune system, and with ready access to nutrients present within the host cell. This intracellular lifestyle also confers an added benefit to UPEC through the development of a filamentous population of cells that exhibit increased resistance to phagocytosis. Differentiation-driven protection is now increasingly recognized as a generalized lifestyle, in other UTI pathogens as well as other genera of bacteria that cause diseases not related to the urinary tract. Investigations into NTHI showed that the nutritional status produces morphological changes that lead to the generation of intracellular communities and improved survival in a chinchilla model of OM. The mechanisms that generate these protective differentiation events requires additional investigation. Whether these are common mechanisms that produce protective filamentation in other bacteria has yet to be resolved. However, the importance of differentiation as a protective mechanism for both

environmental bacteria as well as pathogens, drives our interest in determining what the mechanisms are underpinning this critical morphological differentiation.

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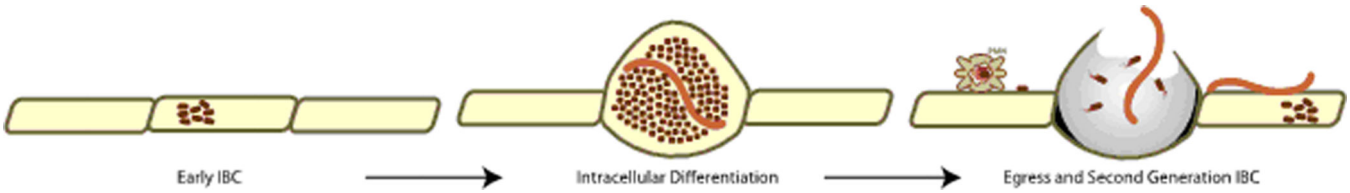

Figure 1.

UPEC intracellular differentiation pathway. UPEC (maroon rods) invade into the cytoplasm of the bladder epithelial cell to initiate the intracellular developmental and differentiation pathway (early IBC). The epithelial cell becomes engorged with UPEC in the coccoid (dark maroon) and filamentous (light maroon) morphologies to form the pod (intracellular differentiation). The pod ultimately lyses, releasing rod and filamentous morphotypes that invade into neighboring naïve cells for persistence (egress and second generation IBC).

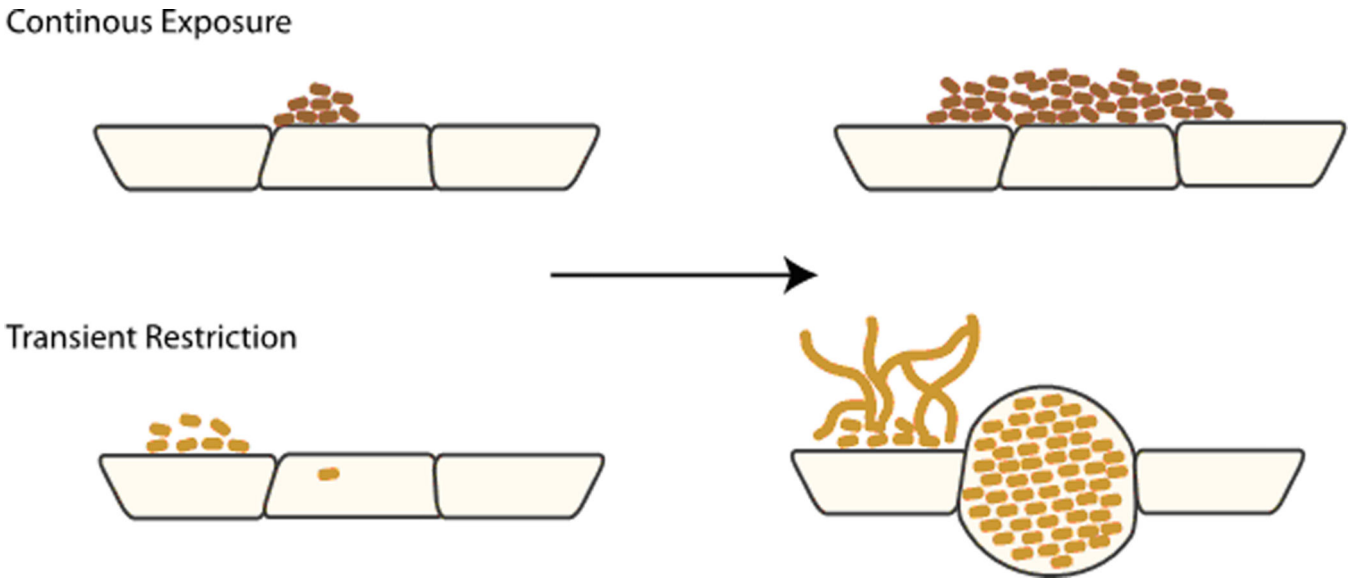

Figure 2.

NTHI differentiation in response to fluctuations in nutritional availability. NTHI IBC formation is dependent upon the nutritional state at the time of infection. Continuously exposed to heme-iron (dark brown rods) form mat-like biofilms on the epithelial surface with limited intracellular development. In contrast, restricted for heme-iron (light brown rods) form a biofilm on the surface that has an open architecture containing filamentous NTHI. In addition, heme-iron restricted NTHI responded to nutritional limitation by forming IBCs within the epithelial cells.

Table 1

Bacterial species	Infection site	IBC	Bacillary-Coccoid	Filament
Campylobacter	Intestine	-	-	Cameron *et al.*, 2012
Escherichia coli		-	Krogfelt *et al.*, 1993	-
Vibrio cholera		-	Krebs & Taylor, 2011	-
Legionella	Lung	-	-	Ogawa *et al.*, 2001
Mycobacterium tuberculosis	Macrophage	-	-	Chauhan *et al.*, 2006
Salmonella		-	-	Rosenberger & Finlay, 2002 Rosenberger *et al.*, 2004
Yersinia		-	-	Ponnusamy & Clinkenbeard, 2012
E. coli	Mammary Gland	Gonen *et al.*, 2007 Mintz *et al.*, 2013	-	-
NTHI	Middle Ear	Szelestey *et al.*, 2013	-	Szelestey *et al.*, 2013
Streptococci	Oral	-	-	Rossetti *et al.*, 2013
Helicobacter pylori	Stomach ulcers	Oh *et al.*, 2005	-	-
Escherichia coli (uropathogenic)	Urinary Tract	Mulvey *et al.*, 1998 Anderson *et al.*, 2003 Justice *et al.*, 2004 Rosen *et al.*, 2007	Justice *et al.*, 2004	Justice *et al.*, 2004 Justice *et al.*, 2006 Rosen *et al.*, 2007
Enterobacter aerogenes		-	-	Rosen *et al.*, 2007
Enterococcus faecalis		Horsley *et al.*, 2013	-	-
Klebsiella pneumoniae		Rosen *et al.*, 2007 Rosen *et al.*, 2008	Rosen *et al.*, 2008	Rosen *et al.*, 2007 Rosen *et al.*, 2008
Proteus mirabilis		-	-	Rosen *et al.*, 2007
Staphyloccocus saprophyticus		Szabados *et al.*, 2008	-	-
Photorhabdus asymbiotica	Wounds	-	-	Wilkinson *et al.*, 2009