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Investigation of *rpoS* and *dps* Genes in Sodium Hypochlorite Resistance of *Salmonella* Enteritidis SE86 Isolated from Foodborne Illness Outbreaks in Southern Brazil

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ABSTRACT

In Rio Grande do Sul, southern Brazil, *Salmonella* Enteritidis is one of the principal microorganisms responsible for foodborne disease. The present study was conducted to compare the sodium hypochlorite resistance of *Salmonella* Enteritidis SE86 with that of other strains of *Salmonella* Enteritidis isolated from different regions of the world and to investigate the involvement of the *rpoS* and *dps* genes in resistance to this disinfectant. We tested five *Salmonella* Enteritidis wild-type (WT) strains isolated from different countries, two mutant strains of *Salmonella* Enteritidis SE86, and two tagged (3XFLAG) strains of *Salmonella* Enteritidis SE86 for their resistance to sodium hypochlorite (200 ppm). The survival of the WT and attenuated strains was determined based on bacterial counts, and tagged proteins (Dps and RpoS) were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with anti-FLAG antibodies. None of the WT strains of *Salmonella* Enteritidis were totally inactivated after 20 min. The SE86 strain lacking *dps* was more sensitive to sodium hypochlorite than was the WT SE86 strain, with a 2-log reduction in counts after 1 min. The RpoS and Dps proteins were actively expressed under the conditions tested, indicating that in *Salmonella* Enteritidis SE86 these genes, which are expressed when in contact with sodium hypochlorite, are related to oxidative stress.

In Brazil, 6,349 official notifications of foodborne outbreaks were recorded from 1999 to 2009, resulting in 123,917 ill people and 70 deaths, and 42.5% the outbreaks were caused by *Salmonella (24)*. Brazil is populated by approximately 190 million people distributed in 27 states. As in other parts of the world, only a small number of foodborne outbreaks are officially reported to regulatory agencies. The great majority (72%) of Brazilian foodborne illnesses cases were reported from only three states of southern Brazil: Rio Grande do Sul (RS; 30%), São Paulo (22%), and Paraná (12%) *(24)*.

Costalunga and Tondo *(9)* reported that salmonellosis accounted for 36% of foodborne disease outbreaks investigated in RS between 1997 and 1999, and Silveira and Tondo *(34)* stated that salmonellosis was at the top of the foodborne disease list of RS in 2000 and 2001. Geimba et al. *(15)* reported that more than 97% of the cases of salmonellosis that occurred in RS from 1999 to 2002 were caused by *Salmonella* Enteritidis. Further studies have revealed that the majority of foodborne outbreaks were caused by a specific strain: *Salmonella* Enteritidis SE86 *(27)*. This microorganism was able to grow faster *(21)* and to adapt better than other *Salmonella* serovars (*Salmonella* Typhimurium and *Salmonella* Bredeney) after exposure to sublethal pH and was more resistant to acid and high temperature *(20)*.

Humphrey *(17)* reported that *Salmonella* possesses a complex regulatory system, which mediates its responses to environmental stresses and can be activated under unfavorable conditions such as extremes in pH and temperature and low levels of oxygen. The sigma factors regulate the specificity of RNA polymerase and gene expression related to environmental stresses *(17)*. SigmaS factor, encoded by the gene *rpoS*, is maximally induced in the early stationary phase and controls the expression of more than 40 genes and operons. The activity of *rpoS* also can be induced in the exponential growth phase by lack of nutrients, heat, osmotic shock, and oxidative stress *(11)*. As part of the regulatory system of *rpoS*, the *dps* gene encodes for a nonspecific DNA binding protein whose expression is induced in the stationary growth phase *(3)*. The *dps* gene is responsible for protecting bacteria against oxidative damage both in vivo and in vitro *(22, 29)*. Dps can be added to the set of evolutionarily conserved antioxidant proteins used by *Salmonella* for resistance to different types of stresses.

Chlorine-releasing compounds have been widely used in the food industry and food services worldwide because of

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their broad spectrum efficacy against microorganisms and their affordability. In Brazil, chlorine is the most commonly used biocide in food industries and food services for disinfection of vegetables, fruits, equipment, and utensils, and 200 ppm is the concentration most widely used, as stipulated by Brazilian regulations *(1)*. Tondo et al. *(36)* found that sodium hypochlorite inactivated biofilms of various *Salmonella* serovars on polyethylene and stainless steel surfaces. These authors determined that *Salmonella* Enteritidis SE86 was more resistant than *Salmonella* Bredeney and *Salmonella* Typhimurium to 200, 400, and 800 ppm of sodium hypochlorite, which suggests that this characteristic of *Salmonella* Enteritidis SE86 could be related to its frequent identification as the cause of salmonellosis. Sodium hypochlorite and hydrogen peroxide inactivate microorganisms by cellular oxidation *(38)*. The present study was conducted to compare the sodium hypochlorite resistance of *Salmonella* Enteritidis SE86 with that of other strains of *Salmonella* Enteritidis isolated from other countries and to investigate the genetic basis of this resistance, which explain the frequent involvement of *Salmonella* Enteritidis SE86 in outbreaks.

MATERIALS AND METHODS

Bacterial strains. The five *Salmonella* Enteritidis strains investigated had been identified as responsible for foodborne outbreaks. Strain SSM2047 was isolated in Albania, strain SSM1813 was isolated in Pakistan, strain SSM4242 was isolated in Morocco, and strain SSM1644 was isolated in Zimbabwe. *Salmonella* Enteritidis SE86 was isolated from a cabbage responsible for an outbreak of salmonellosis in RS, Brazil, in 1999. This strain was characterized with phenotypic and genotypic methods by Geimba et al. *(15)* and Oliveira et al. *(27, 28)* and has the same genotypic profile as *Salmonella* Enteritidis strains involved in more than 95% of the investigated salmonellosis cases in RS from 1999 to 2006 *(28)*. Strains SSM2047, SSM1813, SSM4242, and SSM1644 are stored in the Laboratorio di Microbiologia from Università di Sassari (Sassari, Italy). Strain SE86 was provided by the Laboratório de Microbiologia e Controle de Alimentos of Instituto de Ciência e Tecnologia de Alimentos (Porto Alegre, RS, Brazil). Until used, all strains were stored at −70°C in Luria-Bertani medium with 40% glycerol. *Listeria monocytogenes* ATCC 7641 was used as a negative control for the sodium hypochlorite resistance experiments.

Mutants of *Salmonella* Enteritidis SE86 (Δ*dps* and Δ*rpoS*) were constructed in the Laboratorio di Microbiologia (Università degli Studi di Sassari, Sassari, Italy) using the method described by Datsenko and Wanner *(10)*. The construct was verified by PCR analysis. The SSM5327 (*dps*::Kan) and SSM5333 (*rpoS*::Kan) mutations were transferred into a clean *Salmonella* Enteritidis SE86 background by P22 transduction.

Expression of the *dps* and *rpoS* genes. *Salmonella* Enteritidis SE86 was tagged with the eight amino acid FLAG epitope tag peptide. Strains SSM5348 (*rpoS*::3XFLAG *cat*::FLAG) and SSM5350 (*dps*::3XFLAG *cat*::FLAG) of serovar *Salmonella* Enteritidis were obtained using the method described by Uzzau et al. *(37)*. The 3XFLAG epitope is a sequence of three tandem FLAG epitopes (22 amino acids). For each tagged mutant, a pair of primers was designed to amplify a 3XFLAG and kanR coding sequence by using plasmid pSUB11 *(37)*. The 3′ ends of these oligonucleotides were complementary to the first 20 nucleotides of the pSUB11 3XFLAG coding region (GACTACAAAGACCATGACGG, forward primers) and to the 20 nucleotides of the pSUB11 priming site 2 (CATATGAATATCCTCCTTAG, reverse primers). The 5′ ends of the oligonucleotides were designed to be homologous to the last 40 nucleotides of each tagged gene, not including the stop codon (forward primers), and to the 40 nucleotides immediately downstream of the gene stop codon (reverse primers). 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 *(37)*.

Evaluation of the resistance to sodium hypochlorite. Four *Salmonella* Enteritidis strains (SSM2047, SSM1813, SSM4242, and SSM1644) were incubated separately in brain heart infusion (BHI; Sigma, St. Louis, MO) at 37°C for about 18 h. After incubation, the cultures were diluted in 0.1% peptone water (pH 7.2) to approximately 10^6 CFU/ml, and 0.1 ml of each suspension was inoculated into 9 ml of 200 ppm of sodium hypochlorite solution supplemented with 0.9 ml of 1% bovine serum albumin *(23)*. After 1, 3, 5, 10, 15, and 20 min at room temperature, 1 ml of each bacterial suspension was serially diluted in tubes containing 9 ml of 0.1% peptone water supplemented with 0.1% sodium thiosulfate. After vigorous vortexing, survival was quantified by plating 20 μl of each appropriate dilution on BHI agar and incubating for 24 h at 37°C. Quantification of survivors was performed according to the technique described by Silva et al. *(33)*. The lower detection limit was 1.69 log CFU/ml, and each experiment was conducted twice, with duplicate counts. *L. monocytogenes* ATCC 7641 was used as a negative control in this experiment. The concentration of free chlorine used in experiments was determined with a chlorine test kit (CHEMetrices, Inc., Calverton, VA).

Western blotting of tagged genes. After exposure to sodium hypochlorite, 1-ml aliquots of the cultures were centrifuged (18,000 × *g*, 10 min, 4°C). Protein extracts were boiled for 5 to 10 min, and an aliquot of each sample was resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for detection of 3XFLAG-tagged proteins by Western blotting. The nitrocellulose membranes were blocked with 5% (wt/vol) nonfat dried milk in phosphate-buffered saline (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween 20, washed, and incubated with mouse anti-FLAG M2-peroxidase (HRP) mAbs (Sigma) diluted 1:1,000. The detection was performed using H_2O_2 and $CoCl_2$. Blots were scanned, and the density of the signals was analyzed with the National Institutes of Health public domain software Image J (http://rsb.info.nih.gov/nih-image/). All relative density values corresponded to arbitrary values from Image J software analysis of corresponding bands from Western blots and were normalized by reference to immunodetection of the control protein catalase. Western blots were evaluated in biological and technical duplicates.

Statistical analysis. All experiments performed to evaluate the survival of microorganisms were repeated at least twice, and all counts were conducted in duplicate. The Tukey test was used to compare the differences between the mean values. The differences were considered significant when P values were less than 0.05.

RESULTS AND DISCUSSION

None of the strains of *Salmonella* Enteritidis were totally inactivated after 20 min of exposure to 200 ppm of sodium hypochlorite (Fig. 1). In general, initial cell counts

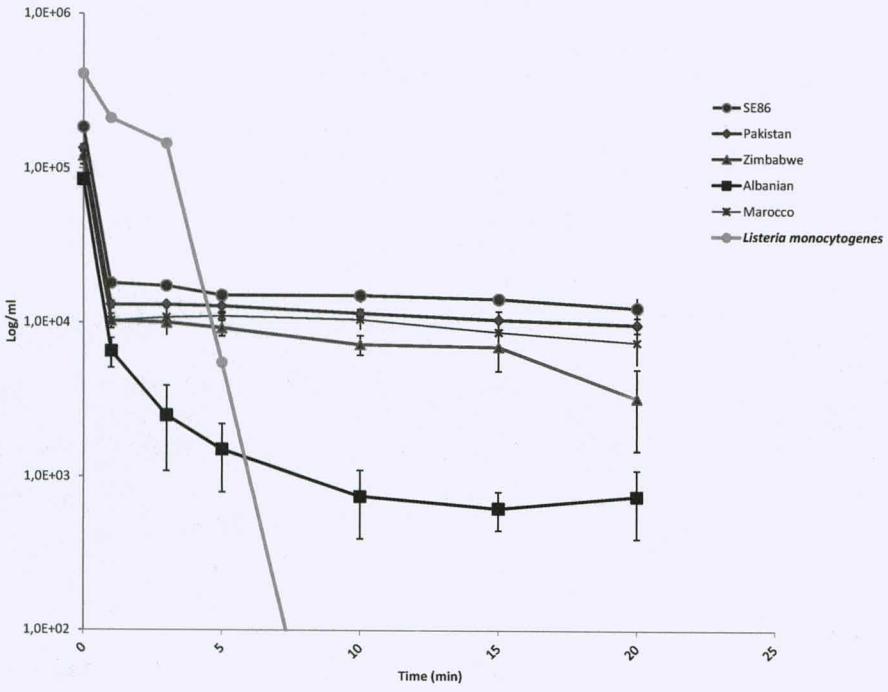

FIGURE 1. *Survival after exposure to 200 ppm of sodium hypochlorite of* Salmonella *Enteritidis strains that have caused food-associated outbreaks in various countries.*

of approximately 10^5 CFU were reduced by 1 log CFU after 1 min of exposure to the sanitizer. However, counts of viable cells remained almost unaltered during the exposure. The strain from Albania (SSM2047) was most sensitive; the viable cell counts were reduced by almost 2.5 log CFU after 20 min. Greater inactivation was reported by Riazi and Matthews *(31),* who found >3-log reductions of *Salmonella* Enteritidis after exposure to 256 ppm of sodium hypochlorite for 20 min.

Various characteristics, e.g., growth rate, thermal and acid resistance *(26),* and resistance to disinfection methods, may contribute to the emergence of a microorganism as a food pathogen. Malheiros et al. *(21)* found that *Salmonella* Enteritidis SE86 multiplied faster than *Salmonella* Typhimurium and *Salmonella* Bredeney in the first 6 h of incubation in homemade mayonnaise. This food was the main vehicle responsible for salmonellosis in RS from 1997 to 2001 *(9, 34).* Malheiros et al. *(20)* also found that *Salmonella* Enteritidis SE86 had a greater capacity for acid adaptation and thermal resistance than did other *Salmonella* serovars after exposure to sublethal pH. Tondo et al. *(36)* found that *Salmonella* Enteritidis SE86 was more resistant to sodium hypochlorite than were other *Salmonella* serovars; however, in our study resistance of *Salmonella* Enteritidis SE86 to sodium hypochlorite was similar to that of other *Salmonella* Enteritidis serovars from other countries. Several researchers have reported very low genetic diversity among *Salmonella* Enteritidis strains *(8, 19).* Pang et al. *(30)* reported that a clone of *Salmonella* Enteritidis has spread worldwide, causing salmonellosis in various countries. This fact could explain the similar resistance to sodium hypochlorite verified in the present study.

Susceptibility of *Salmonella* Enteritidis SE86 mutants to sodium hypochlorite. The effect of sodium hypochlorite on the survival of wild-type (WT) *Salmonella* Enteritidis SE86 and *Salmonella* Enteritidis SE86 mutants is shown in Figure 2. The *Salmonella* Enteritidis SE86 mutant lacking *rpoS* had behavior similar to that of WT SE86, with counts of 10^5 CFU after 20 min of exposure to the sanitizer. These results are in contrast to those of Dukan and Touati *(13),* who investigated the behavior of an *rpoS*-deficient *Escherichia coli* mutant. This strain was much more sensitive to hypochlorous acid. However, unlike Dukan and Touati, we tested our strains against sodium hypochlorite rather than hypochlorous acid. In aqueous environments, a balance is maintained between these two forms (collectively referred to as free chlorine), deionized hypochlorous acid (HOCl) and hypochlorite ions (ClO_2). The ratio of these forms depends on pH and temperature. HOCl is the more reactive of the two forms *(13).*

As expected, *Salmonella* Enteritidis SE86 lacking *dps* was significantly more sensitive to sodium hypochlorite ($P < 0.05$) than was the WT SE86, with a 2-log reduction after 1 min of exposure and a 3-log reduction after 20 min of exposure. In several studies with *E. coli* strains lacking *dps,* the authors concluded that when this gene is not present, the cell suffers more oxidative damage, suggesting that this gene is involved in protecting DNA against this type of damage *(2, 3, 5, 7, 13, 25).*

The Dps protein in *Salmonella* Typhimurium has up to 95% homology with the Dps protein in other enteric gram-negative bacteria, including *E. coli.* The nucleotide sequence of the promoter region of *Salmonella dps* shares 95% identity with the promoter region of *E. coli dps,* suggesting that the *Salmonella dps* promoter may behave similarly to the *E. coli dps* promoter *(39).* Altuvia et al. *(3)* found that bacterial strains lacking *dps* have impaired oxidative stress responses and are unable to survive when there is insufficient food for long periods.

As a control, we exposed the mutant strains to peptone water without sodium hypochlorite (results not shown),

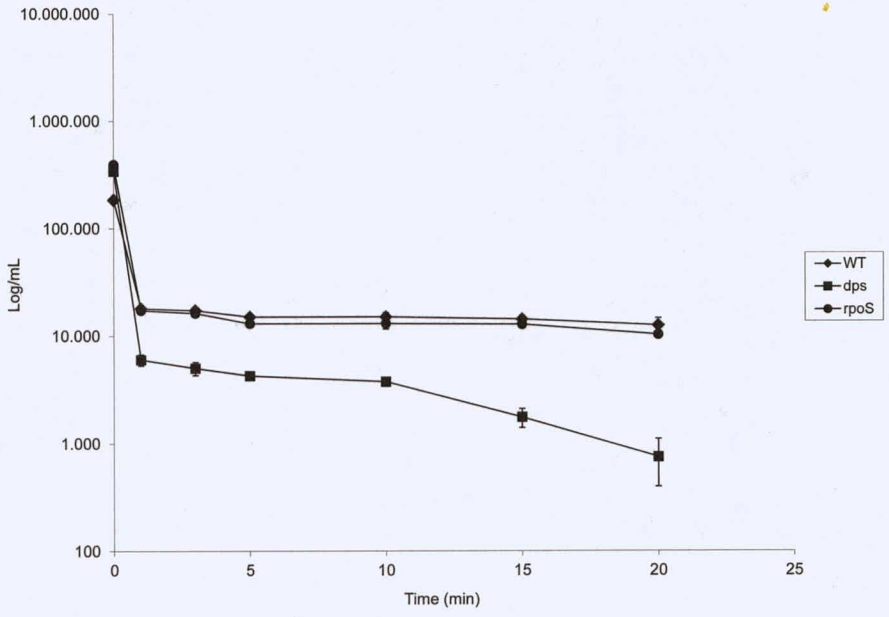

FIGURE 2. *Survival of* Salmonella *Enteritidis SE86 mutants exposed to 200 ppm of sodium hypochlorite.*

using the same times and conditions, to determine the influence of these genes on the sensitivity to sodium hypochlorite. The sensitivity of the mutant strains was similar to that of the WT strain, suggesting that the *rpoS* and *dps* genes are involved in sodium hypochlorite resistance.

Immunodetection of epitope-tagged proteins. Chlorinated compounds have a wide spectrum of activity, affecting the cell membrane, inhibiting enzymes involved in the metabolism of glucose, causing damage to DNA, and oxidizing cellular proteins *(32)*. The phenotypic characteristics of a pathogen are determined by their genetic makeup, and determinants of virulence may be present in the chromosome, usually encoded in pathogenicity islands, plasmids, and bacteriophages *(14)*. Audia et al. *(6)* and Dong et al. *(12)* found that the expression of the *rpoS* gene is related to the exposure of food pathogens to sublethal stress factors, such as temperature, acid, and oxidation.

To investigate the involvement of tagged mutants in resistance to disinfectants, bacterial strains were exposed to sodium hypochlorite for various periods. The expression of the RpoS protein in response to exposure to 200 ppm of sodium hypochlorite was confirmed by Western blot (Fig. 3). The expression of RpoS remained virtually constant for the first 10 min of exposure, briefly increased after 15 min, and then decreased after 20 min. These results indicate that the *rpoS* gene is induced during exposure to sodium hypochlorite and probably coordinated the expression of other genes involved in stress responses.

In several studies, high oxidative stress resistance was entirely mediated by SigmaS *(7, 13, 35)*. Induction of numerous stress resistance genes in the stationary phase depends on the Sigma factor, which is encoded by *rpoS*. The *rpoS* gene regulates the expression of DNA repair enzymes, such as the exonuclease xthA, the methyl transferase ada, and the nonspecific DNA binding protein Dps *(18)*. Dps was actively expressed under the conditions tested in our experiments (Fig. 4). Dps also had greater expression than rpoS in bacteria exposed to the same conditions, especially in the first minutes of exposure. Dps is a cytoplasmic protein that protects cellular DNA against damage caused by oxidative stress due to exposure to hydrogen peroxide, HOCl, and acid *(13, 29, 39)*. Young et al. *(39)* found that when *Salmonella* was exposed to multiple stresses, including oxidative stress, a DNA binding protein in the stationary phase (Dps) was one of the main overexpressed proteins. The positive control used in the

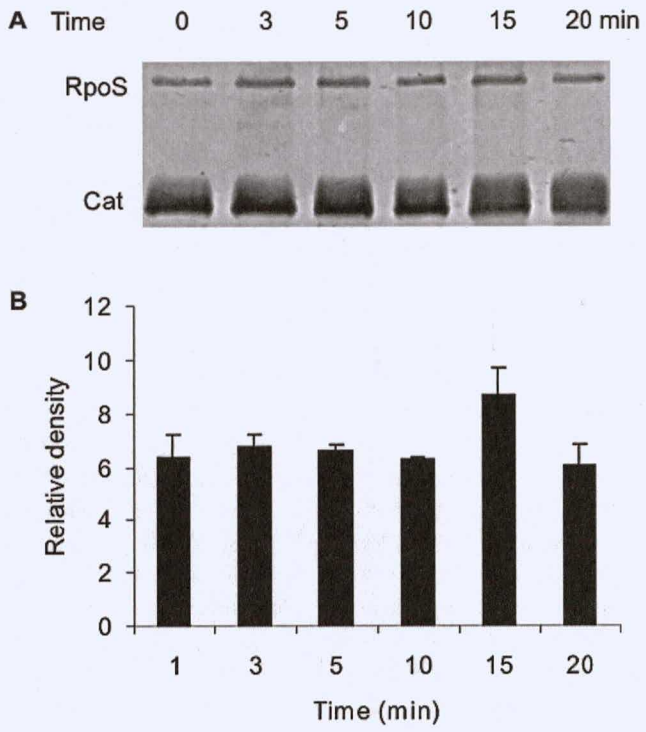

FIGURE 3. *Immunodetection of 3XFLAG-tagged proteins in* Salmonella *Enteritidis SE86 (*rpoS*::3XFLAG* cat*::3XFLAG). (A) Western blot of 3XFLAG-tagged proteins, RpoS and catalase (Cat), as the control. (B) Densitometric analysis of bands from immunodetection of RpoS protein (all values of relative density correspond to arbitrary values from Image J software analysis normalized in reference to immunodetection of control protein Cat).*

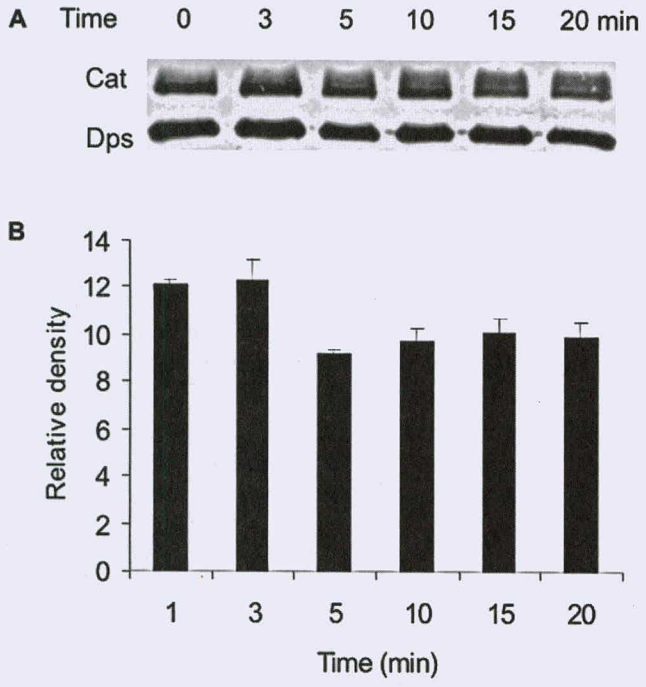

FIGURE 4. *Immunodetection of 3XFLAG-tagged proteins in* Salmonella *Enteritidis SE86 (*dps*::3XFLAG* cat*::3XFLAG). (A) Western blot of 3XFLAG-tagged proteins Dps and catalase (Cat), as the control. (B) Densitometric analysis of bands from immunodetection of Dps protein (all values of relative density correspond to arbitrary values from Image J software analysis normalized in reference to immunodetection of control protein Cat).*

present study, the *cat* gene, was detected at all times and under all conditions analyzed, as described by others *(16, 37)*. As a negative control, mutant strains were exposed to peptone water without sodium hypochlorite (Fig. 5) to evaluate the difference in protein expression.

To our knowledge, the present study is the first in which sodium hypochlorite resistance has been compared among *Salmonella* Enteritidis strains involved in foodborne outbreaks in different parts of the world. This study is also the first to include investigation of the involvement of *Salmonella* Enteritidis *dps* and *rpoS* in the resistance to sodium hypochlorite. The *Salmonella* Enteritidis strains from different countries had similar responses to sodium hypochlorite. Focusing on *Salmonella* Enteritidis strain SE86 isolated in southern Brazil, we found that the *rpoS* and *dps* genes were important for survival after exposure to sodium hypochlorite, indicating the involvement of these genes in responses to oxidative stress. These results suggest that *rpoS* and *dps* may be important factors in the frequent involvement of *Salmonella* Enteritidis SE86 in salmonellosis outbreaks in southern Brazil.

The *Salmonella* Enteritidis SE86 mutant without *dps* survived less well than did the WT SE86 and the SE86 mutant without *rpoS*, suggesting that *dps* is more involved in the oxidative stress response than is *rpoS*. Other researchers have found that *rpoS* in *Salmonella* Enteritidis is involved in acid resistance *(4)*. More studies are necessary to understand the role of these genes in the behavior of *Salmonella* Enteritidis SE86.

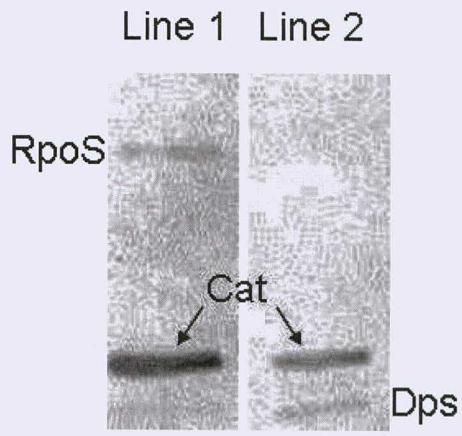

FIGURE 5. *Immunodetection of 3XFLAG-tagged proteins. Line 1,* Salmonella *Enteritidis SE86 (*rpoS*::3XFLAG* cat*::3XFLAG) exposed to peptone water for 10 min; line 2,* Salmonella *Enteritidis SE86 (*dps*::3XFLAG* cat*::3XFLAG) exposed to peptone water for 10 min.*

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