THE ROLE OF BACTERIAL MOLECULAR CHAPERONES IN PATHOGEN SURVIVAL WITHIN THE HOST

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Infection is a bimodal process, involving a complex of various stress factors for both pathogen and host. Bacterial molecular chaperones are important in cell not only under normal, but also under stress conditions, such as infection, inflammation or similar events. In the present paper, the role and the mechanism of bacterial chaperones for pathogen survival within the host cells are discussed.

Bacteria activate evasion mechanisms, including increased production of molecular chaperones, in order to protect themselves against the host and to control the infection. The response of bacteria to a variety of environmental stressors in the infectious process is described in the first section. A number of bacteria that have adapted to an intracellular life style within macrophages, which is highly stressful for bacteria, the different molecular chaperones and their role for pathogenic bacteria survival in the host are presented next. The case of *Brucella,* a model of complex intracellular parasitism that can survive and replicate inside the macrophages and placental trophoblasts, with a focus on molecular chaperones, protecting bacteria from the phagosomal environment and helping intracellular replication is highlighted further.

Key words: molecular chaperones, bacteria, infection, macrophage.

INTRODUCTION

Molecular chaperones perform important functions in protein folding, unfolding or translocation, in the assembly and disassembly of protein complexes, in reversing polypeptide unfolding and preventing protein aggregation, as well as in repairing proteins that have been damaged or misfolded by stress (1-6). Under normal conditions, molecular chaperones are present at low concentrations in cells, but under stress conditions they accumulate to high levels (7, 8) and therefore enable cells to survive. Thus, chaperones are important in both normal and stressed cells.

During infection, the molecular chaperones production increases in both pathogen and host cells (9-12). When entering the host from the environment, a pathogen is confronted with several changes, some of which are highly stressful.

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These include temperature, pH and pO_2 changes (13, 14). Moreover, the pathogen is exposed to natural host resistance mechanisms, *i.e.*, phagocytosis by professional phagocytes (15). Some bacteria are intracellular parasites because they are able to invade eukaryotic cells. To protect itself against the host, the pathogen activates various evasion mechanisms, including increased production of molecular chaperones (16-20).

Bacterial chaperones play an important role in protein secretion, while indirectly contributing to bacterial virulence (21-23). There is evidence which supports the hypothesis that molecular chaperones of bacteria behave as direct virulence factors (24). Other effects of bacterial molecular chaperones on host cells include cell-cell signaling and promoting apoptosis (24).

In the present paper, the involvement of bacterial chaperones in survival mechanisms of pathogens within the host is discussed.

BACTERIAL INFECTION AND STRESS RESPONSE

The first protein-folding molecular chaperone discovered was Cpn60 (25). Since the identification of this protein as molecular chaperone, in 1988, many more proteins with known or putative molecular chaperone functions have been discovered and the term currently applies to 25 families of proteins (Table 1).

Table 1

Prokaryotic molecular chaperone and stress protein families (by Henderson *et al.*, 2006) (23)

Family	Name	Function
Chaperonin	GroEL, GroES	Folding of proteins within cage structure
Thioredoxin	Trx, DsbA to DsbE, glutaredoxin	Protein thiol-disulfide exchange
Small Hsps	IbpA, IbpB	Adsorption of unfolded chains to prevent stress aggregation
Peptidyl-prolyl isomerases	Cyclophilins, FKBPs, parvulins	Isomerization of peptide bond preceding proline
GrpE	GrpE	ADP/ATP exchange factor interacting with DnaK/DnaJ
Hsp40/DnaJ	DnaJ, CbpA, RcsG	Hsp70 cochaperones regulating Hsp70 activity
Hsp70	DnaK, Hsc66, BiP, mitochondrial Hsp70, etc	Prevention of aggregation of unfolded protein chains
Hsp90	HtpG	Regulation of assembly of signal transduction proteins
Hsp100	ClpA, ClpB, ClpC, ClpX, ClpY	Disassembly of oligomers and aggregates
Prefoldin nascent chain-associated complex	Prefoldin	Binding to nascent chains as they emerge from ribosomes

During the last decade there have been a number of reports supporting the hypothesis that inducible molecular chaperones, produced both by bacteria and hosts, function as intracellular, cell surface or extracellular signals, which are involved in the control of the infectious process. This suggests that infection, among other things, is a contest of stress mechanisms with a multitude of unexpected evolutionary twists and turns.

Changes in any cell environmental conditions result in molecular chaperone synthesis. Infection is stressful for both the pathogen and the host. The response of bacteria to a variety of environmental stressors has been studied and reviewed previously (26-29).

The first step in bacterial infection is the interaction between bacteria and host epithelial cells or extracellular matrix. This interaction influences the stress protein synthesis in bacteria. The microarray analysis of *Porphyromonas gingivalis*, which causes periodontal disease, showed that, when the bacterium was cultured on human epithelial cell line Hep-2, the mRNA levels of Hsp40, Cpn60, Hsp70, Hsp90 as well as various peptidyl-prolyl isomerases (PPIs) and members of the thioredoxin family increased significantly (30). Another bacterium, *Neisseria gonorrhoeae*, interacts with epithelial cells and induces *rpoH*, a homologue of sigma-32 factor (σ^{32}), whose regulon contained the genes encoding the chaperones Cpn60 and Cpn10. Attempts to inactivate *rpoH* were unsuccessful, but construction of a strain that conditionally expressed *rpo*H showed that, although σ^{32} is not necessary for adherence, it is crucial for epithelial cell invasion by gonococci (31). In *Haemophilus ducreyi*, the GroEL protein is associated with the bacterial surface, suggesting a possible involvement in the attachment to host cells (32).

Once the bacteria passed the epithelial barrier, they will encounter the immunological big defenders, such as the macrophages or neutrophils. A number of organisms have adapted to an intracellular life style within macrophages (18, 33, 34).

There is evidence that bacteria develop an enhanced cell stress response when they encounter host cells. In their turn, host cells respond in a similar manner when exposed to bacteria or their products (35-37). Exposure of the intestinal epithelial cell line Caco-2 to *Salmonella enterica serovar Enteritidis* stimulates production of Hsp70 and Hsp90 (36). There is also preliminary evidence that Hsp70 overexpression inhibits the ability of cells to be activated by LPS (lipopolysaccharides) (37).

Inactivation of *Campylobacter jejuni* DnaJ resulted in a mutant, which grew in culture but not *in vivo*, being unable to colonize chickens (38). Knockout of the *dnaK-dnaJ* operon in *Salmonella enterica serovar Typhimurium* resulted in a mutant which did grow in culture, albeit at a lower rate. However, bacteria lacking DnaK/DnaJ did not survive and replicate in cultured macrophages or in cultured epithelial cells and failed to colonize mice. This is the first evidence that this operon is involved in the invasion of epithelial cells (39).

It has been also found that the α-crystallin gene *acr2* is the most upregulated gene in *Mycobacterium tuberculosis* subject to heat shock or after uptake into macrophages (24).

While the importance of molecular chaperones for survival in the host holds true for a variety of intracellular pathogens, molecular chaperones induction seems to be less relevant for some other pathogens, including *Listeria monocytogenes* (40). The impact of molecular chaperones on bacterial survival in the host is different in various infections.

INTRACELLULAR BACTERIA SURVIVAL INSIDE THE MACROPHAGES

The microorganisms invading tissues are first and foremost exposed to phagocytes. In many tissues, macrophages are dedicated to the elimination of foreign particles by phagocytosis. For this purpose, macrophages display a wide array of phagocytic and inducible microbicidal functions that could be explained by oxygen-dependent and oxygen-independent mechanisms. The oxygen-dependent mechanism consists of reactive oxygen molecules (*i.e.*, superoxide anion, hydroxyl radicals, hypochlorite ions, hydrogen peroxide, singlet oxygen) generation, within a phagosome and nitrogen intermediates, through the oxidative burst. Oxygen-independent mechanisms include the acidification of the phagosome to pH 5.5 and the release of antimicrobial cationic peptides called defensins, as well as many degradative enzymes concentrated within a large lysosomal compartment (41). Bacteria that readily attract phagocytes and that are easily ingested and killed are generally unsuccessful as pathogens. In contrast, most bacteria that are successful as pathogens interfere to some extent with phagocytes activity or avoid their attention.

Bacterial pathogens have developed numerous and various strategies to avoid phagocytic engulfment and killing, and in the case of intracellular pathogens, to ultimately multiply inside these cells (18, 34). Most of them are aimed at blocking one or more steps in phagocytosis, thereby halting the process. Bacteria can avoid phagocytes in various ways: a) avoiding the contact with phagocytes (*e.g.*, *M. tuberculosis*); b) the inhibition of phagocytic engulfment by some components of the bacterial cell surface (cell wall, fimbriae, a capsule) (*e.g.*, *Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Staphylococcus aureus*); c) survival and replication in a degradative phagolysosomal environment; many bacteria that are intracellular parasites of macrophages (*e.g.*, *Mycobacterium, Brucella, Listeria*) usually destroy macrophages in the end, but the mechanisms are not completely understood; d) products of bacteria that kill or damage phagocytes (aggressins, hemolysins, streptolysin, leukocidin, exotoxin A; and e) other antiphagocytic strategies used by bacteria (*e.g.*, *M. tuberculosis* that inhibits lysosomal fusion using mycobacterial sulfatides modified lysosomes).

Macrophages are obviously one of the most stressful environments for bacteria (27), even for those that have evolved to survive within these cells. Some bacteria are intracellular parasites because they are able to invade eukaryotic cells (*e.g.*, *M. tuberculosis, Mycobacterium leprae*, *Brucella* species, *L. monocytogenes*, *S. typhi*, *Shigella dysenteriae*, *Yersinia pestis*, *Legionella pneumophilla*, *Rickettsiae*, *Chlamydia*) (34).

An example of the magnitude of the stress response induced in *M. tuberculosis* within macrophages is that transcription rate of the *acr* gene encoding Hsp20 increases 800-fold in infected mice (33).

The importance of molecular chaperones for pathogen survival in a stressful environment is illustrated by the experiments with a mutant of the intracellular pathogen *S. typhimurium*, which overexpresses Hsps (16). This mutant was shown to be resistant to a variety of oxidizing agents and heat. On the opposite, mutants of *S. typhimurium* with specific hsp gene defects are highly susceptible to killing by activated macrophages and also express decreased virulence *in vivo* (42, 43).

Regulated expression of *dnaK* under oxidative stress seems to be used by bacterial pathogens to stand the respiratory burst of phagocytes (32, 19). This is the case of *Brucella*, which is a model of complex intracellular parasitism that can survive and replicate inside the macrophages and placental trophoblasts. The interactions of *Brucella* with cultured macrophages from a variety of hosts including cattle (44, 45), humans (46) and mice (47, 48) have been examined, and these bacteria display an impressive ability to survive and replicate within these cells.

CHAPERONES INVOLVED IN BACTERIAL SECRETION

The role of molecular chaperones in the bacterial secretory pathway was demonstrated by various authors (21-23, 49).

Proteins which interact with membranes need to be maintained in an unfolded state, and the DnaK and GroEL chaperones may play a part in this. Secreted proteins face a dual challenge, in that they must not only be kept unfolded (and not allowed to interact with other unfolded proteins) (50) but also targeted to the translocation machinery in the membrane (21).

The main chaperone carrying out this dual process is the chaperone SecB. *In vitro* evidence supports the hypothesis that SecB prevents folding of its bound substrate proteins and maintains them in a translocation competent state (51, 52). SecB has a high affinity for SecA, the main component of the membrane translocation complex, and once protein translocation has begun through this complex, SecB is released and it is available to bind another nascent protein (53). There is functional overlap between SecB and DnaK chaperones, in that overexpression of the DnaK and DnaJ chaperones can allow the growth of *secB*-deficient *E. coli* on an enriched medium (54); GrpE is also required for this complementation (55).

The key to successful bacterial colonization and persistance in many animal hosts, as well as for the formation of many productive symbiotic relationships are the contact-dependent type III secretion systems (TTSS) of bacteria (56), which have been specialized in bacterial proteins translocation directly into the host cell. The majority of pathogenic TTSS substrates are bound to a so-called "secretion chaperone" in the bacterium before delivery into the host (57). These are small proteins that bind to one or more virulence factors. The role of these molecules is to target the virulence factors to the pathogenic TTSS (58), in order to maintain the virulence factor substrate of the TTSS in a "secretion-competent" state, and to prevent inappropriate interactions, which could induce aggregation in the bacterium.

The "secretion chaperones" have been divided into two classes based on their properties of binding a single or multiple virulence factors (59, 22, 23, 60). Spa15 of *Shigella*, InvB of *Salmonella*, SycN-YscB and SycE of *Yersinia* are examples of molecular chaperones secretion into bacteria. Bacterial strains that lack a certain chaperone are usually impaired in the release of the corresponding virulence factor, which is either prematurely degraded or accumulates in the bacterium (61, 57). However, several virulence factors, such as *Yersinia* YopM and *Salmonella* SopE, are not thought to require chaperones.

THE ROLE OF BACTERIAL MOLECULAR CHAPERONES IN VIRULENCE

It is supposed that the infection might be built on a foundation of chaperones. There is evidence which supports the hypothesis that bacterial molecular chaperones act as direct virulence factors. A number of bacteria appear to use specific molecular chaperones as adhesins. In order to do this, the bacteria must secrete these proteins and they must attach to the cell surface. There is now considerable evidence that bacteria express a number of molecular chaperones on the cell surface and can release them into the extracellular milieu to act as signaling virulence factors (Table 2).

So far, nine bacteria (Table 2), including various organisms, such as *Borrelia burgdorferi, Clostridium difficile, Helicobacter pylori,* and *S. typhimurium* (24), have been reported to utilize cell surface Hsp60 and Hsp70 as adhesins.

Besides, it was shown that bacterial chaperones, such as Cpn60 and Hsp70, have cell-cell signaling properties, being able to modulate the activity of host cells. The molecular chaperones produced by pathogenic bacteria, having extracellular signaling functions, include: 1) Cpn60 proteins of *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *E. coli*, *H. pylori*, *M. tuberculosis*, *M. leprae*; 2) Hsp70 proteins of *M. tuberculosis*, *Mycobacterium paratuberculosis*, and *E. coli*; 3) Hsp90 protein of *H. pylori*, the peptidyl-prolyl isomerase of *H. pylori*, and 4) Cpn10 protein of *M. tuberculosis* (24). It has been also demonstrated that *M. tuberculosis* Cpn10 is secreted when the bacterium is present in the macrophage phagolysosome (62).

Table 2

Molecular chaperones involved in bacterial secretion

Molecular chaperone	Bacterium	Cell surface or secreted	Biological activity
Cpn60	*A. actinomycetemcomitans*	Both	Osteolytic factor and cytolytic factor
	Bartonella bacilliformis	Secreted	Mitogen
	Haemophilus ducreyi	Cell surface	Adhesin
	Helicobacter pylori	Cell surface	Adhesin
	Borrelia burgdorferi	Cell surface	Adhesin
	Clostridium difficile	Cell surface	Adhesin
	Legionella pneumophila	Cell surface	Adhesin
	Mycobacterium smegmatis	Cell surface	
	Mycobacterium avium	Cell surface	Adhesin
	Salmonella enterica serovar Typhimurium	Cell surface	Adhesin
Hsp70	*Coxiella burnetti*	Cell surface	Adhesin
	Enteropathogenic E. coli	Cell surface	Adhesin
	Helicobacter pylori	Cell surface	Adhesin
	Legionella pneumophila	Cell surface	Adhesin
	Mycobacterium avium	Cell surface	Possible adhesin
Hsp20	*Helicobacter pylori*	Cell surface	Adhesin
PPI	*Helicobacter pylori*	Secreted	Induces epithelial cell apoptosis
	Rickettsia prowazekii	Cell surface	

One of the effects of bacterial chaperones on host cells is apoptosis promotion, which is likely to inhibit the host antibacterial response (24). *H. pylori* promotes apoptosis of the gastric epithelial cell population, causing atrophic gastritis and gastric dysplasia associated with infection (63).

Mutagenesis screening experiments of *S. pneumoniae* provided the first clue that ClpC is involved in virulence (64) and homeostatic regulation (65). The *clpP* isogenic mutant of *S. pneumoniae* is more susceptible to macrophage killing and defective in colonization of the murine nasopharynx and survival in the murine lung (66). The oral gram-positive *Streptococcus mutans* exhibited reduced growth

under stress conditions and a lower capacity to form biofilms when the *clpP* gene was inactivated (67).

The Clp ATPases have also been found in the studies of virulence genes in both *S. typhimurium* (68) and *S. aureus* (69).

Finally, the *clpB* gene of *Francisella novicida* was identified in a mutational screening analysis aiming to identify mutants unable to grow in macrophages *in vitro* (70).

BRUCELLA PARADIGM FOR INTRACELLULAR LIFE

Bacteria of the genus *Brucella* are the agents causing brucellosis, a worldwide zoonosis that affects a broad range of mammals, including livestock and humans (71). Due to its high infectivity, *Brucella* has recently been classified as a potential agent of biological warfare (72). *Brucella* virulence mostly resides in its ability to enter, survive and replicate within phagocytic and non-phagocytic cells (placental trophoblasts), among which macrophages are a major target in infected mammals (71). Because it can be cultivated *in vitro* and survives in the environment for limited periods of time, *Brucella* is considered a facultative intracellular pathogen. However, this bacterium should rather be viewed essentially as an intracellular pathogen if one considers that mammalian cells are the privileged niche of its multiplication, and mammals are the primary, if not the only, pathogen reservoir. *Brucella* has experienced a long standing coevolution with its favored niche, the mammalian cell, making this pathogen exceedingly well adapted to the intracellular environment. This is demonstrated by the ability of *Brucella* to control its own intracellular trafficking to avoid lysosomal degradation (73), multiply extensively within a host cell without restricting basic cellular functions (74) or inducing programmed cell death (75).

Brucella has evolved some strategies to escape the bactericidal mechanisms associated with phagocytosis by: *(1)* avoiding fusion of its membrane-bound compartment (*Brucella*-containing vacuole (BCV)) with lysosomes (73), precluding bacterial killing by the host; *(2)* expressing an unconventional LPS that, in addition to high endotoxicity, protecting *Brucella* from antimicrobial cationic peptides and complement deposition (76), playing a role in the entrance and early survival inside macrophages (77); *(3)* producing cyclic glucans, that have been shown to modulate maturation of BCVs in order to avoid fusion with lysosomes (78); *(4)* expressing a type IV secretion system, VirB, which has an essential role in intracellular survival and replication (18); and *(5)* changing the pattern of protein expression in response to various environmental conditions present inside the host cell (Fig. 1).

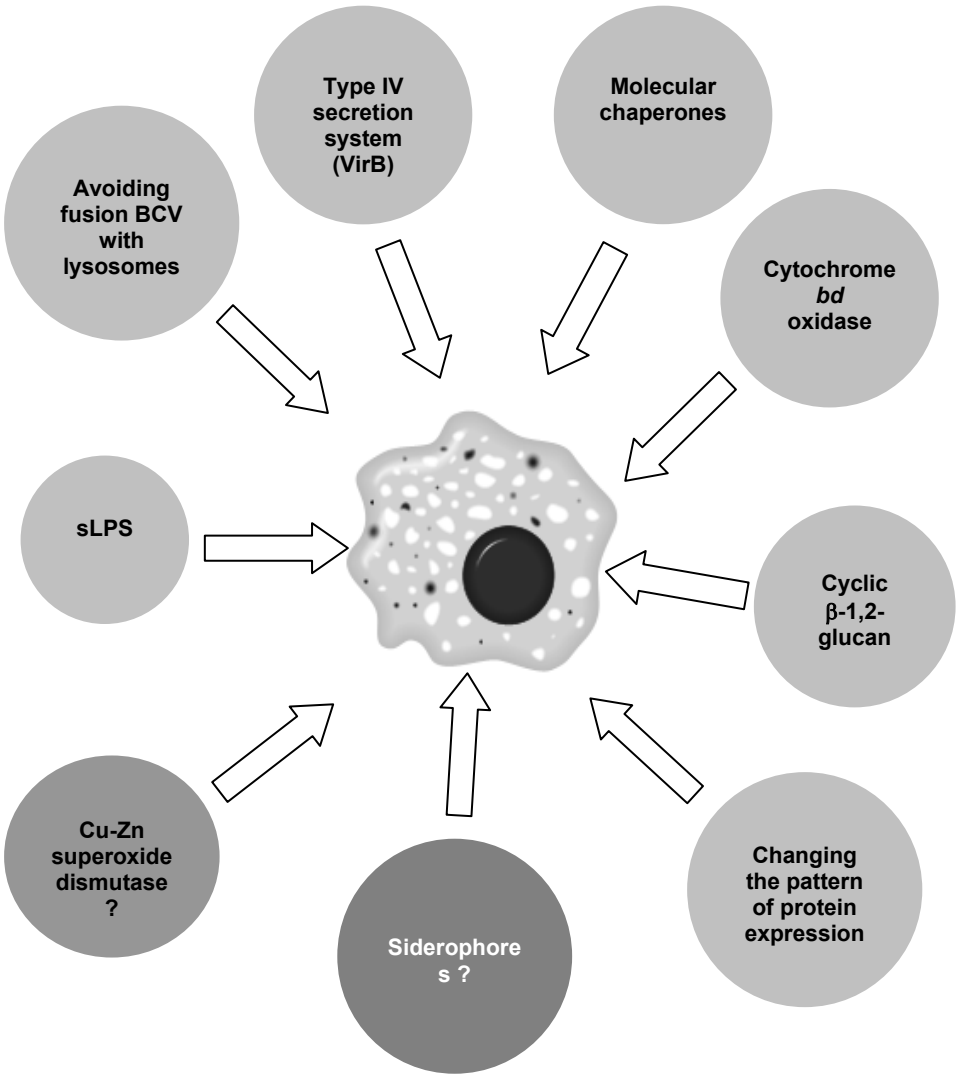

Fig. 1. – *Brucella* strategies to escape the bactericidal mechanisms of macrophages.

Some studies revealed that, as with other bacterial pathogens, various stress conditions (*e.g.*, phagocytosis and acid shock) alter the synthesis of proteins in *Brucella abortus*, *Brucella melitensis* and *Brucella suis* (79-81). In addition to variations in the expression level of 73 proteins, repression of 50 proteins and

induction of 24 new proteins occur during the growth of *B. abortus* within macrophages (80). Acidic and oxidative conditions, as well as nutritional and heat stress, induce the synthesis of "new" bacterial proteins. However, the quantity of these "new" molecules produced *in vitro* is not equivalent to the amount expressed within macrophages (80).

The important role of the molecular chaperone DnaK in intramacrophagic growth of *B. suis* and its acid-induced expression was already described (82). Previous data (82) suggested that DnaK from *B. suis* may play an essential role as a part of protein repair systems, protecting bacteria from the hostile environment encountered in the phagosome. Another hypothesis is that DnaK may be directly involved in the folding and proper localization of virulence factors, as intracellular multiplication is abolished in the null mutant. Insertional inactivation of *Brucella dnaK* and *dnaJ*, coding for the stress molecular chaperone DnaK and DnaJ respectively, have led to the conclusion that DnaK, but not DnaJ, is required for bacterial growth at 37°C (19). Experiments performed with both mutants at 30°C demonstrate that the *Brucella dnaK* mutant survived, but failed to multiply within phagocytes, whereas the parental strain and the *dnaJ* mutant multiplied normally.

B. suis null mutants for ClpATPase chaperonins behave similarly to the wild type strain, indicating that ClpA by itself is dispensable for intracellular growth (83).

Among the proteins induced in *Brucella* spp. in response to the environmental stress conditions are the GroEL molecular chaperones (84).

HF-1 (RNA chaperone host factor-1) is another stress protein involved in their resistance to oxidative conditions found within vacuoles. *B. abortus* mutants of this molecular chaperone do not replicate in macrophages, but initially multiply in mice. It is likely that this stress protein, normally required for the stationary growth, is also necessary during intracellular replication (58).

CONCLUSIONS

In addition to protein folding, bacterial chaperones have many other functions important in the cells under exposure to environmental stress. Some bacteria that survive within host phagocytes have evolved strategies to escape the bactericidal mechanisms associated with phagocytosis. The importance of molecular chaperones for bacteria survival within the host has been shown in a variety of intracellular pathogens, including bacteria of the *Brucella* genus. Under stress conditions caused by infection, the molecular chaperones production increases in both pathogen and host.

Identification of new aspects of the bacterial chaperones involvement in the bacteria–host interaction will undoubtedly constitute a major step in understanding the molecular mechanisms developed by bacteria during the long-standing evolution with their mammalian hosts.

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