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Inducible Acid Tolerance Response in *Shigella sonnei* and *Shigella flexneri*

[1]A. Abdul-Aziz, [1]M.F.F. Abdullah and [2]N.H. Hussain
[1]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450, Shah Alam, Selangor Darul Ehsan, Malaysia
[2]Institute of Science, Universiti Teknologi MARA, 40450, Shah Alam, Selangor Darul Ehsan, Malaysia

Corresponding Author: A. Abdul-Aziz, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450, Shah Alam, Selangor Darul Ehsan, Malaysia

ABSTRACT

Acid Tolerance Response (ATR) in *Shigella sonnei* and *Shigella flexneri* was induced by pre-exposing the mid exponential phase cultures at sub lethal pH of 4.5 for 90 min before challenging them in lethal pH of 3.0. The ATR was exhibited by both species with *S. flexneri* displaying a higher tolerance to lethal acid killing. Analyses of protein profiles of both unadapted and adapted cells using 2-dimensional gel electrophoresis (2D-PAGE) revealed that the acid adapted cells of *S. sonnei* demonstrated differential expression of 19 Acid Stress Proteins (ASPs). Five of these proteins were synthesized *de novo*, 11 were upregulated while three other proteins were repressed. In *S. flexneri*, two proteins were synthesized *de novo*, four upregulated and two proteins were repressed. Two of these proteins with estimated molecular weights of 14.1 and 15.1 kDa and isoelectric points (pI) of 5.50 and 5.58, respectively were found to be consistently induced in both species. An inducible ATR in *Shigella* may protect the bacteria from environmental pH stress and aid in their pathogenesis. The detection of proteins important for ATR may present novel targets for antimicrobial intervention.

Keywords: Acid tolerance response, acid stress proteins, *Shigella sonnei*, *Shigella flexneri*

INTRODUCTION

Shigella is the primary causative agents of shigellosis, a bacillary dysentery which is transmitted directly through faecal-oral routes or indirectly through faecal-contaminated food and water. They are neutrophiles thus the bacteria will need to first overcome the acid stress mechanisms employed by the human body before they can establish an infection and disseminate. These include the acidic environment of the stomach, with pH value as low as 2.0 (Verdu *et al.*, 1994), the volatile fatty acids in the small intestine with pH around 4.0-4.5 (Gahan and Hill, 1999) and the acidic interior of phagolysosomes (Oh *et al.*, 1996; Rathman *et al.*, 1996). However, studies have shown that microorganisms have the ability to adapt to environmental stress. Microbial cells if first pre-exposed or adapted to sub-lethal dose of acid will subsequently survive better when challenged to lethal dose as compared to the unadapted cells. Such phenomenon of inducible ATR has been observed in a number of enteric pathogens (Foster and Hall, 1991; Goodson and Rowbury, 1989; Karem *et al.*, 1994; Murphy *et al.*, 2003; Tiwari *et al.*, 2004). Nevertheless, most of these studies were done on the stationary phase of the bacteria cells where cells would naturally induce a general stress response that provides resistance against most environmental challenges.

Few studies have been conducted on actively growing exponential phase cells which lack the general stress response. Enteric pathogens in contaminated foods and drinks can potentially be in an exponential phase of growth. Most food are acidic, either naturally or due to added preservatives, thus providing a favorable environment for adaptation of the microorganisms which upon consumption may protect them from the acid stress environment in the stomach, small intestines and phagocytes. Moreover, the inducible ATR also involves changes in the gene expression of the cells (Audia *et al.*, 2001; Foster and Hall, 1990) whereby, the synthesis of some cellular proteins are upregulated or repressed while some are synthesized *de novo* as part of the survival mechanism to protect the cells from subsequent damages.

The aims of the present study were to determine whether ATR could be induced in both *S. sonnei* and *S. flexneri* and to investigate the differences in their tolerance response. Although, a closely related study has been documented whereby the survival of both *Shigella* species in acidic fruit juices was determined (Bagamboula *et al.*, 2002), the present study took a different approach by using inorganic acid instead. The synthesis ASPs during the adaptation period would also be observed which may further serve as novel cellular targets for the development of new antimicrobial compounds.

MATERIALS AND METHODS

Both *S. sonnei* S37 and *S. flexneri* S307 were clinical isolates obtained from the Institute of Medical Research (IMR) Kuala Lumpur and the cultures were maintained on Nutrient Agar (NA) at 37°C.

Preparation of mid-exponential cells culture: One loopful of a 24 h culture was inoculated into 100 mL of NB pH 7.0 and grown at150 rpm, 37°C for 24 h. The cultures were then diluted (1:50) in fresh NB and further grown to mid exponential phase (OD_{600} of 0.05). The cells were harvested, washed twice in NB by centrifugation at 7,000 rpm for 10 min and the pellets were resuspended in 10 mL NB at pH 7.0. These cell suspensions (approx 10^7 cells mL^{-1}) were used in all subsequent experiments as mid-exponential cell cultures.

Acid sensitivity of *Shigella sonnei* and *Shigella flexneri*: The sensitivity of both *Shigella* species to different acid concentrations was studied to determine their lethal and sub-lethal pH values. The lethal acid pH is referred to the external pH value that instantly killed and reduced the viability of the bacterial population to less than 1% within the 3 h exposure while the sub-lethal pH is defined as the pH that inhibits growth and reduced the viability of the cells by 30-50% at the end of the exposure period.

Mid-exponential cells were inoculated into a series of flasks containing 100 mL NB and adjusted to an initial OD_{600} of 0.05. One milliliter sample was taken immediately, diluted in PBS and plated to determine the initial viable count. The pH of each culture was then adjusted with HCl. Following that, the cultures were incubated at 150 rpm, 37°C. Samples were then taken every 30 min and the cell count was determined.

Induction of ATR: The ability of *S. sonnei* and *S. flexneri* to induce tolerance to lethal acid was carried out by first pre-exposing the mid-exponential phase cells of both species to a sub-lethal acid pH value for 90 min (equivalent to approximately two doublings) before challenging them to the lethal pH value.

The adapted cells of the *Shigella* species were prepared by exposing the mid-exponential cells to the sub-lethal pH 5.0 at 37°C, 150 rpm for 90 min while, the unadapted cells remained at pH 7.0. The initial cell count (t_0) was determined at the end of the exposure period before the adapted cells were challenged to lethal pH of 3.0 to test for acid tolerance. A sample of the cells was immediately taken (t_1) and the cultures were further incubated with samples taken at every 10 min over a period of 40 min. Each experiment was performed in three repeats.

Two-dimensional SDS-PAGE: The acid adapted and unadapted cells were harvested separately and washed twice in PBS and sterile distilled water. A volume of 50 µL of the packed cells were lysed in lysis buffer (8 M urea, 2% CHAPS, 0.002% bromophenol blue, 0.1% protease inhibitor solution (Sigma, USA) using a sonicator. (Sartorius, Germany). The resulting lysate was centrifuged at 13,000 rpm, 5°C to remove cell debris and the clarified protein sample was immediately used or stored at -80°C. Protein concentrations were determined using a Bicinchoninic acid protein assay kit (Sigma, USA) according to the manufacturer's instructions.

An aliquot in of each cell lysate containing 15 µg of proteins were resolved using two-dimensional SDS-PAGE (ZOOM Benchtop Proteomic System, Invitrogen, USA) according to the manufacturer's instructions. The isoelectric focusing was performed on a broad range strip of pH 4-7 using a protocol of 175 V for 15 min, followed by voltage ramping from 175-2000 V in 45 min and a final 2000 V for 30 min. The second dimension PAGE was performed on a pre-cast Bis-Tris 4-12% gradient gel at 200 V for 50 min. The gels were then stained using Invitrogen's SilverQuest silver staining kit and analysed using Imaxia's Gelfox software.

The 2D proteins profiles of *S. sonnei* and *S. flexneri* were first compared to a partially annotated 2D gel image of *S. flexneri* 2a strain 2457T (Liao *et al.*, 2003). Several clear matching protein spots were chosen as landmark and used to estimate the relative density, Molecular Weight (MW) and pI values of other spots in the gels. The Gelfox software was used to compare the proteins profiles between unadapted and acid adapted cells of both species. These proteins were identified preliminarily by comparing their MW and pI values to virtual protein spots on an *in silico* 2D gel image of *S. flexneri* 2457T (ARGONNE National Lab (USA) Gelbank, http://gelbank.anl.gov). For each protein spot of interest, corresponding spots within a range of ±5% of the MW and ±0.1 pI units from the *in silico* virtual gel were chosen. Preliminary identification of the protein spots was done using the database at the website. The list was then filtered based on the potential of these proteins in contributing towards stress mechanism, excluding hypothetical proteins or other proteins without known functions.

RESULTS AND DISCUSSIONS
Acid sensitivity and ATR of *Shigella sonnei* and *Shigella flexneri*: Both mid-exponential cells of *S. sonnei* and *S. flexneri* were rapidly killed at pH below 4.0 (Fig. 1a-b). Approximately 30 and 70% of the *S. sonnei* populations remained viable after 3 h at pH 4.5 and 5.0, respectively. *Shigella flexneri* appeared to have a higher acid resistant and able to grow normally at pH 5.0 while about 40% of the cells survived at pH 4.5. Hence, pH 3.0 and 4.4 was chosen as the lethal and sub-lethal pH, respectively for both species.

Gorden and Small (1993) reported that most *Shigella* species were resistant to extreme acid conditions as they were able to survive exposure to pH 2.5 for at least two hours. However, in the present study, both cells of *S. sonnei* and *S. flexneri* were unable to survive at pH lower than 3.5 where the cells died almost instantly. The difference could probably be due to the different phase
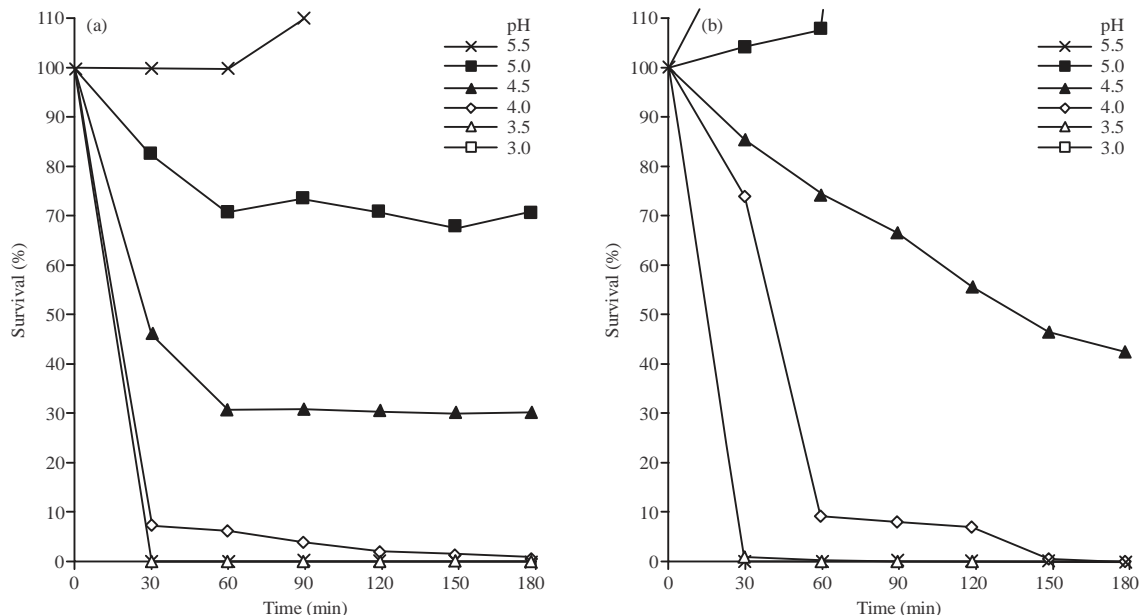
Fig. 1(a-b): Mid-exponential cells of (a) *Shigella sonnei* and (b) *Shigella flexneri* grown in various pH. Cells were sampled at 30 min interval for 3 h to determine viable cell counts

of the cultures used. Studies have shown that resistance was observed when the bacteria reached at least the late-exponential phase with full expression of acid resistance expressed in the stationary phase (Benjamin and Datta, 1995; Gorden and Small, 1993). The acid survival ability in stationary phase of *S. flexneri* was reported to be associated with the sigma factor σ^s encoded by *rpoS* (Nunoshiba and Demple, 1994; Waterman and Small, 1996). In contrast, the mid-exponential phase used in the present study is a phase where cells were actively growing due to the abundance of nutrients in the environment. Such condition will unlikely to induce any general resistance system thus these cells are less resistant when exposed to extreme acid conditions. *Shigella flexneri* also appear to have a better ability to survive in acidic conditions. This could possibly due to ability of the *S. flexneri* membrane to block extracellular protons from entering the cells, more efficient buffering capacity or a better proton-pump system to discharge extra protons outside the cells.

Both *S. sonnei* and *S. flexneri* displayed an inducible ATR as shown in Fig. 2a-b. Adapted cells of both strains survived better as compared to the corresponding unadapted cells ($p < 0.05$) at every sampling time point. *Shigella flexneri* cells displayed a stronger ATR, with higher survival percentages as compared to cells of *S. sonnei* at all time points ($p < 0.05$). After 40 min of acid challenge, 1% of the adapted *S. flexneri* cells remained viable which is 250,000-fold higher than the unadapted cells ($p < 0.01$). However, at this similar point, only about 0.005% of the adapted *S. sonnei* cells remained viable which is 700-fold higher than the unadapated cells ($p < 0.01$). At the end of the 90 min exposure the number of viable declined to similar levels in both adapted cells and unadapted cells. Thus, the ATR conferred by adaptation is only able to protect the adapted cells effectively for the first half-hour of the acid challenge. A stronger ATR observed in *S. flexneri* which could be due to a greater tolerance towards acid. While, most studies were done on stationary phase cells, this is probably the first attempt to compare the ATR of these pathogenic *Shigella* species in a complex medium at mid-exponential phase.
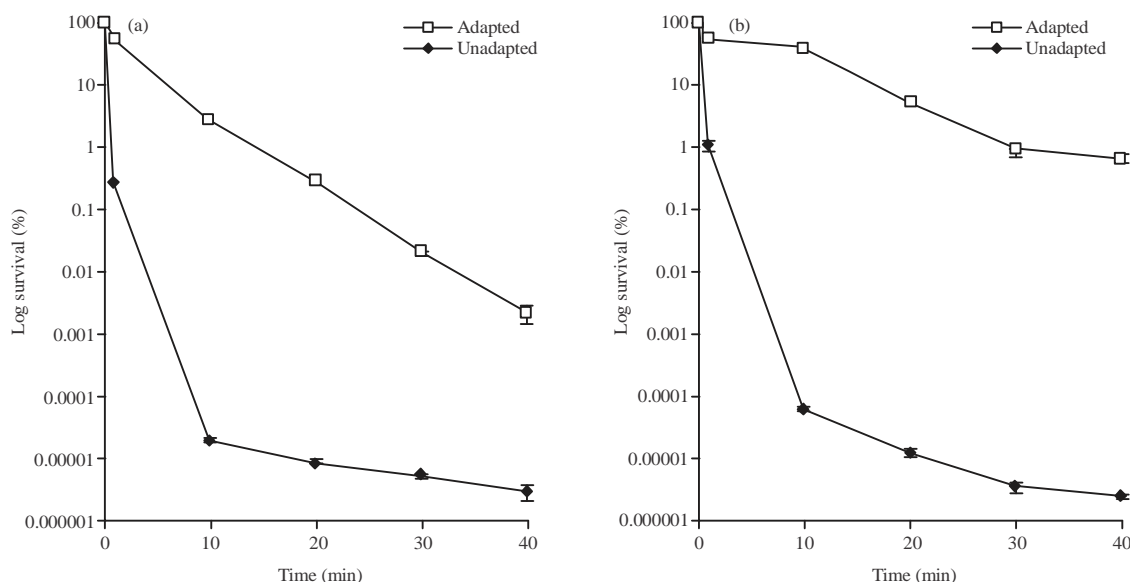
Fig. 2(a-b): Induction of ATR in *Shigella* at pH 4.5. Mid-exponential cells of (a) *Shigella sonnei* and (b) *Shigella flexneri* were adapted by pre-exposing to a sub-lethal pH of 4.5 for 90 min and challenged at a lethal pH of 3.0. Error bars indicate the standard errors of the means

2-D gel analysis: In *S. sonnei*, a total of 139 matched spots were detected from both gels while the unmatched spots were not included in the analysis (Fig. 3a-b). The density data revealed that 19 of these spots showed at least a two-fold increase or decrease in their concentrations in the adapted cells which could also be verified by visual comparison of their density. Of these, five were novel proteins expressed only in the adapted cells while the expression of 11 proteins was induced or over-expressed and the three remaining proteins were repressed in these cells. In *S. flexneri*, 134 protein spots could be matched the (Fig. 4a-b) whereby, eight of these were differentially expressed in the adapted cells which consist of two novels, four induced and two repressed proteins.

By comparing the MW and pI of protein spots to a 2D virtual gel, a few possible ASPs candidate were identified. It is interesting to note that two of the novel proteins spots found in acid adapted cells of *S. sonnei* (Fig. 3a, spots 4 and 5) were also synthesized *de novo* in acid adapted cells of *S. flexneri* (Fig. 4a, spots 1 and 2), with predicted Molecular Weights (MW) of 14.1 and 15.1 kDa and isoelectric points (pI) of 5.50 and 5.58, respectively. Thus, these proteins are probably strong candidates involved in ATR mechanism of *Shigella* as they were present in adapted cells of both species. Preliminary identification showed strong possibility of the first protein being TolR, an inner membrane protein, while the second one could be either osmotically induced protein OsmC, a heat shock protein ibpA or a membrane bound ATP synthase, AtpC.

Two systems were found to be involved in acid resistance of stationary phase *Shigella* grown in minimal medium (Bearson *et al*., 1997). However, recent studies using *E. coli* K-12 mutants revealed that none of these systems are involved in the development of acid tolerance in exponential phase cells grown in rich medium (Seputiene *et al*., 2006). This suggests a different survival mechanisms are involved in both exponential and stationary cells of *E. coli* which could also be the case in *Shigella* as they are closely related. The involvement of the inner membrane
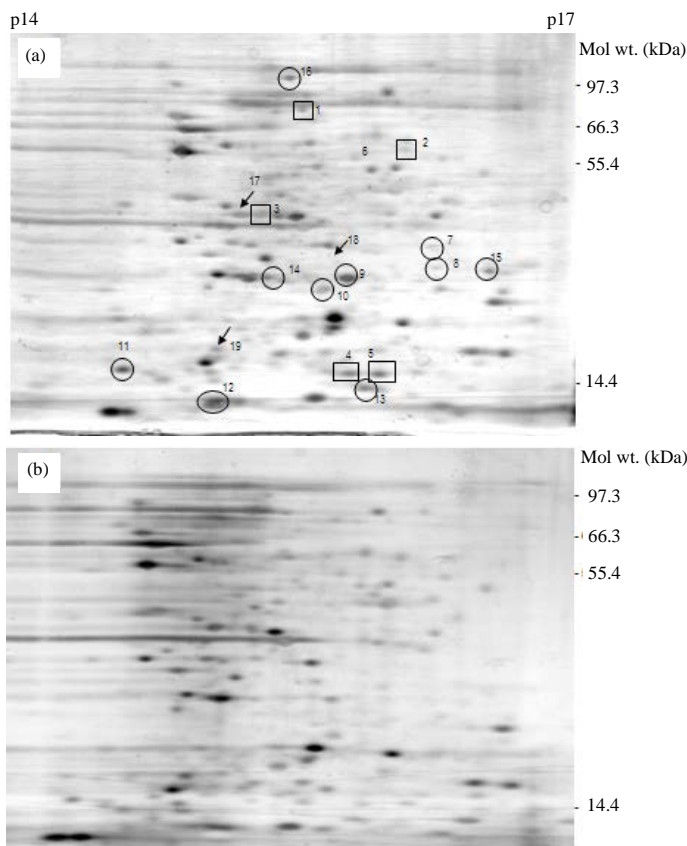
Fig. 3(a-b): The 2D gel of total protein extracts from (a) Acid adapted and (b) Unadapted *Shigella sonnei* cells. The rectangles in (a) indicate proteins synthesized *de novo,* circles indicate over-expressed proteins while arrows indicate repressed proteins as compared to the unadapted cells

protein TolR has also been speculated in other studies. In *S. typhimurium,* the acid adapted cells were found to be capable of altering the outer membrane protein profile of the bacteria (Leyer and Johnson, 1993) while, studies on *E. coli* cells suggest that modification of the lipid bilayer in cell membrane helps to decrease the proton permeability thus protecting the cells from acid shock (Chang and Cronan, 1999). This is supported by Booth *et al.* (2002), who suggested that adaptive response to acid is responsible in changing the membrane permeability. Tol R is one of the proteins known to be responsible in maintaining the structural integrity of the outer membrane of bacteria cells (Lazzaroni *et al.,* 1999). It is postulated here that *Shigella* could employ a similar passive mechanism of modifying the cell membrane during exposure to mild acid thus decreasing the proton permeability, making it more resistant to extreme acidic conditions.

A second passive mechanism that could be employed is by increasing the buffering capacity towards excessive H^+ by cellular constituents (Hill *et al.,* 1995). Some bacteria may have specialized systems in the cell membrane which are activated upon adaptation to mild acidic conditions (Seputiene *et al.,* 2006). These include the induction of proteins which bind external protons more effectively or help H^+ ions to access the sites of binding inside the cell membrane or cytoplasm thus
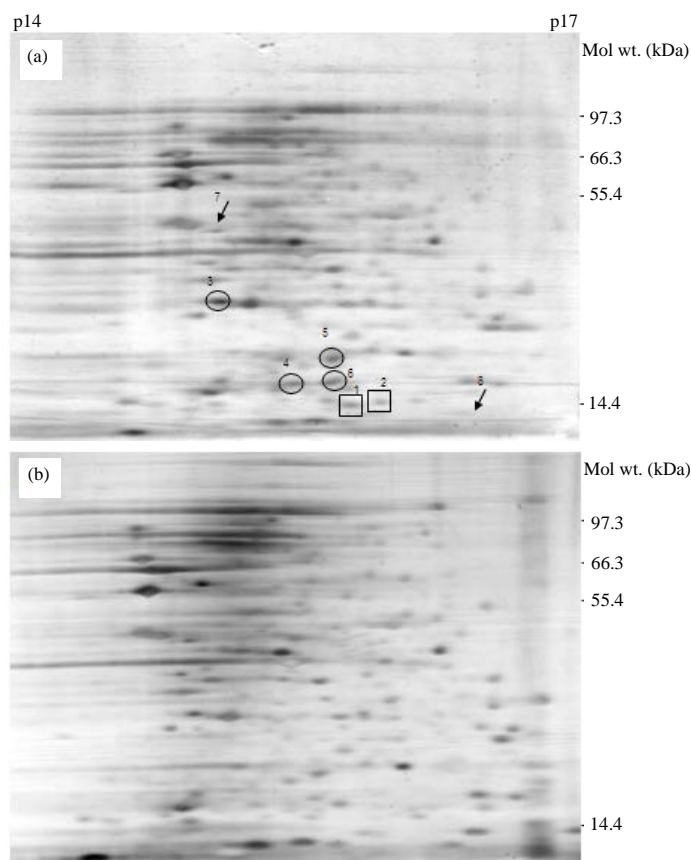
Fig. 4(a-b): The 2D gel of total protein extracts from (a) Acid adapted and (b) Unadapted *Shigella flexneri* cells. The rectangles in (a) indicate proteins synthesized *de novo,* circles indicate over-expressed proteins while arrows indicate repressed proteins as compared to the unadapted cells

contributing to the acid resistance. In the present study, the induction of AtpC, an ATP synthase protein in ATR could probably involved in transporting the excess H^+ in these species. In *L. monocytogenes*, the synthesis of sub-unit b of ATP synthase was also speculated to play a similar role in removing extra H^+ from the cytoplasm (Phan-Thanh *et al.*, 2000).

A possible role for exponential phase ATR in *Shigella* infections: *Shigella* is noted for their uniquely small infectious dose whereby only 10-500 cells are sufficient to cause infection which is attributed to the general stress response. The primary natural habitat for *Shigella* is the human gut. Once expelled from the human body, the bacteria is postulated to enter into stationary phase where it remains 'dormant' until transmitted to another suitable host possibly via contamination. Food contaminated with *Shigella* could be acidic and able to initiate growth under favorable conditions and enters the exponential phase. Hence, the involvement of the ATR mechanism that allows survival in acid could be a significant factor in their ability to cause infections. Clinically, *S. flexneri* causes a more severe diarrhea compared to *S. sonnei*. It is possible that a stronger ATR mechanism in *S. flexneri* contribute towards their virulence by allowing larger numbers of cells to survive in the intestinal environment. However, a direct link between ATR and virulence in this species remains to be established.

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