International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

Study of the effect of a BolA mutant (SL1344) in *Salmonella Typhimurium* on in vitro proliferation in permissive HeLa cells.

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Abstract

Salmonellosis is one of the foodborne zoonotic diseases that annually causes approximately 153 million enteric illnesses and 57,000 deaths due to diarrhea. The main objective of this study is to elucidate the role of the BolA gene in the virulence of S. *Typhimurium* in permissive non-phagocytic HeLa cells through gene inactivation. PCR was performed to amplify the BolA virulence gene using the St bolApkd primer. The generated bacterium is the SL1344 strain, where the Kanamycin resistance gene replaces the bolA gene. The results obtained demonstrate successful inactivation of the bolA gene, resulting in a gradual decrease in the number of bacterial cells after 10 hours of infection, reaching a minimum at 24 hours post-infection. This outcome suggests the need to validate its reproducibility, paving the way for potential future solutions in the inactivation of the wild-type bacteria.

Keywords: salmonellosis, mutant, proliferation, HeLa cells, bolA gene, virulence.

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1. Introduction

Salmonella Typhimurium is a bacterium that can cause salmonellosis through the consumption of contaminated animal-origin foods (meat, eggs, milk). It is a Gram-negative bacillus, facultative anaerobe, mobile by peritrichous flagella, with a size of 1 to 3 µm in length and 0.5 to 0.7 µm in diameter, belonging to the Enterobacteriaceae family [1]. The transmission of *Salmonella Typhimurium* mainly occurs through the fecal-oral route, i.e., by ingesting contaminated fecal matter. This can happen through the consumption of contaminated food, water, or direct contact with infected animals. Symptoms of *Salmonella Typhimurium* infection include diarrhea, abdominal cramps, fever, nausea, and vomiting [2]. These symptoms generally appear within 6 to 72 hours after exposure to the bacterium [3]. The serological identification of Salmonella relies on the characterization of somatic and flagellar antigens. Indeed, 99.8% of human-origin isolates belong to serotypes of the subspecies enterica, including *Salmonella Typhimurium*. The situation is further complicated by the emergence of antibiotic-resistant strains. However, its main reservoir is the intestine of a large number of animals, including farm birds, leading to the dissemination of these microorganisms in the environment [4]. The seasonality of salmonellosis shows a predominance in the summer months, motivated by the increase in environmental temperature that would facilitate the proliferation of the bacterium in food [5].

In the European Union, salmonellosis accounts for the largest number of confirmed cases of foodborne infections, with 131,468 cases [6]. In the United States, Salmonella leads among foodborne illnesses. In Spain, as well as in the rest of Western countries, most salmonellas are produced by *Salmonella Enteritidis*, followed in incidence by *Salmonella Typhimurium* and to a lesser extent by other serotypes [7]. The large-scale genome organization of Salmonella is similar to that of Escherichia coli: Groisman and ochman, 1994; Sanderson and al. 1995, with which it shares 80% DNA homology and 90% protein homology [8].

In bacteria in general, a genetic mutation in the parent cell will be transmitted to daughter cells and can exchange genetic material among themselves. However, one of the potential effectors is the BolA protein, which affects different pathways directly related to virulence [9; 10]. This occurs through homologous recombination following horizontal transfer through conjugation, transduction, or transformation. Salmonella Typhimurium contains two type III secretion systems. This system allows certain Gram-negative bacilli to secrete and inject proteins pathogenicity in eukaryotic host cell cytosol [11]. Our objective is to study the effects of in vitro infection of generated Salmonella BolA mutants in non-phagocytic permissive HeLa eukaryotic cells, the dynamics of intracellular proliferation, and their variability. Experimental studies were carried out.

2. Materials and methods

2.1. Biological materials

- **Bacterial Strain and Genetic Modifications**

The bacterial strain employed in this study is the SL1344 strain, a non-pathogenic variant in humans. Notably, the bolA gene in this strain has been replaced with the Kanamycin resistance gene. This genetic modification serves as a crucial aspect of our investigation into the role of the BolA gene in the virulence of Salmonella Typhimurium.

- **Plasmid and Recombinase System**

The plasmid utilized in this study is PKD46, characterized by its thermosensitive nature and the expression of the λ phage recombination system, inducible by Arabinose. This plasmid facilitates controlled genetic manipulations, contributing to the precision of our experimental design.

Primer Sequences for BolA Gene Inactivation

The primers employed for the inactivation of the bolA gene are as follows:

St bolApkd13left:

Gccagcataaacagagcgactaacggaaagagaagtttttgtgtaggctggagctg cttc

St bolApkd13right:

Gccgttttcacaattgtcggctaatttttctctgaatactattccggggatccgtcgaac

These primers have been designed to specifically target and initiate the inactivation process of the bolA gene, a pivotal step in our investigation. The selected cell lines for cultivation are permissive epithelial cells derived from human carcinoma, specifically HeLa cells (model cells for the virulence of these bacteria in vitro).

2.2. Generation of Mutants

Genetic Inactivation Technique of Chromosomal Genes Using PCR Fragments: This method allows us to replace a chromosomal sequence with an antibiotic resistance gene (kanamycin), generated through PCR. Oligonucleotides with corresponding homologous extensions of the substitute gene are utilized. PCR products, digested with DpnI (a restriction enzyme requiring N6-methyladenine in the recognition sequence), were employed to transform SL1344 competent cells through electroporation, utilizing the pKD46 plasmid (λ phage Red recombinase). After plating on LBK30 agar plates and incubating at 37°C, a selection of mutant clones was carried out for further analyses to confirm the success of the transformation using confirmation primers [12].

2.3. Techniques Used

• **Electroporation**: This method facilitates DNA uptake by inducing membrane permeabilization through an electric discharge, optimizing transformation [13].

• **Intracellular Proliferation**: Following a minimum infection time of 30 minutes, mutants were collected at intervals of 2 hours, 6 hours, 10 hours, and 24 hours. Subsequently, the results were compared with those obtained in the parental strain SL1344 (positive control).

• **Immunolabeling**: In the case of cytoskeleton labeling, samples were incubated again with a solution of Alexa Fluor-488 or Alexa Fluor 660 phalloidin-diluted in PBG for approximately 30 minutes.

2.4. Proliferation Estimation Tool

• At each infection time point, samples are collected to estimate bacterial proliferation within infected cells in P24 wells.

• The intracellular proliferation index was estimated by dividing the number of viable intracellular bacteria at a specific post-infection time, which could be 2, 4, 6, 10, and 24 hours.

3. Results and Discussion

3.1. BolA Gene Inactivation

Figure (1) illustrates the verification results of the replacement and inactivation of the bolA gene in SL1344. To accomplish this, a virulent Salmonella Typhimurium strain (SL1344) was utilized to construct a BolA mutant strain. The confirmation oligos, with a melting temperature close to 50°C, possess the ability to identify sequences external to the immediate vicinity of the genetic region, thus enabling the amplification of larger sizes compared to those obtained by PCR amplification of the kanamycin resistance gene PKD13.

3.2. Infection in Permissive HeLa Cells by the Wild-Type Strain

The illustration in Figure (2) visually depicts the progression of infection within HeLa cells after an 8-minute exposure to the SL1344 bacteria. To facilitate differentiation, the cell has been labeled with green phalloidin, while bacteria and lipopolysaccharide (LPS) are distinctly labeled with red Alexa dye. It is worth noting that the observed fine red dots are likely attributed to the presence of vesicles containing LPS, whereas the larger red dots are likely representative of the bacteria themselves. Within just two hours after the initiation of infection, it is observed that the permissive HeLa cell undergoes nearly complete invasion by the bacteria, given that bacteria unable to penetrate the cellular barrier are effectively eradicated by gentamicin administration.

3.3. Effect of BolA Mutant

Figure (3) illustrates the results of infection and intracellular proliferation in HeLa cells by the wild-type strain and the BolA mutant. It is evident from this graph that concerning in vitro infection occurring in permissive cells, the HeLa cell line appears to exhibit reduced affinity for infection and subsequent intracellular proliferation compared to the wild-type SL1344 and ΔbolA parental lines. However, the disparity in infectivity and proliferation among these cell lines is not particularly substantial. It is noteworthy that the recovery of bacterial cells throughout the infection assay begins to show remarkable disparities after 6 hours of infection, ultimately peaking 12 hours post-infection. Following this peak, a noticeable decline in the number of recovered bacterial cells becomes evident 24 hours after infection. Furthermore, it is crucial to accurately interpret specific aspects of cellular colonization, especially when it comes to the ability to identify and quantify bacterial proliferation on an individual cell basis.

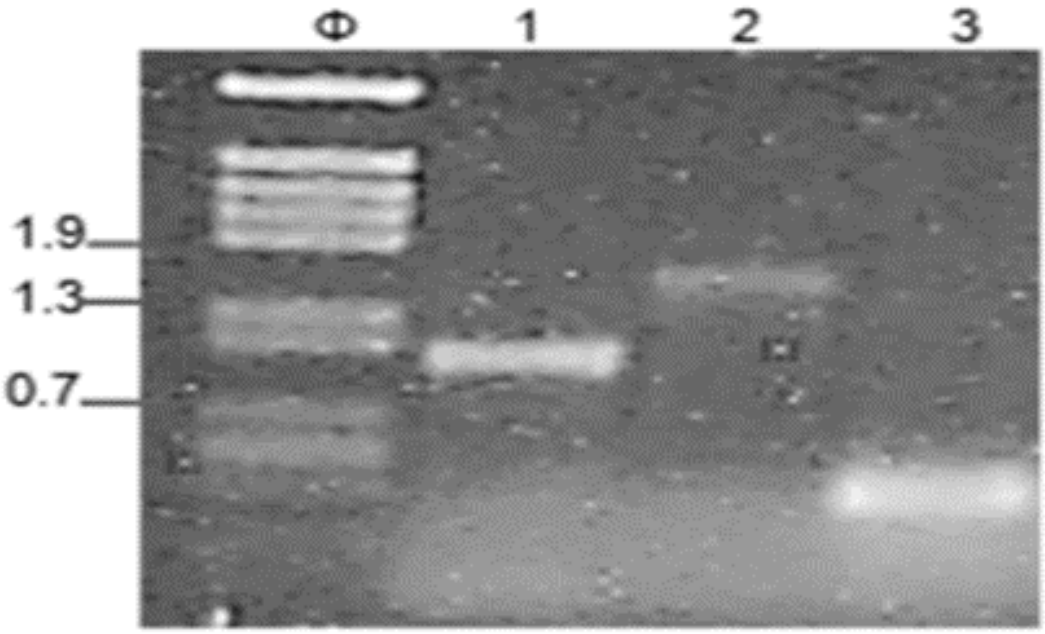

Figure 1. Verification of Replacement and Inactivation of the bolA Gene in SL1344, (Φ): Molecular weight marker. (1): Mutant DNA obtained with primers cfL/k2. (2): Mutant DNA obtained with primers L/R. (3): Control using confirmation primers BolAcfL/cfR amplified on SL1344 DNA.

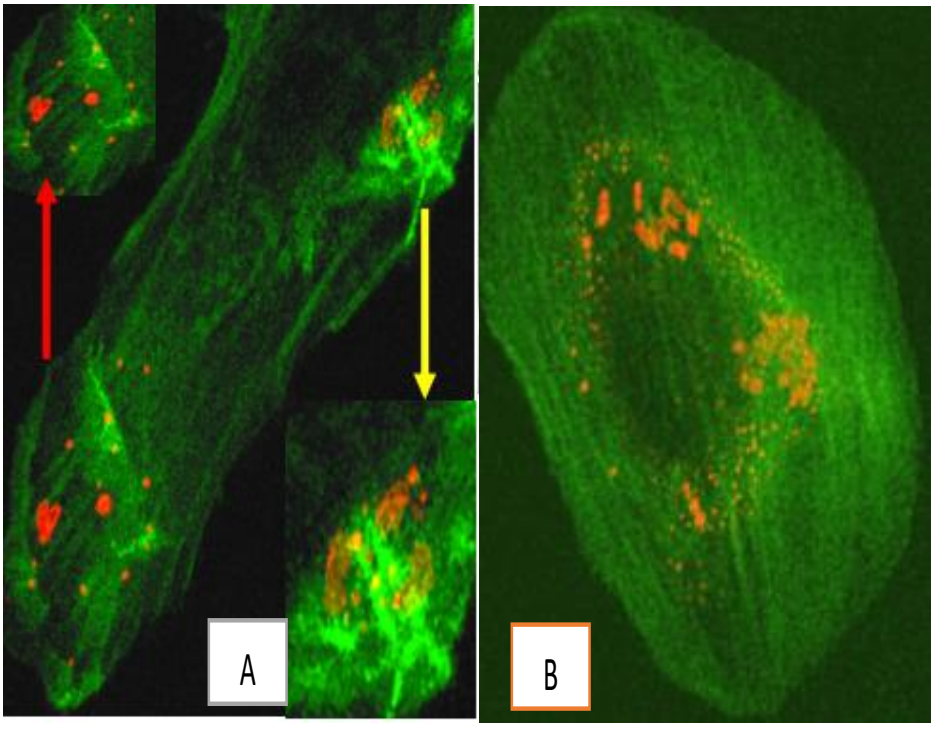

Figure 2. (A): HeLa cell captured at 8 minutes of infection with SL1344 bacteria. (B): HeLa cell captured at 120 minutes of infection with SL1344 bacteria.

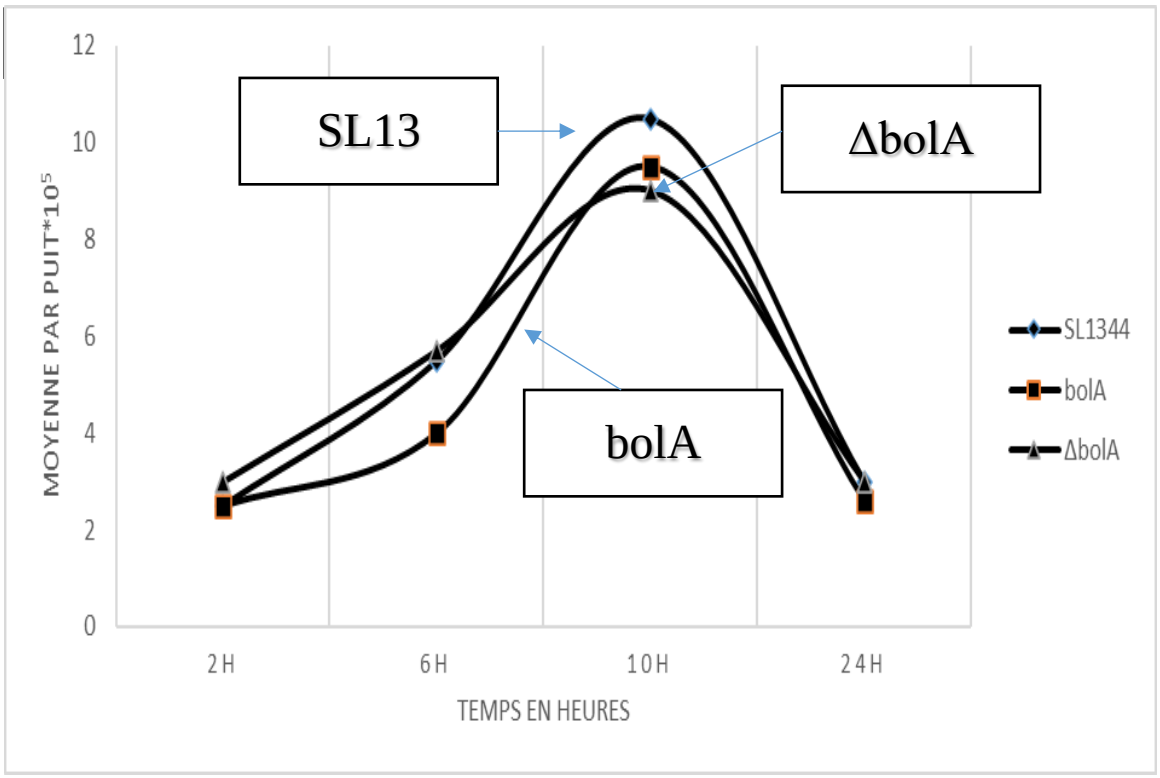

Figure 3. Results of the Infection Test and Intracellular Cellular Proliferation in the HeLa Cell Line for SL1344, bolA, and ΔbolA

This becomes even more significant in cases of persistent situations, where observations at the overall culture level reveal that the number of bacteria recovered after infection shows no increase, unlike what is observed in permissive cells such as HeLa cells. Conversely, in these persistent situations, the number of bacteria remains relatively constant for an extended period, spanning several days, before gradually starting to decrease. Additionally, it is important to note that mutant clones recovered following infection exhibit a diminished capacity for infection compared to normal cells, as evidenced by their reduced ability to infiltrate and persist within the eukaryotic cell. The absence of BolA leads to a defective virulence capacity, likely connected to the remarkable impact of this protein on *S. Typhimurium* [14]. The studied gene, bolA, plays a crucial role in the complex regulation of cell morphology and cell division [15]. When BolA is inactivated under unfavorable growth conditions, such as in stationary phase, aberrant cell morphology emerges as a consequence [16]. This observation underscores the indispensable role of BolA in maintaining normal cell morphology and highlights the disastrous consequences that can arise from its inactivation in challenging growth environments. This function of the bolA gene extends beyond Escherichia coli, demonstrating similarity across various organisms, including *Salmonella Typhimurium.* Our results effectively demonstrate the ability *of Salmonella Typhimurium* to proliferate within HeLa cells. This is consistent with studies conducted by [17]. Another study investigated the interaction of *Salmonella Typhimurium* with HeLa cells using electron microscopy and revealed that bacteria adhered to the cell surface, particularly to microvilli, and entered cells through a phagocytosis process [18].

4. Conclusions

The study we conducted remains a continuation of the development of the importance of this gene in biological processes such as cell division and peptidoglycan biosynthesis. Therefore, our confirmation was on the presence of mutations in non-essential synthesis of the wall or in morphogenic, proteins could exert a significant influence on the lasting infection and virulence (multiplication) *Salmonella typhimurium* in the intracellular medium.

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