Original Article

Expression of *ompR* gene in the acid adaptation and thermal resistance of *Salmonella* Enteritidis SE86

Ana Carolina Ritter[1], Donatella Bacciu[2], Lucélia Santi[3], Salvatore Rubino[2,4], Sergio Uzzau[2], Eduardo César Tondo[3]

[1] *Instituto de Ciência e Tecnologia de Alimentos, Laboratório de Microbiologia e Controle de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil*
[2] *Dipartimento di Schienze Biomediche, Università Degli Studi di Sassari, Sassari, Sardegna, Italy*
[3] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil*
[4] *Department of Infection and Immunity, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia*

Abstract

Introduction: The objective of this study was to evaluate the involvement of the *ompR* gene in the acid adaptation and thermal resistance of *S.* Enteritidis SE86**,** responsible agent of more than 95 % of investigated food-borne diseases, throughout the last decade in Southern Brazil. In this study, we constructed a mutant strain of *S.* Enteritidis SE86 (Δ*ompR*) that was attenuated by a knockout technique. The OmpR protein expression was determined in a tagged (3XFLAG) strain of *S.* Enteritidis SE86.

Methodology: The mutant strains were cultivated separately in nutrient broth and nutrient broth supplemented with 1% glucose (NBG) to induce acid adapted cells. The organisms were exposed to different temperature such as 37 ºC, 52 ºC, and 60ºC. The survival of the SE86 wild type (WT) and attenuated strain was determined by bacterial count, and the tagged protein (*ompR*::3XFLAG cat::FLAG) was detected by SDS-PAGE and immunoblotting with anti-FLAG antibodies.

Results: Results showed that when exposed at 52ºC, the acid-adapted SE86 WT cells were completely inactivated after 300 minutes; however, non-adapted cells (WT and Δ*ompR*) and acid-adapted Δ*ompR* demonstrated higher thermal sensitivity, since they were completely inactivated in 240 minutes. At 60ºC, the acid-adapted SE86 Δ*ompR* also demonstrated higher sensitivity that SE86 WT, being totally inactivated after 15 minutes, while the WT cells were inactivated in 20 minutes.

Conclusion: The acid adapted cells showed increased expression of OmpR when exposed to 52 ºC and 60ºC, this confirmed the requirement of acid adaptation for *S.* Enteritidis SE86 to resist elevated temperatures.

Key words: *Salmonella* Enteritidis; *ompR*; thermal resistance; acid adaptation

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Introduction

Salmonella is one of the most common causes of human food poisoning worldwide. *Salmonella enterica* encompasses more than 2,600 serotypes [1]; of which *Salmonella* serovars Enteritidis Typhimurium are most commonly involved in salmonellosis [2].

Both outside of the host and during infection, *Salmonella* encounter numerous environmental stressors, which can trigger physiologic responses which may be potentially lethal to *Salmonella*, and the survival of these microorganisms depends on their ability to adapt or respond to these stressors [3]. Stress adaptation of *Salmonella* cells can be induced in the exponential and stationary phases. During both phases, an increased resistance to acid and thermal stress can occur [4]. In order to promote acid adaptation, *Salmonella* serotypes express two low-pH-inducible systems known as "Acid Tolerance Response" (ATR), which are classified based on the growth phase at which each becomes induced. Most studies have focused the log-phase ATR system to be induced when exponentially growing cells suddenly undergo a rapid transition to low pH [5]. More than 50 acid shock proteins (ASP) are produced during this response [6]. The regulatory gene *rpoS,* encoding an alternative sigma factor, is required for log-phase acid tolerance and for control of the production of clusters of ASPs [6, 3].

The second ATR system, related to the stationary-phase ATR, is induced by exposing stationary-phase cells to low pH [5,7]. It is distinct from the general stress response system that is induced by entry into the

stationary phase regardless of the culture pH. The general stress response system requires stationary-phase induction of the alternative sigma factor, while the acid-induced stationary-phase ATR does not [6,3]. Thus, OmpR is a stationary-phase ASP. RpoS, responsible for the stationary-phase induction of many proteins, was not required for this induction [5]. The importance of OmpR for the optimal functionality of the stationary-phase ATR in *Salmonella* is well known, but further studies are necessary to clarify which OmpR-regulated genes are involved in this adaptive response [6].

Our previous studies demonstrated that acid adaptation capacity of *S.* Enteritidis involved in salmonellosis have occurred in southern Brazil [8-11]. Even though different *Salmonella* serovars have been isolated from food in this country, it was observed that a specific *S.* Enteritidis strain (named SE86) was the human infection predominant *Salmonella* strain in the last decade in Rio Grande do Sul (RS) state, in southern Brazil [12]. During *in vitro* studies, SE86 has demonstrated an increased acid and thermal resistance after acid exposure [9,10]. In an *in vivo* study, the intestinal invasion ability of SE86 and *S.* Typhimurium (ST99) untreated or subjected to acid adaptation was compared. Results demonstrated that the acid-adapted SE86 strain showed higher cell counts in the ileum-caecal junction than the ST99 strain, suggesting that acid adaptation influenced the virulence of this microorganism [11]. Based on these data, the aim of this study was to evaluate the involvement of *ompR* gene in the acid adaptation and thermal resistance of *S*. Enteritidis SE86.

Methodology

Bacterial strains

The *S.* Enteritidis SE86 strain was isolated from cabbage involved in an outbreak of salmonellosis which occurred in RS State, Brazil, in 1999. This bacterium was characterised by phenotypic and genotypic methods by [13,14], and showed the same genotypic profile of *S*. Enteritidis strains involved in more than 95% of the investigated salmonellosis cases in RS state between 1999 and 2006 [15]. The SE86 strain was provided by the Laboratory of Food Microbiology of Food Science and Technology Institute - ICTA/UFRGS, Brazil. Before experiments, all strains were stored at -70ºC in lysogeny broth (LB) medium with the addition of 40% glycerol. The mutant of *S*. Enteritidis SE86 (Δ*ompR*) was constructed in the Laboratorio di Microbiologia of Università di Sassari, Italy, using the knockout method, described by Datsenko and Wanner [16]. The construct was verified by PCR analysis. The SSM 5337 (*ompR*::Kan) mutation was transferred into a clean *S*. Enteritidis SE86 background by P22 transduction.

Expression of the ompR gene

S. Enteritidis SE86 was tagged with the 8aa FLAG epitope tag peptide. Strain SSM5358 (*ompR*::3XFLAG cat::FLAG) of serovar *S.* Enteritidis was obtained using the method described by Uzzau *et al.* [17]. The 3XFLAG epitope is a sequence of three tandem FLAG epitopes (22aa). For the tagged mutant, a pair of primers was designed to amplify the 3XFLAG- and kanR-coding sequences by using plasmid pSUB11 [17]. The 3' ends of these oligonucleotides were complementary to the first 20nt of the pSUB11 3XFLAG coding region (GACTACAAAGACCATGACGG, forward primer) and to 20nt of the pSUB11 priming site 2 (CATATGAATATCCTCCTTAG, reverse primer). The 5' ends of the oligonucleotides were designed to be homologous to the last 40nt of each tagged gene, not including the stop codon (forward primer), and to the 40nt immediately downstream of the stop codon (reverse primer). The CAT protein was used as an internal marker because it is very stable. A constitutively-expressed epitope-tagged gene such as *cat* was used as a positive control or internal reference [17].

Acid adaptation

For acid adaptation, strains were cultivated in nutrient broth supplemented with 10g/L glucose (NBG) (Merck, Darmstadt, Germany). The cultures were maintained for 18 hours at 37°C in static condition according to Tetteh and Beuchat [18]. Each strain was also incubated in nutrient broth (NB) (Synth, São Paulo, Brazil) without glucose, at the same conditions, in order to produce non-adapted *Salmonella* cells [9]. After 18 hours of incubation, the pH of the cultures were measured using a pH meter (PHTEK).

Determination of thermal resistance

Aliquots of 1ml (approximately 8 log CFU ml^{-1}) of the acid-adapted and non-adapted cultures were transferred to Erlenmeyer flasks containing 99ml of pre-warmed NB kept in a water bath (Schott CT 52, Mainz, Germany). The flasks were incubated at temperatures of 37 ºC and 56ºC for 300 minutes and 60°C for 20 minutes. Aliquots of 1ml were taken and

serially diluted in 9ml of 1g l-1 peptone water (Vetec, Rio de Janeiro, Brazil). After vigorous vortexing, 20µl of appropriate dilutions were plated by the drop culture method on BHI agar (Merck, Darmstadt, Germany) and incubated for 24 hours at 37ºC. The quantification of survivors was carried out according to the method described by Silva [19]. The lowest detection limit of acid survivors was 1.69 log CFU ml^{-1}, and each experiment was carried out at least twice, with duplicate counts [9].

Western blotting of tagged gene

After the exposure to the specific temperatures described above, 1ml aliquots were taken and centrifuged (18000 *g*, 10 minutes, 4ºC). Protein extracts were then boiled for 5–10 minutes, and an aliquot of each sample was resolved by 12% SDS-PAGE for detection of the 3XFLAG-tagged proteins by western blotting. The nitrocellulose membranes were blocked with 5% (w/v) non-fat dried milk in PBS containing 0.05% Tween 20, washed and incubated with mouse anti-FLAG M2-peroxidase (HRP) mAbs (Sigma Aldrich, St. Louis, USA) that had been diluted 1:1000. The detection was carried out using H_2O_2 and $CoCl_2$.

Statistical analysis

All of the survival experiments were repeated at least twice, and all counting results were performed in duplicate. The Tukey's test was carried out in order to compare the differences between the mean values. The differences were considered significant with p values < 0.05.

Results

pH reduction during acid-adaptation

Salmonella grown in NBG was considered acid-adapted, while *Salmonella* cultivated in NB was considered non-adapted. Before inoculum, the initial pH values of NBG were 6.60 ± 0.02 and after *S.* Enteritidis SE86 growth, the final pH values presented mean values of 4.49 ± 0.02. The pH values of NB without glucose before and after inoculum were 6.80 ± 0.03.

Susceptibility to different temperatures of non-adapted and acid-adapted ΔompR S. Enteritidis SE86

After four hours of exposure at 37°C, acid-adapted SE86, with or without the *ompR* gene, demonstrated higher counts when compared with non-adapted SE86. The difference observed between counts was almost 2 log CFU (Figure 1) although no significant differences were observed ($p < 0.05$).

Figure 2 demonstrates the effect of acid adaptation on the survival of SE86 wild type (WT) and SE86 mutant *ompR* exposed to 52ºC for 300 minutes. Acid-adapted SE86 WT cells were completely inactivated after 300 minutes, however, non-adapted cells (WT and Δ*ompR*) and acid-adapted Δ*ompR* demonstrated higher thermal sensitivity (and a significant difference ($p < 0.05$)), since they were completely inactivated in 240 minutes.

At 60ºC, the acid-adapted SE86 Δ*ompR* and non-adapted cells also demonstrated higher thermal sensitivity than SE86 WT (Figure 3), both being totally inactivated after 15 minutes, while the WT cells were inactivated after 20 minutes.

Immunodetection of epitope-tagged OmpR protein of non-adapted and acid-adapted SE86 at different temperatures

In order to verify the expression of OmpR protein as a consequence of acid adaptation and thermal resistance, the *ompR* gene of *S.* Enteritidis SE86 was tagged and subjected to the experiments described above. The expression of the OmpR protein, in response to the exposure to 37ºC, 52ºC and 60ºC was confirmed by western blot (Figure 4, 5 and 6).

After exposure for 60, 120 and 180 minutes at 37ºC, acid-adapted and non-adapted cells expressed similar amounts of OmpR protein (Figure 4). However, a higher expression of the OmpR protein was observed in acid-adapted SE86 *ompR* tagging than non-adapted cells during the exposure at 52ºC and 60ºC (Figures 5 and 6).

Figure 1. Survival of nonadapted *Salmonella* Enteritidis SE86 Wild Type (●) and acid-adapted *Salmonella* Enteritidis SE86 Wild Type (■), nonadapted *Salmonella* Enteritidis SE86 lacking *ompR* (▼) and acid-adapted *Salmonella* Enteritidis SE86 lacking *ompR* (▲) exposed to 37º C in Nutrient Broth.

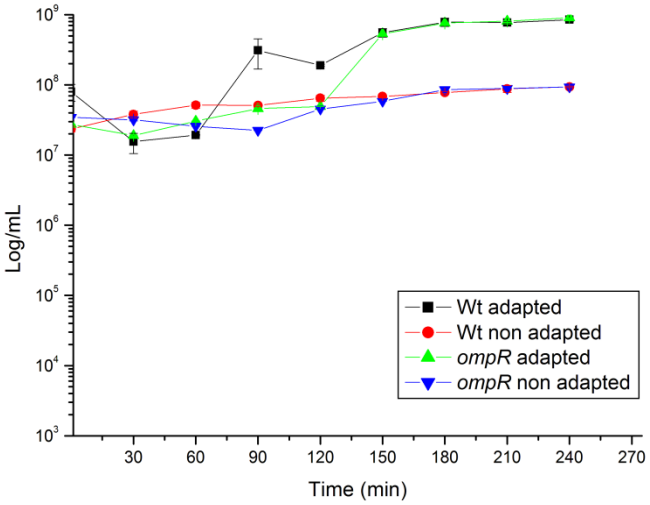


Figure 2. Survival of nonadapted *Salmonella* Enteritidis SE86 Wild Type (●) and acid-adapted *Salmonella* Enteritidis SE86 Wild Type (■), nonadapted *Salmonella* Enteritidis SE86 lacking *ompR* (▼) and acid-adapted *Salmonella* Enteritidis SE86 lacking *ompR* (▲) exposed to 52º C in Nutrient Broth.

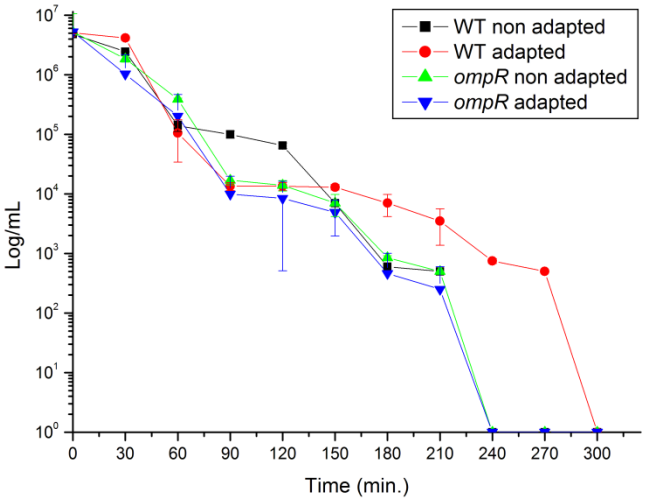


Figure 3. Survival of nonadapted *Salmonella* Enteritidis SE86 Wild Type (●) and acid-adapted *Salmonella* Enteritidis SE86 Wild Type (■), nonadapted *Salmonella* Enteritidis SE86 lacking *ompR* (▼) and acid-adapted *Salmonella* Enteritidis SE86 lacking *ompR* (▲) exposed to 60ºC in Nutrient Broth.

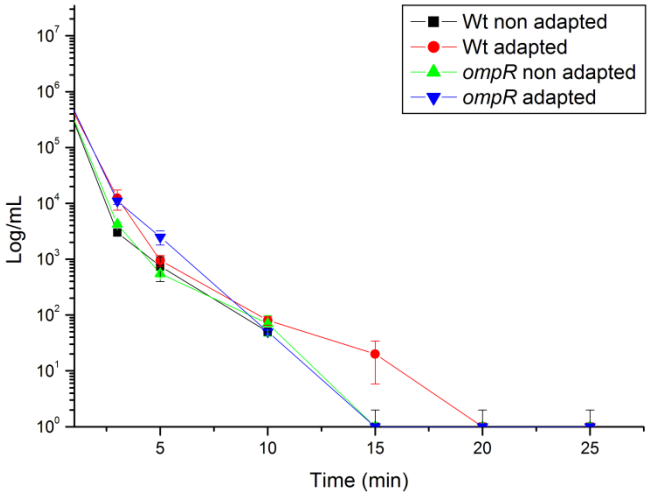


Figure 4. Western blot of *S.* Enteritidis SE86 3xFLAG-tagged OmpR protein and catalase Cat protein as control (OmpR::3xFLAG cat::3xFLAG) exposed to 37º C.

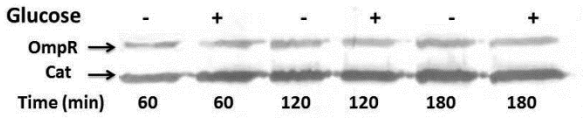


Figure 5. Western blot of *S.* Enteritidis SE86 3xFLAG-tagged OmpR protein and catalase Cat protein as control (OmpR::3xFLAG cat::3xFLAG) exposed to 52º C.

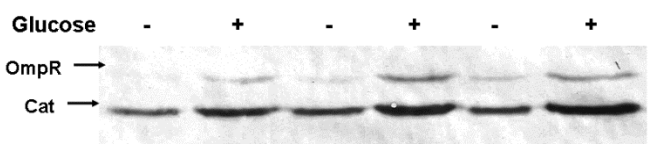
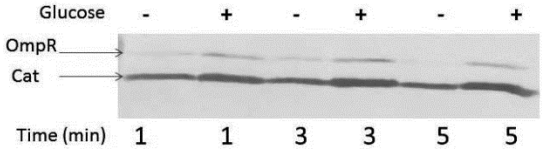


Figure 6. Western blot of *S.* Enteritidis SE86 3xFLAG-tagged OmpR protein and catalase Cat protein as control (OmpR::3xFLAG cat::3xFLAG) exposed to 60º C.



Discussion

Several virulence factors are involved in the adaptive response to environmental factors because pathogens are exposed to a variety of stressors. In this study we confirmed the involvement of the OmpR protein in the acid adaption of *Salmonella* Enteritidis SE86, a fact that has already been reported by other studies with different *Salmonella* serovars. As an example, Bang *et al*. [5] observed that acid adaptation induced the production of OmpR, which, in its phosphorylated state, could trigger the expression of diverse genes necessary for the acid-induced stationary-phase ATR of *S.* Typhimurium. The *ompR* gene is known to associate the acid adaptation with the increased acid shock resistance of *Salmonella* (specifically serovar Typhimurium) since several groups of acid shock proteins (ASPs) are induced during ATR in order to prevent or repair the macromolecular damage caused by acid stress [4]. An extensive research effort has been made in the last few decades to identify and characterise these stress proteins in *S.* Typhimurium, and several regulatory genes controlling the expression of different subsets of ASPs have been described, including the alternative sigma factor RpoS, the iron regulator Fur, the two-component signal transduction system PhoP/PhoQ and the OmpR response regulator [20]. Most of the identified ASPs are involved in cellular regulation, molecular chaperoning, energy metabolism, transcription, translation, the synthesis of fimbriae, regulation of cellular envelopes, colonisation, and virulence [21].

In the present study, we exposed acid-adapted and non-adapted mutants of SE86 (lacking *ompR*) to 52°C and 60°C, and also observed the expression of OmpR, demonstrating the involvement of this protein with thermal resistance. Interestingly, at 52 ºC and also at 60 °C, the acid-adapted mutants of SE86 demonstrated similar behaviour of non-adapted SE86 cells, which were completely inactivated after 240 and 15 minutes, respectively (Figure 1 and 2). The acid-adapted SE86 WT demonstrated the same behaviour as SE86 studied by Malheiros *et al.* [9], being inactivated after 300 minutes of exposure at 52ºC. Several studies have demonstrated that acid adaptation increases the thermal resistance of *Salmonella* [9,22,23]; however, most authors have attributed this behaviour to the heat-shock proteins (HSPs), responsible for the thermal shock resistance of *Salmonella*. Classical HSPs are molecular chaperones (DnaK, DnaJ, GroEL and GroES) or adenosine 5′-triphosphate-dependent proteases (ClpC, ClpP, ClpX and FtsH), and these proteins are usually controlled by the classic heat-shock sigma factor (σH) and an extracytoplasmic response regulated by the extracytoplasmic function (ECF) sigma factor (σE) [3-24-25-26] Interestingly, several σH- and σE-regulated genes encoding cytoplasmic and periplasmic chaperones and proteases appear to contribute to *Salmonella* virulence. The molecular mechanisms of σH and σE activation differ, but both mechanisms allow for a rapid response to the thermal stress [3].

The expression of the OmpR protein was observed in *ompR*-tagged SE86, which was exposed to the same conditions of acid adaptation and thermal resistance as the mutant SE86 lacking *ompR.* Furthermore, we could observe an increasing expression of OmpR in acid-adapted cells when compared to non-adapted cells (Figures 4 and 5). At 37°C there was no difference between treatments, which led us to believe that higher temperatures (such as 52ºC and 60ºC) are related to the increased OmpR expression. Two genes regulated by OmpR, *ompF* and *ompC*, have already been demonstrated to be related to high temperature exposure [27]. These authors evaluated the expression of *Serratia marcescens ompF* and *ompC* genes in the presence of different environmental factors, including osmotic pressure, temperature, pH and salicylate, and concluded that high temperature followed by pH were responsible for the predominant expressions of these genes.

In conclusion, the results of the present study show that the *ompR* may involved in the thermal resistance, after acid-adaptation, of SE86. However, further Two-Dimensional (2D) Gel Electrophoresis, proteomic studies and sequencing comparison are needed to confirm the involvement of other genes regulated by the OmpR protein with this behaviour.

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Corresponding author
Ana Carolina Ritter
Universidade Federal do Rio Grande do Sul
Instituto de Ciência e Tecnologia de Alimentos, Laboratório de Microbiologia e Controle de Alimentos
Porto Alegre, Rio Grande do Sul, Brasil.
Email: anacarolina.ritter@gmail.com

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