The Alternative \(\sigma\) Factor KatF (RpoS) Regulates Salmonella Virulence

FC Fang, SJ Libby, NA Buchmeier, PC Loewen, J Switala, J Harwood, and DG Guiney

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The alternative σ factor KatF (RpoS) regulates Salmonella virulence

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FERRIC C. FANG†‡, STEPHEN J. LIBBY*, NANCY A. BUCHMEIER‡, PETER C. LOESEN§, JACEK SWITALA§, JULIA HARWOOD*, AND DONALD G. GUINEY*

*Department of Medicine, Division of Infectious Diseases, University of California, San Diego Medical Center, 225 Dickinson Street, San Diego, CA 92103; ‡Department of Pathology, University of California, San Diego School of Medicine, La Jolla, CA 92039; and §Department of Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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ABSTRACT Nutrient limitation is a critical signal in Salmonella virulence gene regulation. The katF (rpoS) gene mediates the expression of the Salmonella spv plasmid virulence genes during bacterial starvation. A katF Salmonella mutant has increased susceptibility to nutrient deprivation, oxidative stress, acid stress, and DNA damage, conditions which are relevant to the intraphagosomal environment of host macrophages. Moreover, the katF mutant has significantly reduced virulence in mice. katF encodes an alternative σ factor of RNA polymerase which coordinately regulates Salmonella virulence.

The plasmid-encoded spv genes are highly conserved among the most virulent non-typhoidal Salmonella serovars: S. typhimurium, S. dublin, S. choleræasuis, and S. enteritidis (1). These genes are required for virulence in mice (2), invasive disease in humans (3), and opportunistic infection in patients with AIDS (4). Recently, we demonstrated (5, 6) that expression of the spv genes is induced by starvation. This starvation-regulated expression is dependent upon SpvR, a transcriptional activator belonging to the LySR family (7). We proposed that nutrient limitation within the phagosomes of host macrophages is the environmental signal triggering expression of the Salmonella plasmid virulence genes (5).

The katF locus (also known as rpoS, appR, and csi2) is a regulator of as many as 32 genes in Escherichia coli (8). Although most bacterial genes show reduced expression during starvation (9, 10), katF-regulated genes are selectively expressed under conditions of nutrient limitation, with maximal expression occurring during the stationary phase of bacterial growth (11, 12). katF has extensive sequence homology with rpoD (encoding σ2) and hipR (encoding σ32), suggesting that katF encodes an alternative σ subunit of RNA polymerase which mediates the transcription of specific genes during starvation (13). The DNA homology between katF and other genes encoding σ factors is greatest in the DNA-binding region, resulting in the classification of katF as a group 2 σ factor (14).

E. coli genes which are positively regulated by katF include katE [hydroperoxidase II (HPII) catalase], xskA (exonuclease III), appA (pH 2.5 acid phosphatase), g12A (glycogen synthase), otsBA (traphosphate synthase), treA (trehalase), osmB (osmotically inducible lipoprotein), and bolA (morphogene) (15–19). In addition, the mcba gene (microcin B17) appears to be negatively regulated by katF (15). Although katF is specifically regulated by carbon starvation, katF mutants of E. coli are more susceptible to a variety of environmental extremes, including starvation (8, 11, 12) as well as oxidative (8, 11), thermal (8, 11), irradiative (20), acid (8, 11) and osmolar (8) stresses.

The stationary phase or starvation-induced expression of the spv plasmid virulence genes closely parallels the pattern of expression of katF-regulated genes. Although katF has not been identified previously in Salmonella, two-dimensional electrophoretic analysis has demonstrated the presence of starvation-induced genes in S. typhimurium (21). In this study, we demonstrate the presence of katF in S. typhimurium, elucidate its role in regulating the expression of the spv plasmid virulence genes, and use a Salmonella katF mutant to establish the role of katF in Salmonella virulence.

METHODS AND MATERIALS

Media. Luria–Bertani (LB) broth (tryptone, 10 mg/ml; yeast extract, 5 mg/ml; NaCl, 10 mg/ml) or M9 minimal medium (Na2HPO4, 7 mg/ml; KH2PO4, 3 mg/ml; NaCl, 0.5 mg/ml; NH4Cl, 1 mg/ml; thiamin, 5 μg/ml; MgSO4, 0.12 mg/ml; glucose, 4 mg/ml) at 37°C were used for all experiments. agar (1.5%) was added to solid medium. Penicillin (250 μg/ml) from Sigma was used for antibiotic selection.

Bacterial Strains and Plasmids. All studies were performed using wild-type S. typhimurium ATCC 14028s or its isogenic katF mutant derivative SF1005. The construction of SF1005 is described in Results. Additional studies used wild-type E. coli NM522 (22) and its isogenic katF::TnlO mutant derivative UM315 (23). pRR10 (from R. Roberts, University of California, San Diego, La Jolla) is a mobilizable RK2-based mini-replicon encoding β-lactam resistance. pFF14 (5) is a pACYC184 derivative encoding a spvB–lacZ translational fusion and chloramphenicol resistance.

Genetic Methods. The polymerase chain reaction (PCR) was used to amplify an internal 600-base-pair sequence of katF from S. typhimurium genomic DNA by a conventional method (24). The primers, 5′-GGCGGTCGCGCATCGTGG-3′ and 5′-CTGGCCAGCCTCAGCTTG-3′, were derived from the published E. coli katF sequence (13). Chromosomal mapping was performed by a recently described rapid mapping method (25); bacteriophage lysates obtained from Mud-P22 inserted at various sites in the S. typhimurium chromosome were slot-blotted onto nitrocellulose and probed with the radiolabeled internal katF fragment. DNA sequencing was performed by the enzymatic method of Sanger et al. (26).

Enzyme Assays. Quantitative catalase assays were performed with an oxygraph as described (18), and activity is expressed as units per mg of dry weight. β-Galactosidase

Abbreviations: HP, hydperoxidase; MMS, methyl methanesulfonate.

†To whom reprint requests should be addressed: University of Colorado Health Sciences Center, Denver, CO 80262.
activity was measured by a conventional method (5) and expressed in Miller units.

Survival Assays. Susceptibility of Salmonella strains to hydrogen peroxide was determined by adding hydrogen peroxide to stationary-phase bacteria in LB broth to a final concentration of 15 mM. Evaluation of prolonged stationary-phase survival was performed in M9 medium on a rotary shaker at 37°C for 6 days. Susceptibility to methyl methanesulfonate (MMS) was determined by adding MMS to stationary-phase bacteria in LB broth to a final concentration of 60 mM. Susceptibility to pH 4 was determined by pelleting stationary-phase bacteria and resuspending the cells in LB broth adjusted to pH 4 with citrate buffer. In each of these experiments, aliquots of bacteria were removed at timed intervals, diluted, and plated onto LB agar for quantitation of viable cells.

Mouse Virulence Assay. Salmonella virulence in 7-week-old female BALB/c mice was determined by oral administration of bacteria via feeding cannula. Inocula were quantified by dilutional plating.

RESULTS

Demonstration of katF in Salmonella. Oligonucleotide primers flanking a 600-base-pair internal region of the E. coli katF gene were used to amplify an internal katF allic sequence of S. typhimurium 14028s by PCR. The amplified fragment was cloned and sequenced, revealing a >90% identity with katF of E. coli. This confirmed that katF is present in Salmonella, that its sequence is highly conserved, and that the amplified internal gene fragment originated from katF. By using a modification of a recently described rapid mapping method (25), katF was located at approximately 57–60 min in the S. typhimurium chromosome (data not shown), corresponding closely with its known location at 59 min in E. coli (18).

Construction of a katF Salmonella Mutant (SF1005). To examine the role of katF in Salmonella, we used a virulent Salmonella strain to construct an isogenic derivative containing an inactive katF allele. A strategy was devised for the mutation of katF in Salmonella by gene interruption. The internal PCR-derived Salmonella katF gene fragment was cloned into a suicide vector based upon the RK2-derivative pRR10 (the vector system will be reported separately), and the katF suicide vector was conjugally transferred into S. typhimurium 14028s. Transconjugants were selected for resistance to penicillin. Brilliant green–sulfadiazine medium was used to counterselect the E. coli donor strain. Insertional inactivation of katF resulted from homologous recombination of the suicide vector into the bacterial chromosome. Disruption of katF was confirmed by Southern hybridization using the katF fragment as a probe (Fig. 1). The katF mutant S. typhimurium strain was designated SF1005.

katF Regulates Catalase Expression in Salmonella. katF is required for HPI catalase expression in E. coli. S. typhimurium SF1005 demonstrated a 78% reduction in stationary-phase catalase activity when compared with the isogenic wild-type strain (Fig. 2). Residual catalase activity is attributable to the HPI catalase, which is controlled by oxyR (27).

A katF Salmonella Mutant Is Susceptible to a Variety of Environmental Stresses. Resistance to oxidative stress may be important in the ability of Salmonella to withstand oxygen-dependent killing mechanisms in phagocytic cells (28). Ninety-eight percent of an inoculum of SF1005 did not survive exposure to 15 mM hydrogen peroxide for 60 min.

Fig. 1. Southern blot analysis of S. typhimurium 14028s and SF1005. Total genomic DNA from S. typhimurium 14028s (wild type) and SF1005 (katF) was isolated, digested with EcoRI or BamHI, electrophoresed through 1% agarose, transferred to nitrocellulose, and hybridized with the 32P-labeled PCR-derived internal katF fragment. The autoradiograph is shown. Lane 1, SF1005 DNA cut with EcoRI; lane 2, 14028s DNA cut with EcoRI; lane 3, SF1005 DNA cut with BamHI; lane 4, 14028s DNA cut with BamHI. Positions of 10.6- and 6.0-kilobase (kb) bands were determined from comparison with molecular size markers. Recombinational insertion of a suicide vector into the katF gene introduces a new EcoRI restriction site and ~4.0 kb of vector DNA. The expected pattern resulting from disruption of katF in SF1005 is confirmed: a single 11-kb EcoRI fragment (lane 2) is converted into two fragments, 8.5 kb and 6.5 kb (lane 1), and a single 6-kb BamHI fragment (lane 4) is converted into a single 10-kb fragment (lane 3).

Fig. 2. Quantitation of catalase activity. Logarithmic and stationary-phase catalase activities were measured with an oxygraph (18) and are expressed as units per mg of dry cell weight.

Fig. 3. Salmonella killing by 15 mM hydrogen peroxide. Hydrogen peroxide was added to stationary-phase S. typhimurium 14028s or SF1005 in LB broth.
growth increased susceptibility murium whereas to rotary conditions found These data indicate that SF1005 is MMS. catalase in DNA repair DNA. nutrient deprivation analogous to stationary-phase S. katF mutants were also found to withstand starvation, oxidative stress, DNA damage, and acid stress. katF is required for Salmonella virulence in mice. The katF mutant SF1005 was evaluated for virulence in BALB/c mice. The oral lethal dose for SF1005 was 1000-fold greater than for the wild-type parent (Table 1), establishing the essential role of katF in Salmonella virulence. The oral lethal dose for plasmid-cured S. typhimurium 14028s was only 10-fold greater than for the wild-type parent.

**DISCUSSION**

katF may be added to phoP (32, 33) and ompR (34) as a coordinate regulator of virulence genes in Salmonella. However, in contrast to these conventional two-component regulatory systems, katF encodes an alternative σ factor (13) that is nonessential for normal exponential cell growth but essential during infection.

Nutrient deprivation appears to be a critical environmental signal triggering the expression of Salmonella virulence genes within the phagosomes of host macrophages (5). Recent experiments indicate that expression of the katF-regulated virulence gene spvB is promptly induced following ingestion of Salmonella by murine macrophages in vitro (J. Fierer, personal communication). The phoP regulatory sys-

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**Fig. 4.** Salmonella starvation survival. Stationary-phase S. typhimurium 14028s or SF1005 cells were maintained in M9 medium on a rotary shaker at 37°C for 6 days. whereas wild-type organisms were unaffected (Fig. 3). The reduced catalase activity of SF1005 may account for its increased susceptibility to hydrogen peroxide.

*Salmonella* proliferation is inhibited by macrophages (29), and there is some evidence that macrophages restrict the growth of phagocytosed organisms by limiting essential nutrients within the phagosome (28). *S. typhimurium* SF1005 was found to have a reduced ability to survive starvation conditions during prolonged stationary phase (Fig. 4) in a manner analogous to *E. coli* katF mutants (8, 11, 12). The ability of SF1005 to survive in M9 minimal medium on a rotary shaker at 37°C for 6 days was 7-fold reduced relative to wild-type 14028s. The ability to withstand conditions of nutrient deprivation may be critical in the characteristic ability of *Salmonella* to survive for prolonged periods within phagocytic cells. The ability to survive under starvation conditions may also be important in the ability of *Salmonella* to persist in the environment.

Since *E. coli* katF regulates the expression of xthA, a gene involved in DNA repair (19), we evaluated the ability of SF1005 to withstand DNA damage induced by the alkylating agent MMS. SF1005 was 40-fold more susceptible than wild type to killing by exposure to 60 mM MMS (Fig. 5) for 60 min. These data indicate that SF1005 is impaired in its ability to repair DNA. This is of particular interest in light of the recent observation that recA mutant *S. typhimurium*, which is deficient in DNA repair, is avirulent and unable to survive in macrophages in vitro (F. Heffron, personal communication).

The intraphagosomal pH of macrophages has been estimated to be 4–5 (30). Therefore, we evaluated the ability of the katF mutant *S. typhimurium* SF1005 to withstand exposure to pH 4. SF1005 was found to be 10-fold more susceptible to this pH reduction after 60 min, when compared with wild-type 14028s (Fig. 6). *E. coli* and *Shigella* katF mutants similarly have been found to be more susceptible to acid stress. In summary, the katF mutant *Salmonella* strain is impaired in its ability to withstand starvation, oxidative stress, DNA damage, and acid stress.

**KatF Regulates spv Plasmid Virulence Gene Expression.** Employing gene fusions and mRNA analysis, we recently demonstrated that the spv plasmid virulence genes of *Salmonella* are selectively expressed during stationary phase or starvation conditions (5, 6). The role of katF in *Salmonella* plasmid virulence gene expression was examined by using the spvB-lacZ translational fusion vector pFF14 (5) transformed into *S. typhimurium* SF1005. Stationary-phase spvB expression was 86% reduced in the katF mutant SF1005 (Fig. 7). A similar reduction in spvB expression was observed in an *E. coli* katF mutant containing the spvB-lacZ vector.

**Fig. 5.** *Salmonella* killing by 60 mM MMS. MMS was added to stationary-phase *S. typhimurium* 14028s or SF1005 in LB broth.

**Fig. 6.** *Salmonella* killing by pH 4. Stationary-phase *S. typhimurium* 14028s or SF1005 cells were pelleted and resuspended in LB broth adjusted to pH 4 with citrate buffer.
katF should provide significant insights into the molecular basis of Salmonella virulence.

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Table 1. Mouse virulence of S. typhimurium 14028s and SF1005

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose, no. of bacteria</th>
<th>Mortality, no. dead/total (%)</th>
<th>log LD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028s</td>
<td>3 × 10^3</td>
<td>1/4 (25)</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>3 × 10^6</td>
<td>7/8 (88)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^7</td>
<td>4/4 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^8</td>
<td>3/8 (38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^9</td>
<td>1/3 (33)</td>
<td></td>
</tr>
<tr>
<td>SF1005 (katF)</td>
<td>3 × 10^6</td>
<td>0/8 (0)</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>3 × 10^7</td>
<td>0/8 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^8</td>
<td>0/8 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^9</td>
<td>2/4 (50)</td>
<td></td>
</tr>
<tr>
<td>14028s (plasmid-cured)</td>
<td>3 × 10^6</td>
<td>0/4 (0)</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>3 × 10^7</td>
<td>3/7 (43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^8</td>
<td>4/7 (57)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10^9</td>
<td>2/2 (100)</td>
<td></td>
</tr>
<tr>
<td>LB broth control</td>
<td>0</td>
<td>0/7 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Virulence in 7-week-old female BALB/c mice was determined by oral administration of bacteria in LB via feeding cannula. Mortality data are shown as the number of dead mice divided by the total number of mice at 21 days after administration. The median lethal dose (LD50) was determined by the method of Reed and Muench (31).