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The Ferritin-Like Dps Protein Is Required for *Salmonella enterica* Serovar Typhimurium Oxidative Stress Resistance and Virulence

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Resistance to phagocyte-derived reactive oxygen species is essential for *Salmonella enterica* serovar Typhimurium pathogenesis. *Salmonella* can enhance its resistance to oxidants through the induction of specific genetic pathways controlled by SoxRS, OxyR, σ^S, σ^E, SlyA, and RecA. These regulons can be found in a wide variety of pathogenic and environmental bacteria, suggesting that evolutionarily conserved mechanisms defend against oxidative stress both endogenously generated by aerobic respiration and exogenously produced by host phagocytic cells. Dps, a ferritin-like protein found in many eubacterial and archaebacterial species, appears to protect cells from oxidative stress by sequestering iron and limiting Fenton-catalyzed oxyradical formation. In *Escherichia coli* and some other bacterial species, Dps has been shown to accumulate during stationary phase in a σ^S-dependent fashion, bind nonspecifically to DNA, and form a crystalline structure that compacts and protects chromatin from oxidative damage. In the present study, we provide evidence that Dps protects *Salmonella* from iron-dependent killing by hydrogen peroxide, promotes *Salmonella* survival in murine macrophages, and enhances *Salmonella* virulence. Reduced numbers of *dps* mutant bacteria in the livers and spleens of infected mice are consistent with a role of Dps in protecting *Salmonella* from oxidative stress encountered during infection.

Complex mechanisms have evolved to allow bacteria to withstand the oxidative stress associated with aerobic life (13). Many regulatory and enzymatic loci required for resistance to reactive oxygen species generated during aerobic metabolism have been found to play an important role in the ability of pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium to cause disease (5, 8, 12, 15, 20–22, 25, 26), suggesting that conserved strategies to resist oxidative stress in the environment can also allow organisms to withstand the respiratory burst of phagocytic cells.

Elegant studies of *Escherichia coli* by Kolter and colleagues first identified a low-molecular-weight protein that accumulates during stationary phase and binds to DNA; the protein was designated Dps (DNA binding protein in stationary phase) (2). Expression of *dps* in *E. coli* has been shown to be regulated by the stationary-phase sigma factor RpoS (σ^{38}), OxyR, and IHF (3). As *E. coli* enters stationary phase, more than 180,000 Dps molecules accumulate within a single organism, making Dps the most abundant protein in the cell (1). Dps-deficient mutant *E. coli* strains are unable to survive long-term starvation (3, 17) and exhibit enhanced susceptibility to oxidative stress (25). *E. coli* Dps is able to form a microcrystalline structure on chromatin in intact stationary-phase cells or in association with purified DNA in vitro (29), leading to the suggestion that Dps physically protects DNA by sequestration. However, the crystal structure of Dps has revealed similarity to the iron storage protein ferritin (18, 30), and Zhao et al. (30) have found that Dps prevents DNA damage in *E. coli* through its capacity to bind Fe(II) and prevent the formation of hydroxyl radicals. Recent studies in *Agrobacterium tumefaciens* and *Mycobacterium smegmatis* indicate that Dps can prevent oxidative DNA damage even in the absence of DNA binding (10, 19).

The contribution of Dps to *S. enterica* pathogenesis has not been investigated previously, although *dps* expression appears to be induced following *Salmonella* ingestion by macrophages (14, 27). In this study, we report the contribution of *dps* to *Salmonella* oxidative stress resistance, survival in macrophages, and virulence in mice.

The ability of *Salmonella* to survive and replicate within host phagocytes is absolutely essential for *Salmonella* virulence (16). Phagocyte-derived reactive oxygen species generated by the NADPH phagocyte oxidase play an important role in innate immunity to *Salmonella* (24, 28), and a number of *Salmonella* mutant strains with enhanced susceptibility to oxidative stress have a reduced capacity to survive in macrophages (9, 12, 21, 26). To determine whether *dps* is required for oxidative stress resistance, a *dps*::*aph* mutation was constructed in *S. enterica* serovar Typhimurium ATCC 14028s using the method of Datsenko and Wanner, in which the *dps* open reading frame was replaced with the *aph* cassette (11). Oligonucleotide primers 5′-TTAATTACCTGGGACACAAACATCAAGAGGATATGAGATTGTGTAGGCTGGAGCCTTC and 5′-TACCTTCCTGCAACTCGAAGTATTCAGGGTAGAGATAGATATTCCGGGGATCCGTCGACC were utilized to create the *dps*::*aph* disruption, and primers 5′-CGGTGCTATACTTATTTTCG and 5′-CTGCGGATTCGCTGCGTTTG were used to confirm the expected insertion mutation. In addition, oligonu-

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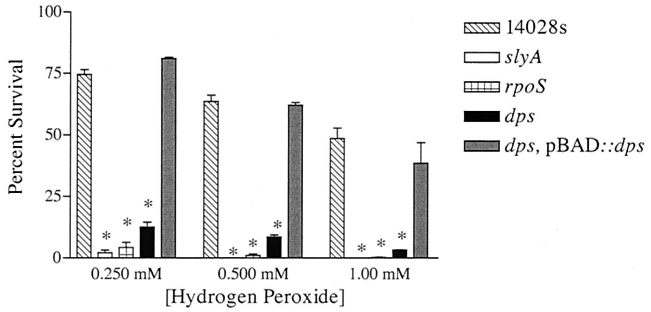

FIG. 1. *dps* mutant serovar Typhimurium has enhanced susceptibility to hydrogen peroxide. Results are expressed as percent survival after 2-h exposure to 0.250, 0.500, or 1.0 mM hydrogen peroxide at 37°C and represent the average percent survival from three independent assays. Error bars represent the standard deviations of three independent experiments. *, $P < 0.01$ by Student's *t* test, compared to wild type.

cleotide primers 5′-GCCAAAACTGAAGCTACAGGTGC CAAGTGCGCACTATGTCAGGAAACAGCTATGACCA TG and 5′-GAATGACCTCTTCCATCTTCCATCTCAGC GATCAGCGCGTCCGCTTTTACAACCAATAACCAAT TC were used to create a *slyA*::*aph* disruption using the method of Datsenko and Wanner (11), and primers 5′-GCTTTAG TTTTAGCCAAAACTG and 5′-ACCGTCTCTCCACGCT AAAC were used to confirm the mutation. The absence of Dps protein in the *dps*::*aph* mutant strain was also confirmed by Western blot analysis using Dps antiserum (provided by R. Kolter) (data not shown). Wild-type *S. enterica* serovar Typhimurium, the isogenic *dps*::*aph* mutant derivative (SL3474), the *dps*::*aph* mutant complemented with plasmid pBAD::Dps (SL3476), an isogenic *rpoS*::pRR10 (Δ*trfA*) mutant (SF1005) (15), and an isogenic *slyA*::*aph* mutant (SL3343) were compared for their susceptibilities to various concentrations of hydrogen peroxide. For Dps complementation using pBAD::*dps*, L-arabinose was added to a final concentration of 0.2%. Bacterial killing by hydrogen peroxide was measured in liquid medium as described by Buchmeier and Libby (7). Briefly, overnight cultures were grown in Luria-Bertani (LB) medium, diluted to 10^6 CFU in phosphate-buffered saline (PBS), and incubated at 37°C with a final concentration of 0.250, 0.500, or 1 mM hydrogen peroxide. Aliquots were removed after 2 h, and the number of viable cells was determined by serial dilution and plating onto LB agar. Percent survival following hydrogen peroxide challenge was calculated for each strain by dividing the number of CFU obtained from incubation in PBS alone by the number of CFU obtained from incubation in hydrogen peroxide. Each assay was repeated at least three times, and standard deviations were calculated and plotted. *Salmonella* carrying a *dps* mutation was found to be 15-fold less viable than wild type at 1 mM H_2O_2 and only slightly more resistant than a *slyA* mutant strain (Fig. 1). The hydrogen peroxide sensitivity phenotype was complemented to near-wild-type levels by providing *dps* on a plasmid in *trans*. Thus, the role of *dps* in the ability of *S. enterica* serovar Typhimurium to withstand killing by hydrogen peroxide is comparable to observations in *E. coli* (23).

As discussed above, the antioxidant actions of Dps have been linked to its ability to sequester iron and prevent Fe(II)-dependent oxyradical formation. We therefore examined the ability of the Fe(II) chelator 2′,2-dipyridyl to inhibit killing of *dps* mutant *Salmonella* by hydrogen peroxide. Twenty-minute preincubation in 1 mM 2′,2-dipyridyl completely rescued the *dps* mutant strain from killing by 2 mM H_2O_2, but it provided only partial protection for *rpoS* or *slyA* mutant strains (Fig. 2).

The intracellular survival of wild-type and mutant *Salmonella* strains was determined in C3H/HeN (*ity*^{r}) primary peritoneal macrophages essentially as described previously (6, 28). Sodium periodate-elicited peritoneal macrophages were harvested from mice and plated at a density of 4×10^5 to 6×10^5 cells/well. Macrophages were infected 24 h later at a multiplicity of infection of 5:1 (bacteria/macrophage) with wild-type, *dps* mutant, or *slyA* mutant serovar Typhimurium cells that were opsonized with normal mouse serum. Extracellular bacteria were killed by the addition of gentamicin (50 μg/ml) to the medium. Macrophages were lysed at specific time points with 0.5% deoxycholate, serially diluted in PBS, and plated onto LB agar to determine the number of surviving bacteria. The percent surviving bacteria was calculated from three separate experiments and averaged. The *dps* mutant strain was found to be impaired in its ability to survive in murine macrophages compared to wild type (Fig. 3), but not as severely as the *slyA* mutant strain. In addition, a *dps* mutant complemented by providing *dps* in *trans* on low-copy-number plasmid pRB3 (4) showed partial restoration of survival inside murine peritoneal macrophages.

The virulence of *dps* mutant serovar Typhimurium was assessed using a C3H/HeN (*ity*^{r}) murine model. Groups of four 8-week-old C3H/HeN *ity*^{r} female mice (Taconic Laboratories, Germantown, N.Y.) were infected intraperitoneally with wild-type, *dps* mutant, or *slyA* mutant bacteria. Overnight cultures of bacteria were diluted in PBS, and 200 μl of each dilution (approximately 1,000 CFU) was administered intraperitoneally using a 25-gauge needle. The inoculum size was confirmed by serial dilution and plating onto LB agar (data not shown). Infected mice were monitored for survival during a 2-week period. Only one of four mice infected with the *dps* mutant died during the course of the experiment, and the remaining mice never showed signs of illness. The virulence assay was

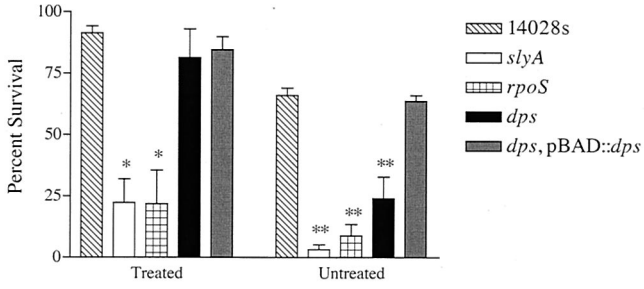

FIG. 2. The iron chelator 2′,2-dipyridyl rescues *dps* mutant *Salmonella* challenged with hydrogen peroxide. Each strain was pretreated with 1 mM 2′,2-dipyridyl or PBS for 20 min and then incubated with 2 mM H_2O_2 for 20 min at 37°C. Viable bacteria were determined by serial dilution and plating on LB agar. The numbers of surviving bacteria are expressed as a percentage of the original inoculum. The average percent survival from three independent experiments is shown, with error bars indicating standard deviations. *, $P < 0.05$; **, $P < 0.001$ by Student's *t* test, compared to wild type.

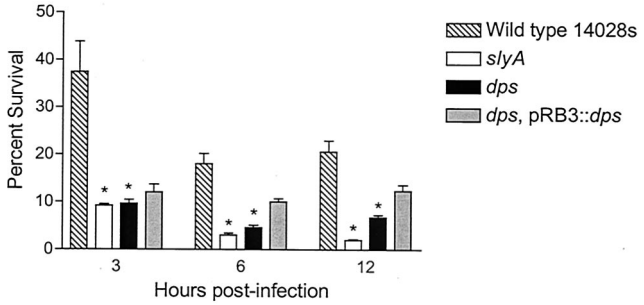

FIG. 3. *dps* mutant *Salmonella* exhibits reduced survival in periodate-elicited murine peritoneal macrophages. Survival of *Salmonella* strains was determined in C3H/HeN (*ity*^{r}) peritoneal macrophages at 3, 6, and 12 h postinfection. Murine peritoneal macrophages were elicited with 5 mM sodium periodate and harvested 4 days later. Macrophages were seeded at approximately 5×10^5 macrophages per well in 48-well plates and infected with *Salmonella* strains at a multiplicity of infection of 5:1 (bacteria/macrophages). Results are expressed as percent survival and represent the average of three independent assays, with error bars indicating standard deviations. *, $P < 0.05$ by Student's *t* test, compared to wild type.

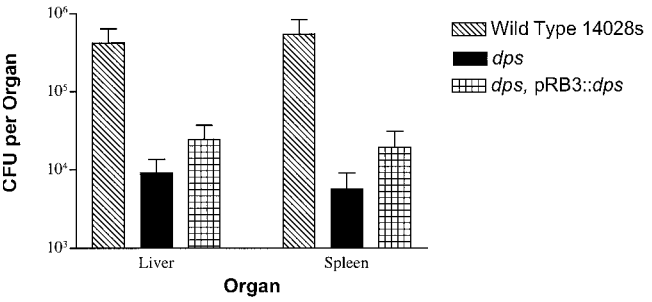

FIG. 5. *dps* mutant *Salmonella* showed reduced survival in vivo. Groups of four mice were infected intraperitoneally with 1,500 CFU of wild-type serovar Typhimurium, a *dps* mutant, or a *dps* mutant complemented by providing *dps* in *trans*. After 5 days, the mice were euthanized and the spleens and livers were recovered. The organs were homogenized in 3 ml of sterile water, serially diluted in PBS, and plated onto XLD agar to enumerate viable bacteria per organ. The results are expressed as CFU per organ, and error bars are included to represent standard deviations. *, $P < 0.02$ as determined by Student's *t* test, compared to wild type.

repeated several times, with virtually identical results (Fig. 4). All mice infected with wild-type *Salmonella* succumbed by 9 days postinfection, but no mice infected with the *slyA* mutant strain died. These results demonstrate the crucial importance of *dps* in the ability of serovar Typhimurium to cause lethal infection in mice.

As intraperitoneal lethality is not the optimum test for virulence, since it is measuring the cumulative growth, survival, and the lethal aspects of the bacteria, quantification of bacteria from mouse tissues was performed. Groups of four mice were infected with 1,800 CFU of wild-type serovar Typhimurium 14028s, a *dps* mutant, and a *dps* mutant complemented by providing *dps* in *trans*. After 5 days, the mice were euthanized and the spleens and livers were recovered and homogenized in 3 ml of sterile water. Enumeration of viable bacteria from each organ was determined by serial dilution in PBS and plating onto xylose-lysine-desoxycholate (XLD) agar. The results are illustrated in Fig. 5. These data demonstrate that a *dps* mutant is required for survival and the attenuation of virulence that is seen in C3H/HeN mice is due to a defect in intramacrophage survival. A *dps* mutant complemented by providing *dps* in *trans* showed a partial recovery in the number of surviving bacteria in the spleen and liver. Incomplete complementation of the macrophage survival and virulence-related phenotypes by pRB3::*dps* may have resulted from aberrant *dps* expression or instability of the pRB3 episomal vector.

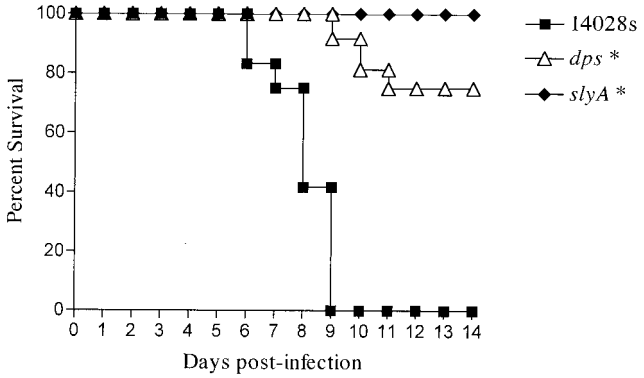

FIG. 4. *dps* mutant *Salmonella* is attenuated for virulence in C3H/HeN (*ity*^{r}) mice. Groups of four 8-week-old C3H/HeN female mice (Taconic Laboratories) were infected intraperitoneally with wild type (14028), an avirulent *slyA* mutant (SL3343), or a *dps* mutant strain (SL3474). Approximate 1,000 CFU of each strain in PBS was administered intraperitoneally. The survival of infected mice from three independent experiments is shown. *, $P < 0.001$ by χ^2 test, compared to wild type.

Resistance to oxidative stress plays an important role in the ability of *Salmonella* to resist killing by host phagocytes and cause a productive infection. Pathogenic microorganisms can resist host-derived reactive oxygen species by avoidance, inhibiting production, production of scavengers, metabolic detoxification, or repair of damage. Iron sequestration is another important mechanism, since intracellular Fe(II) can catalyze the formation of highly toxic oxyradicals from hydrogen peroxide.

The ferritin-like protein Dps has been found in many eubacterial and archaebacterial species. Similar to ferritin, Dps can sequester iron atoms to prevent their participation in the formation of toxic reactive oxygen species (30). Additionally, some Dps homologues appear to condense chromatin into a microcrystalline array that may physically protect DNA from damage (29). In the pathogenic gram-negative bacterium *Salmonella*, *dps* expression is induced following internalization of the bacteria by macrophages (14, 27). The results of our studies unequivocally demonstrate a role of *S. enterica* serovar Typhimurium *dps* in oxidative stress resistance and virulence. The most likely mechanism is the sequestration of iron and prevention of iron-dependent oxidative DNA damage. Dps can be added to the list of evolutionarily conserved antioxidant proteins employed by *Salmonella* to resist killing by host phagocytes.

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