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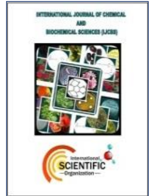

# Study of the effect of a BolA/DacA double mutant (SL1344) in Salmonella typhimurium on in vitro proliferation in the non-permissive NRK cells

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**Abstract**

Salmonellosis, a foodborne zoonotic disease, is accountable for an astonishing 153 million cases of enteric illnesses and approximately 57,000 deaths from diarrhea annually. In light of this, the main objective of the present study is to elucidate the impact of a double mutant Bola/DACA on the proliferation of bacteria within a non-permissive NRK cell. The analysis of the results reveals a noticeable decrease in the number of bacteria that proliferate after a 24-hour period of bacteria-host cell interaction. It is important to note, however, that despite this decrease, the virulence of the bacteria persists, although to a lesser extent, as evidenced by a marked decrease in the quantity expressed in colony forming units (CFU). These compelling results serve as a catalyst for efforts to generate mutants carrying multiple mutations that are likely to have a very pronounced effect on virulence. By expanding the repertoire of mutant strains, it becomes possible to target virulence mechanisms more comprehensively, which could lead to the development of effective strategies to combat the pathogenicity of Salmonella.

**Keywords:** Salmonella, mutant; proliferation, NRK cells, virulence.

**Full length article** **Corresponding Author*, e-mail: taimani1971@gmail.com

## 1. Introduction

*Salmonella typhimurium*, a particularly feared bacterium, is responsible for causing salmonellosis, a formidable disease resulting from the consumption of contaminated animal-derived foods such as meat, eggs, and milk. This small organism, classified as a Gram-negative bacillus, is characterized by its facultative anaerobic nature, its mobility due to its peritrichous flagella, and its modest dimensions of 1 to 3 um in length and 0.5 to 0.7 um in diameter [1]. She is part of the prestigious Enterobacteriaceae family. The spread of Salmonella Typhimurium primarily occurs through the fecal-oral route, which involves the ingestion of contaminated fecal matter. This could occur because of consuming contaminated food, contaminated water, or direct contact with animals carrying diseases. The symptoms of Salmonella Typhimurium infection include diarrhea, abdominal pain, fever, nausea, and vomiting [2]. Usually, these symptoms appear within a period of 6 to 72 hours after coming into contact with the bacteria [3]. The serological identification of Salmonella is based on the differentiation of somatic and flagellar antigens. Indeed, 99.8% of the samples from humans are classified into the serotypes of the subspecies enterica, particularly Salmonella typhimurium. The situation becomes even more complex with the emergence of antibiotic-resistant strains. However, the intestines of various animals, including domesticated birds, serve as the predominant habitat for these microorganisms, thereby contributing to their dispersion in the environment [4]. Salmonellosis exhibits a seasonal predominance during the summer months, facilitated by the increase in environmental temperatures, which provide conducive conditions for the bacteria to multiply in food [5]. In Europe, salmonellosis is responsible for the highest number of foodborne illnesses, with 131,468 confirmed cases. In the United States, salmonellosis is the foremost cause of foodborne illnesses. [6]. In Spain, as well as in other Western countries, the majority of cases of salmonellosis are caused by Salmonella enteritidis, followed in frequency by Salmonella typhimurium, with a lesser incidence for other serotypes [7]. The genome of Salmonella exhibits a similar organization to that of Escherichia coli, with a homology rate of 80% at the DNA level and 90% at the protein level [8]. In the field of bacteria, it is widely acknowledged that any genetic mutation occurring in the mother cell will inevitably be passed down to its offspring, the daughter cells. Furthermore, these daughter cells are not only capable of inheriting genetic material from their mother cell, but they can also exchange genetic material amongst themselves. Salmonella typhimurium possesses two type III secretion systems. These systems play a central role in the pathogenicity of certain Gram-negative bacilli, allowing them to secrete and then inject proteins responsible for inducing pathogenicity into the cytosol [9]. Our objective is to study the effects of in vitro infection of a double mutant generated from Salmonella, BolA/dacA, in non-permissive NRK cells on intracellular proliferation and their variability.

## 2. Materials and methods

### *2.1 Biological materials*

The bacterial material discussed in this study is the competent cell. SL1344. The plasmid utilized in this study is PKD46, a thermosensitive plasmid. In order to determine if the generated mutants affect the process of infection and intracellular proliferation, infection assays were conducted in non-permissive NRK cells.

### *2.2. Obtention de mutants*

Genetic inactivation technique of Chromosomal genes using PCR fragments [10-13]. This method enables us to replace a chromosomal sequence with an antibiotic resistance gene (kanamycin), generated by PCR, using oligonucleotides with homologous extensions corresponding to the gene to be substituted. The PCR products digested by DpnI (a restriction enzyme that requires N6 methyl adenine in the recognition sequence) were utilized to transform SL1344 competent cells (trans glycosylase deficient) through electroporation using the pKD46 plasmid (lambda red recombinase). After LBK30 plating and incubation at 37 °C, the selection of mutant clones was carried out for further analysis to confirm the successful transformation using confirmation primers. Two mutants were employed, Mutant SL1344/bolA and the dacA mutant.

### *2.3 Utilized Techniques*

In order to determine whether the generated mutants affect the process of infection and intracellular proliferation, infection assays were carried out in non-permissive NRK cells. The minimum post-infection time is 30 minutes, during which the contact between the bacterium and the cell is facilitated. The mutants were collected for their subsequent analysis at different time points of infection (2 hours; 6 hours; 10 hours and 24 hours), comparing the results obtained with those obtained in the parental strain SL1344 as the positive control for the results.

**Immunostaining:** In the case of cytoskeleton staining, re-incubate the samples with a solution of Alexa Fluor-488 phalloidin or Alexa Fluor 660 phalloidin diluted in PBG for approximately 30 minutes.

### *2.4 Proliferation Estimation Tool*

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

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

## 3. Results and Discussion

### *3.1. Infection of non-permissive NRK cells by the sl1344 strain.*

The figure (1) displays a photograph taken 15 minutes after the infection of a non-permissive cell by the SL1344 strain. It results in the formation of initial LPS vesicles upon contact. Indeed, the vesicles are stained with red anti-Rabbit antibodies and the cell cytoskeleton with green phalloidin. However, a modification in the structure of the contact membrane has been observed. The figure (2) depicts the results following a 30-minute infection of the non-permissive NRK cell by the SL1344 bacterial cell. Furthermore, only two cells are infected. Internalized bacteria and the production of initial LPS vesicles (lipopolysaccharide), a specific bacterial product characterize this infection. The alteration of the bacterial entry surface into the host cell is also observed. In addition, the intracellular bacterium is subject to significant selective pressures from the host cell, which is also evident in the recapture with a high frequency of mutator phenotype bacteria due to disruptions in the mutS gene in cell infection that allow bacterial proliferation. Both figures (3) display two photographs taken after 6 hours of infection. Indeed, it is observed that LPS vesicles are dispersed throughout the cell. However, an absence of bacteria close to the cell's environment. However, two localized infection zones have been identified, representing the two sites of bacterial penetration into the host cell. The internalized bacterium is located within the vacuole and begins to proliferate. In NRK cells, there is observed a limitation in proliferation and long-term survival. It is worth mentioning that the important virulence factor of Salmonella typhimurium is its ability to replicate intracellularly within epithelial cells [14].

### *3.2. Infection and intracellular proliferation in NRK cell line*

The figure (4) displays the results of proliferation of wild-type and double mutant bacterial strains. Indeed, the recovery of bacterial cells throughout the infection assay is nearly equal between SL1344 and ΔbolA dacA. Furthermore, a significant decrease in the number of recovered bacterial cells is observed at 24 hours post-infection. The double mutant appears to have an impact on the infection process, as it does not exhibit a significant infection affinity and intracellular proliferation compared to the wild-type SL1344. The absence of BoLA and DacA results in defective virulence capacity, which is closely linked to the significant impact of their protein on S. Typhimurium, as indicated in the study by [15]. In our research, our focus has been on the study of the BoLA and DacA genes, as they play a crucial role in the complex regulation of cell morphology and cell division, as highlighted by [16]. In unfavorable growth conditions, such as those encountered in the stationary phase, these two structures become inactive, resulting in the emergence of aberrant cellular morphology, as indicated in [17]. Our findings reveal the remarkable ability of the bacterium Salmonella typhimurium to multiply within NRK cells. This observation was confirmed by previous investigations conducted by [18]. Furthermore, another study utilized electron microscopy to investigate the interaction between Salmonella typhimurium and NRK cells, revealing that the bacteria adhere to the cell surface, especially to the microvilli, and enter the cells through the process of phagocytosis, as demonstrated in [19]. Furthermore, this interaction has proved to be very pronounced and evident.

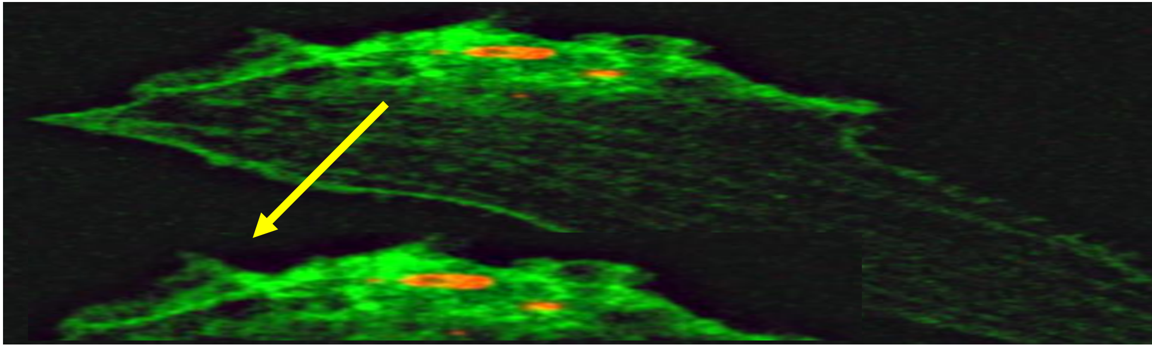

**Figure 1.** Cell captured after 15 minutes of infection by the SL1344 bacterium in NRK cell, the cell is labeled with green phalloidin, the bacterium and LPS are labeled in red Alexa

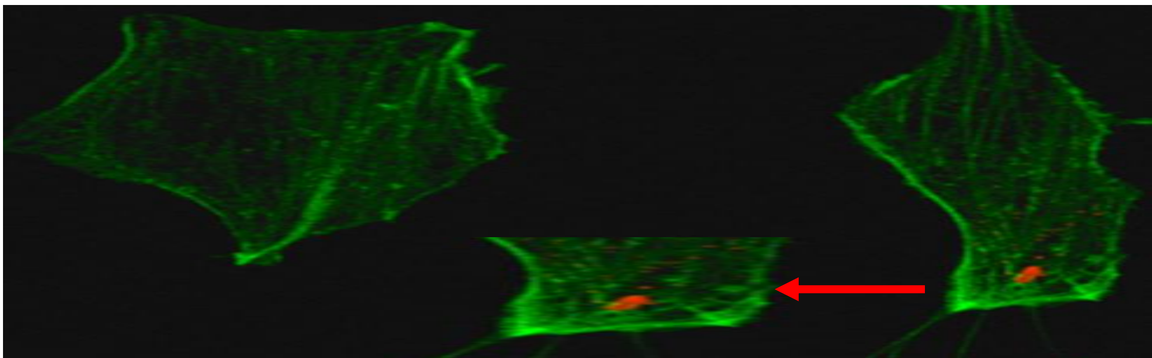

**Figure 2**. Cell captured 30 minutes post-infection by the bacterial cell SL1344 NRK, with the cell labeled in green phalloidin, and the bacteria and LPS labeled in red Alexa. The arrow indicates an expansion of the infection area.

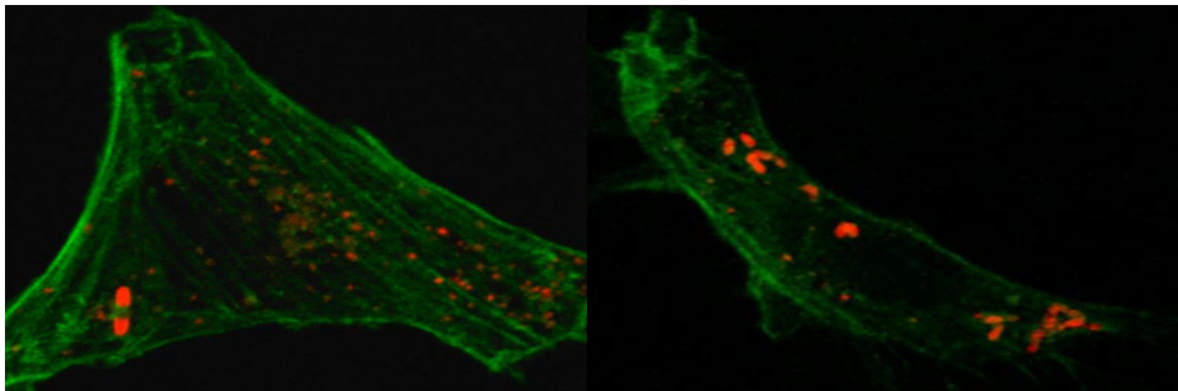

**Figure 3.** Cell captured at 360 minutes post-infection of SL1344 bacteria in NRK cell, image processed using Huygens deconvolution program, cell is labeled with green phalloidin, bacteria and LPS are labeled with red Alexa.

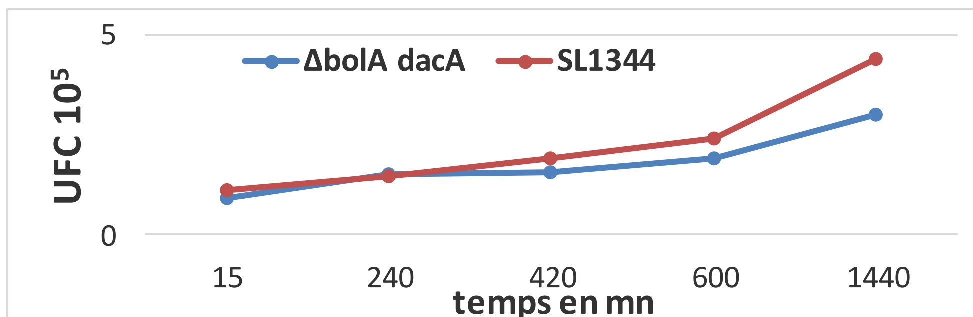


**Figure 4**. Results of infection and intracellular proliferation assay in NRK cell line for SL1344 and double mutant strains.

## 4. Conclusions

In conclusion, it has been demonstrated that the absence of the BoLA and dACa genes has a detrimental effect on the virulence capacity of S. Typhimurium, which can be attributed to the significant influence of their protein. In addition, these genes play a critical role in the regulation of cell morphology and division, especially under adverse growth conditions. Furthermore, our research confirms the ability of the Salmonella typhimurium bacterium to proliferate in NRK cells, as previously indicated by other studies. Furthermore, electron microscope analysis revealed the specific interaction between the bacteria and NRK cells, with the bacteria attaching to the cell surface, particularly to the microvilli, and then entering the cells through phagocytosis. Overall, these observations have illuminated the complex mechanisms underlying the pathogenicity of Salmonella typhimurium. In conclusion, these findings shed light on the intricate processes involved in the virulence of this bacterium. The study we conducted represents a contribution to the understanding of the significance of these genes in biological processes such as cell division and peptidoglycan biosynthesis. Therefore, our confirmation of mutations in the non-essential synthesis of the cell wall or in morphogenic proteins could have a significant impact on the sustained infection and virulence (multiplication) of Salmonella typhimurium in the intracellular environment.

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