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Short communication

Genome analysis reveals insights into high-resistance and virulence of *Salmonella* Enteritidis involved in foodborne outbreaks

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ABSTRACT

Salmonella enterica serovar Enteritidis strain SE86 has been associated with several foodborne diseases occurring in Southern Brazil, becoming and important causative agent of human salmonellosis. In this work, the complete genome of the bacterium *Salmonella* Enteritidis SE86 was sequenced using the Illumina MiSeq platform. An *in silico* analysis of the SE86 genome was performed in order to compare it with different *Salmonella* strains as well as to investigate the presence of stress-resistance and virulence genes. This strain showed a variety of genes that can be involved in antimicrobial and biocide resistance, acid and thermal resistance as well as virulence and adhesion. These genetic features could explain its increased resistance and the prevalence of this strain in foodborne outbreaks in Southern Brazil.

1. Introduction

Salmonellosis is one of the most common food-borne diseases in the world, accounting for 93.8 million cases of foodborne illness and 155,000 deaths per year (Oh and Park, 2017). In the USA, this pathogen causes about 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths annually (CDC, 2019). In Brazil, during the period of 2000 to 2017, 12,503 food outbreaks were recorded, and *Salmonella* spp. was the main etiological agent identified in these outbreaks (Brasil, 2018).

The genus *Salmonella* belongs to the family *Enterobacteriaceae* and forms a complex group of bacteria, classified in two species (*S. enterica* and *S. bongori*), six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*) and over 2600 serotypes (Andino and Hanning, 2015). Among these serovars, *S.* Typhimurium and *S.* Enteritidis have been more frequently associated with human salmonellosis (WHO, 2019). The human cases of *Salmonella* Enteritidis increased in Europe during the period 2013–2017, and were normally associated with the consumption of contaminated egg products and poultry meat (EFSA, 2018).

In Southern Brazil, more specifically in the State of Rio Grande do Sul (RS) a specific strain named *S.* Enteritidis SE86 was responsible for > 95% of the investigated salmonellosis occurring from 1999 to 2013 (Geimba et al., 2004; Tondo and Ritter, 2012; Tondo et al., 2015). This strain was accurately studied, demonstrating a greater capacity for adaptation and survival compared to other strains or serotypes of *Salmonella*, especially regarding its resistance to acid, heat, sanitizers and antimicrobials (Capalonga et al., 2014; Ritter et al., 2014; Ritter et al., 2018). A clone relationship among *S.* Enteritidis responsible for foodborne outbreaks occurring in Southern Brazil, including SE86 and related strains, and *S.* Enteritidis found on poultry samples has been suggested. This clonal relationship was suspected, but not proved, for > 10 years, based on epidemiological results collected by the Brazilian Ministry of Health (Borges et al., 2017).

Even though these studies have contributed to explain the possible reasons why the strain SE86 was involved in several salmonellosis outbreaks in Southern Brazil, the whole genome sequencing of this strain was not performed to date. This information would be a valuable tool making possible to understand phenotypic characteristics and their possible origins in the SE86 genome. Thus, the main objective of this study was to explore the genome of the *Salmonella* Enteritidis SE86 and to relate its more relevant properties with previously studied phenotype characteristics.

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2. Materials and methods

2.1. Strain and growth conditions

The *S*. Enteritidis SE86 strain was isolated from a cabbage responsible for a salmonellosis outbreak occurring in RS State, Brazil, in 1999. This bacterium was characterized by phenotypic and genotypic methods and showed the same genotypic profile of *S*. Enteritidis strains involved in > 95% of the investigated salmonellosis of RS State during 1999 to 2013 (Tondo et al., 2015). The strain was cultivated in brain heat infusion (BHI; Oxoid, Basingstoke, UK) broth for 24 h at 37 °C.

2.2. Genome sequencing and annotation

S. Enteritidis SE86 total DNA was extracted with phenol-chloroform following usual procedures and purified using a Genomic DNA Clean & Concentrator (Zymo Research, Irvine, CA, USA). DNA fragment libraries were further prepared with 50 ng of DNA using a Nextera XT DNA sample preparation kit and sequenced using an Illumina® MiSeq System (2 × 250 paired-end reads with the Illumina v2 reagent kit) (Illumina, San Diego, CA, USA), followed by quality-based read trimming. The quality of generated sequences was evaluated using FastQC. The sequences with bases having a Phred quality score < 20 were trimmed with the aid of Geneious software (version 10.2.3). The paired-end sequence reads were assembled into contigs with SPAdes 3.9.0 (Bankevich et al., 2012), following confirmation by mapping reads to contigs generated by SPAdes using the Geneious software (version 10.2.3).

NCBI Prokaryotic Genome Annotation Pipeline (PGAAP) was employed to identify coding sequences (CDS) based on the best-placed reference protein set. Similarly, to aid the gene prediction and annotation, *S*. Enteritidis SE86 genome was analyzed by RAST (Rapid Annotation Subsystem Technology) webservice. Genes of interest had their annotation refined manually. The genome sequence is available at the database under de accession number NZ_CP019681.1.

2.3. Genome comparisons

Graphic genome comparisons were performed by using BRIG package (Alikhan et al., 2011), as well as the BLAST tool from the NCBI database, taking as reference the complete available genome from *Salmonella enterica* subsp. *enterica* serovar Enteritidis str. P125109 (GenBank accession AM933172.1) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis str. ATCC BAA-708 (GenBank accession CP025554.1). To calculate the Average Nucleotide Identity (ANI) between the genome of SE86, P125109 and ATCC BAA-708 strains, was used the JSpecies (Richter et al., 2016) software. Additionally, the genome of strain SE86 was subjected to the ResFinder 3.1 Server (Zankari et al., 2012) to check for mutated and acquired antimicrobial resistance genes.

3. Results and discussion

3.1. Genome comparisons and annotation

The SE86 genome consists of a chromosome of 4,685,718 bp, with an overall GC content of 52.2% and 4615 predicted coding sequences of which 132 correspond to pseudogenes, according to NCBI Prokaryotic Genome Annotation Pipeline. Comparatively, the reference isolate (P125109) is 130 bp longer than *S*. Enteritidis SE86 and 7332 bp longer than *S*. Enteritidis ATCCBAA-708. The properties of the genome are summarized in Table 1.

Average Nucleotide Identity (ANI) was calculated among SE86, P125109 and ATCC BAA-708. SE86 *vs* P125109 presented 99.81% of identity, and SE86 *vs* ATCC BAA-708 presented 99.08% of identity. The comparison of SE86 genome with the other genomes included in this work is shown in Fig. 1.

Table 1
General genome features of *S*. Enteritidis SE86, *S*. Enteritidis P125109 and *S*. Enteritidis ATCCBAA-708.

Feature	*S*. Enteritidis SE86	*S*. Enteritidis P125109	*S*. Enteritidis ATCC BAA-708
Size (bp)	4,685,718	4,685,848	4,678,516
Genes (total)	4615	4424	4852
Protein coding genes (CDS)	4615	4318	4518
Pseudogenes	132	133	211
rRNA (5S/16S/23S)	8/7/7	8/7/7	8/7/7
tRNA	83	84	86

Some important genes like *fliH*, *sapA* and *emrE* demonstrated difference between SE86, P125109 and ATCC BAA-708 genomes. These genes, especially *emrE* (Multidrug transporter EmrE) can be one of the most responsible for the resistance of *S*. Enteritidis SE86 in relation to other serovars as already mentioned in several studies as well as further discussed in this article.

The RAST webservice analysis provided a subsystem where the SE86 genes were grouped (Fig. S1). A total of 176 genes were associated with stress response, which contains oxidative stress and cold-response compounds, 107 genes for virulence, disease and defense (being that 74 for resistance to antibiotics and toxic compounds), 274 genes for cell wall and capsule. The relationships between genome findings and previous information about the phenotypical behavior of *S*. Enteritidis SE86 regarding stress response, virulence and defense will be discussed below.

3.2. Antimicrobial and biocide resistance

The presence of several genes associated with resistance to antimicrobials and biocides was found in the genome of SE86 strain (Tables 2 and S1). The resistance of *S*. Enteritidis SE86 to tetracycline and streptomycin was previously reported (Geimba et al., 2005; Oliveira et al., 2006) and could be explained by the dissemination of *tetR* and *aadA* genes. The *tetR* repressor controls the expression of tetracycline efflux pumps, which tightly regulates the *tetA* mRNA expression (Møller et al., 2016). The *aadA* gene encodes for an aminoglycoside adenylyltransferase that inactivates streptomycin and spectinomycin and is generally placed on gene cassettes localized into integrons (Stern et al., 2018). These resistance genes have been probably selected due to tetracycline and streptomycin utilization, as they are common antimicrobials used for veterinary purposes (Liljebjelke et al., 2017).

According to the ResFinder server, the SE86 genome contains chromosomal point mutations in *gyrA* gene (*gyrA* p.S83F mutation) promoting nalidixic acid and ciprofloxacin resistance. In addition, the server has detected acquired antimicrobial resistance gene to aminoglycoside. The investigation about antibiotic resistance of *S*. Enteritidis isolated from foods involved with salmonellosis in Southern Brazil revealed that the resistance to nalidixic acid and ampicillin increased in the period from 1999 to 2012 (Oliveira et al., 2006; Capalonga et al., 2014). SE86 genome contains the genes coding for tripartite efflux systems EmrAB-TolC and AcrAB-TolC, which confers resistance to antibiotics such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-(trifluoro-methoxy) phenylhydrazone (FCCP), 2,4-dinitrophenol and nalidixic acid. The resistance to quinolones has been also attributed to changes in membrane permeability causing a reduction in their intracellular concentration (Ballesté-Delpierre et al., 2014). Quinolones have been extensively employed in animal feed and are known to select for quinolone-resistant *Salmonella* in animals. At the same time, quinolones are one of the few existing therapies for severe *Salmonella* infections, particularly in adults (Tondo and Ritter, 2012). The expression of *acrAB* is controlled by *acrR*, the local repressor of *acrAB*, and the global regulators *marA* (*marRAB*), *soxS*

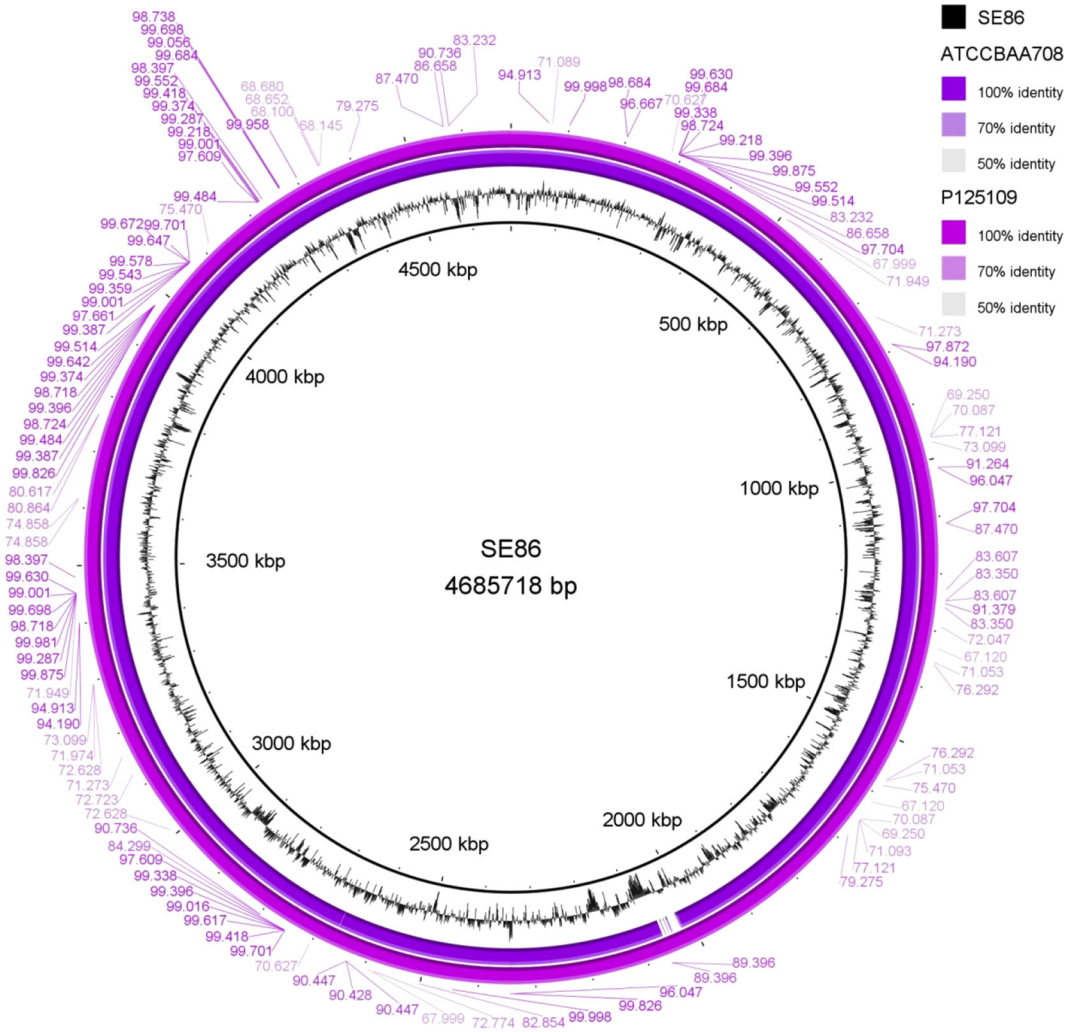

Fig. 1. Whole genome sequence analysis of *Salmonella* Enteritidis SE86 and comparison with other strains. Circular diagram of the SE86 genome showing from in to out the homology based on BLASTn analysis with strains BAA708 and P125109.

(*soxRS*) and *ramA*, all genes found in the genome of *S*. Enteritidis SE86.

Biocides are widely used as disinfectants to sanitize equipment, utensils and facilities surfaces in the food industry. However, the extensive exposure to biocides can cause bacterial adaptation, also increasing antibiotic resistance (Romero et al., 2017). Previous studies demonstrated that the SE86 strain was resistant to different sanitizers commonly used in food industry (Tondo et al., 2010), which might be associated the presence of several genes related to biocide resistance in SE86 genome (Table S1). The resistance of strain SE86 to sodium hypochlorite was compared with different *S*. Enteritidis strains isolated in Albania, Zimbabwe, Morocco and Pakistan. The strains were submitted to 200 mg/L sodium hypochlorite and SE86 demonstrated to be significantly more resistance than the other strains tested (Ritter et al., 2012).

The most frequently genes found in *Escherichia* sp. and *Enterobacter* sp. increasing resistant to disinfectants and antibiotics were *qacED1* and *qacA/B* (Márquez et al., 2017), belonging to the group of quaternary ammonium compounds (QACs). Two QAC resistance genes, namely

Table 2
Genes associated with resistance and virulence in the genome of *S*. Enteritidis SE86.

Feature	Genes (locus ID)[a,b]
Drug resistance	*emrAB* operon (B0663_14490, B0663_14495), *katG* (B0663_21270), *acrA-acrB-acrZ-tolC* (B0663_08260, B0663_08265, B0663_03900, B0663_17580), *marA-marB-marR* (B0663_08260, B0663_08265, B0663_02700), *floR* (B0663_23250), *tetR* (B0663_02910), *bcrC* (B0663_16630), *gidB* (B0663_09645), *ramA* (B0663_02915), *acrD* (B0663_02400), *mdfA* (B0663_17580), *qacE* (B0663_22360), *osmC* (B0663_08050), *acrEF/envCD* (B0663_17575), *soxS/ompX* (B0663_21985), *addA* (B0663_09645), *ybjC* (B0663_04390)
Thermal and acid resistance	*dnaK* (B0663_00055), *dnaJ* (B0663_00060), *grpE* (B0663_14150), *hscAB* (B0663_13700), *rpoS* (B0663_15060), *hns* (B0663_02385), *ompR* (B0663_16750), *nhaC* (B0663_08085), *phoPQ* (B0663_08140), *groL* (B0663_22315)
Adhesion	*fimH* (B0663_00115), *sefB* (B0663_23075), *lpfA* (B0663_18880), *fimA* (B0663_02750), *stdA* (B0663_15660), *bapA* (B0663_13575), *hin* (B0663_05670), *csgA* (B0663_10300), *shdA* (B0663_13545)
Virulence	*pipB* (B0663_05110), *ssap* (B0663_08795), *sifA* (B0663_09855), *sopA/sopD* (B0663_12920), *hilD* (B0663_14815), *fliB* (B0663_05675), *fliZ* (B0663_05685)

[a] Detailed functional description of gene/coded protein is provided as electronic Supplementary material.
[b] Locus ID according to NCBI accession number NZ_CP019681.1.

qacE and *mdfA*, were identified in SE86 genome. The *qacE* gene is located in the 3′-CS of class 1 integrons in Gram-negative bacteria, suggesting that exposure to QACs could also select for antibiotic resistance associated with such integrons (Zou et al., 2014; Romero et al., 2017). In addition, all biocide-tolerant *Escherichia* sp. and *Enterobacter* sp. isolates carried efflux pump genes *acrB* and *mdfA*, being that cells expressing MdfA are substantially more resistant to a diverse group of cationic or zwitterionic lipophilic compounds and to chemically unrelated, clinically important antibiotics (Márquez et al., 2017).

Sodium hypochlorite is recognized as a powerful oxidant and at high doses, can lead to DNA damage, oxidize cellular proteins and inhibit enzymes involved in glucose metabolism (Ritter et al., 2012). The genes of transcription factors *oxyR* and *soxR* present in SE86 genome (Table S1) are linked to bacterial responses to reactive chlorine species (RCS) (Gray et al., 2013). Genes regulated by *oxyR*, one of the best-characterized redox-sensitive transcription factors known to date, are strongly upregulated after HOCl treatment of *E. coli* and *S. enterica*. The *soxR* (*soxRS* operon), together with *marA* (also present in the SE86 genome) can activate *acrAB* expression, as previously discussed. Furthermore, the regulation of the SoxRS regulon is mediated by the conversion of the SoxR protein to an active form, which induces the transcription of *soxS*. The SoxS protein bind to the promoter region of many genes associated with response to oxidative damage (Gray et al., 2013; Sheplock et al., 2013).

3.3. Acid and thermal resistance

Salmonella needs to surpass some barriers such as low stomach pH, limited nutrient availability to survive and colonize the human body, and a high body temperature (42 °C) in the case of poultry birds (Dawoud et al., 2017). The acid and the thermal resistance of the strain SE86 were observed in phenotypical studies (Malheiros et al., 2008), and this resistance could be explained by the presence of several genes involved in the response to acid and thermal stress in the genome (Table 2).

The comparison of strain SE86 with *S*. Typhimurium and *S*. Bredeney strains (either acid-adapted or non-adapted) exposed to different pH and temperatures revealed that SE86 had a better capacity for acid adaptation and more thermal resistance than other strains tested (Malheiros et al., 2008). In another study, strains SE86 and *S*. Typhimurium ST99 (either acid-adapted or non-adapted) were exposed to simulated gastric fluid pH 1.5, and then inoculated in germ-free adult male Wistar rats, which were observed under aseptic conditions for twelve days. Acid-adapted SE86 had a significant higher survival rate than non-adapted SE86, non-adapted ST99 and acid-adapted ST99 in the simulated gastric fluid. The *in vivo* experiments showed higher counts of acid-adapted SE86 in the ileum-cecal junction as compared with the other treatments. Despite all strains were able to rapidly multiply in germ-free animals, more intense mortality was caused by acid-adapted SE86, suggesting that acid adaptation influenced the virulence of this microorganism (Perez et al., 2012).

After revealing this phenotypic behavior, the *ompR* gene was investigated in *S*. Enteritidis SE86, showing increased expression when the strain was exposed to 52 °C and 60 °C (Ritter et al., 2014). This result indicates that acid adaptation is required for *S*. Enteritidis SE86 to resist elevated temperatures. Another genes involved in acid and thermal resistance were found in SE86 genome, such as *dnaK*, *dnaJ*, *grpE*, *hscAB*, *rpoS*, *hns*, *nhaC*, *phoPQ*, *groL* and their functions are listed in the Table S2.

3.4. Virulence and adhesion

Salmonella has settled a number of mechanisms to modulate the production of virulence factors in response to natural environmental stresses. In order to establish a successful infection in the broad-host-range, *Salmonella* utilize a wide variety of virulence factors, encoded by a large number of genes distributed along its chromosome and in mobile genetic elements. The genome sequence of *S*. Enteritidis SE86 revealed the presence of many virulence genes (Tables 2 and S3) related with flagella, host colonization factors (fimbriae, adhesins/invasins), and biofilm-related proteins.

The high adhesion capability of SE86 on different surfaces has been described, including stainless steel (AISI 304 or 316), polyethylene and metal welds (Casarin et al., 2014; Tondo et al., 2015; Casarin et al., 2016). These phenotypic characteristics may be related with *fimH*, *lpfA*, *sefB*, *stdA*, *bapA* genes, present in SE86 genome and previously reported by other works about the formation of *Salmonella* biofilms (Jonas et al., 2007; Fàbrega and Vila, 2013; Berne et al., 2015).

Regarding the virulence of SE86, previous studies demonstrated that acid-adapted SE86 was capable to causing more intestinal morphological abnormalities and showed higher counts in the ileum-cecal junction of germ-free adult mice as compared with other strains (Perez et al., 2012). Besides that, the histopathological analyzes revealed greater severity of the infection caused by SE86. In addition, the ability of *Lactobacillus acidophilus* LA10 to exert antagonistic effects against SE86 in the gastrointestinal tract of conventional mice was investigated, but a significant number of SE86 cells were able to colonize the gastrointestinal tract of mice, specifically in the colon and ileum (Scapin et al., 2013). In the SE86 genome, the presence of *pipB*, *sifA*, *sopA*, *SopS*, *hild* and *fliC* genes might explain the abilities of this strain to colonize and succeed within the host. These and so many other genes are located within the so-called *Salmonella* pathogenicity islands (SPIs), horizontally acquired genomic segments known to contribute to *Salmonella* pathogenesis (Fàbrega and Vila, 2013).

4. Conclusion

The genome of *S*. Enteritidis SE86 presents a variety of genes that can be associated with antimicrobial and biocide resistance, acid and thermal resistance as well as virulence and adhesion. The analysis of this genome permitted to confirm the phenotypic characteristics that SE86 strain has demonstrated in previous studies over the last years. The data provided will help to understand why this pathogen continues to be the major cause of outbreaks in Brazil, in addition to helping us to understand the role of the resistances demonstrated by this peculiar strain in the occurrence of these outbreaks.

Declaration of Competing Interest

Authors declare no conflicts of interest regarding this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108269.

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