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PNAS 1992;89:11978-11982
doi:10.1073/pnas.89.24.11978

This information is current as of October 2006.

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Notes:

The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence

(starvation/pathogenesis/*spv*/plasmid/gene regulation)

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Communicated by Robert K. Selander, September 25, 1992 (received for review August 6, 1992)

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. choleraesuis*, 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 during 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 σ^{70}) and *htpR* (encoding σ^{32}), 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], *xthA* (exonuclease III), *appA* (pH 2.5 acid phosphatase), *glgA* (glycogen synthase), *otsBA* (trehalose 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[¶] 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 μ g/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 μ g/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::Tn10* 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'-GCGCGTCGCGCACT-GCGTGG-3' and 5'-CTGGCCAGCACTTCACGCTG-3', were derived from the published *E. coli katF* sequence (13). Chromosomal mapping was performed by a modification of 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, hydroperoxidase; MMS, methyl methanesulfonate.

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[¶]Small, P. & Falkow, S., Abstracts of the 92nd General Meeting of the American Society for Microbiology, May 26–30, 1992, New Orleans, LA, #B74.

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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 methane-sulfonate (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* allelic 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 con-

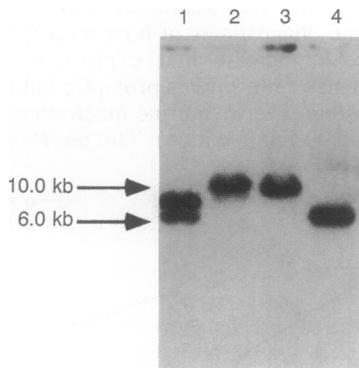


Fig. 1. Southern blot analysis of *S. typhimurium* 14028s and SF1005. Total genomic DNA from *S. typhimurium* 14028s (wild type) and SF1005 (*katF*) was isolated, digested with *EcoRI* or *BamHI*, electrophoresed through 1% agarose, transferred to nitrocellulose, and hybridized with the ³²P-labeled PCR-derived 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 10.0- 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).

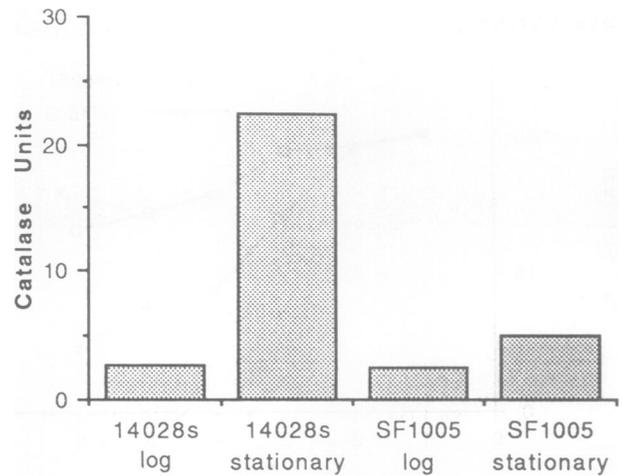


FIG. 2. Quantitation of catalase activity. Logarithmic and stationary-phase catalase activities were measured with an oxygraph (18) and are expressed as units per mg of dry cell weight.

taining 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 HPII 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,

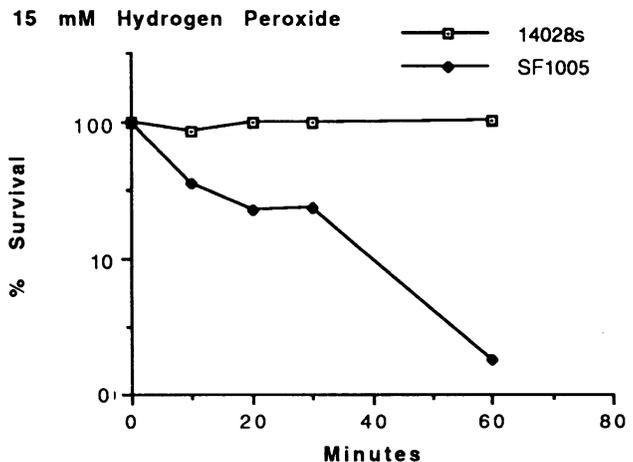


FIG. 3. *Salmonella* killing by 15 mM hydrogen peroxide. Hydrogen peroxide was added to stationary-phase *S. typhimurium* 14028s or SF1005 in LB broth.

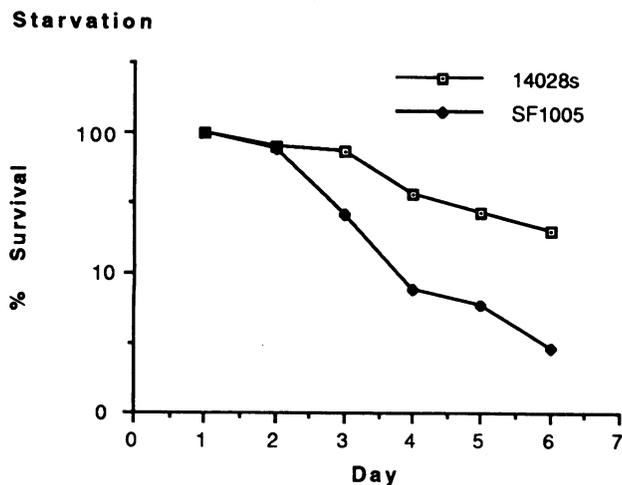


FIG. 4. *Salmonella* starvation survival. Stationary-phase *S. typhimurium* 14028s or SF1005 cells were maintained in M9 medium on a rotary shaker at 37°C for 6 days.

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

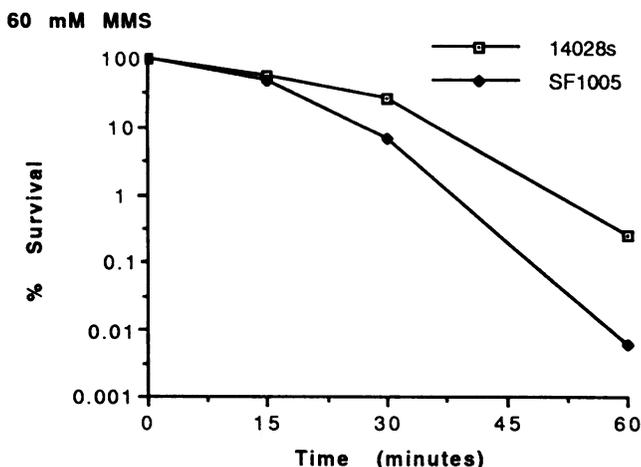


FIG. 5. *Salmonella* killing by 60 mM MMS. MMS was added to stationary-phase *S. typhimurium* 14028s or SF1005 in LB broth.

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.

***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 100-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-

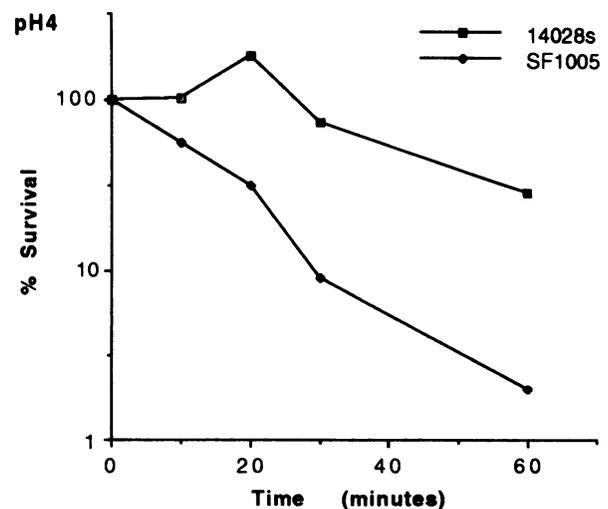


FIG. 6. *Salmonella* killing by pH 4. Stationary-phase *S. typhimurium* 14028s or SF1005 cells were pelleted and resuspended in LB broth adjusted to pH 4 with citrate buffer.

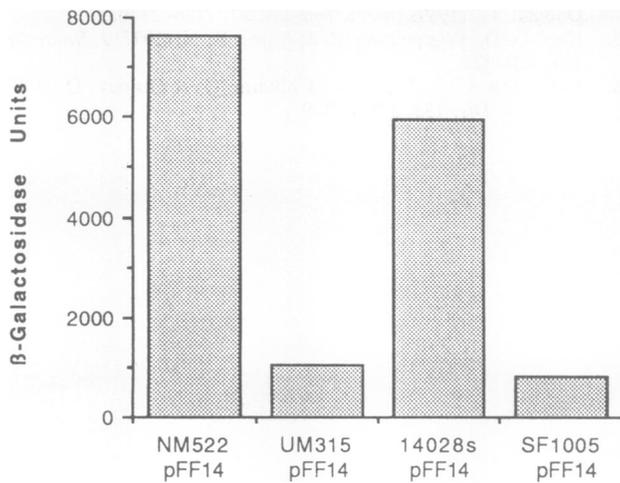


FIG. 7. Plasmid virulence gene expression. Stationary-phase β -galactosidase expression from the *spvB-lacZ* fusion on the plasmid pFF14 (5) was measured for *E. coli* NM522 (wild type), *E. coli* UM315 (*katF*), *S. typhimurium* 14028s (wild type), and *S. typhimurium* SF1005 (*katF*).

tem, which is independent of *katF*, has been implicated in the resistance of *Salmonella* to macrophages and may also be influenced by environmental signals related to starvation (35).

Genes regulated by *katF* not only respond to starvation but are required for the resistance of *Salmonella* to starvation and other environmental stresses which are found within phagosomes of host phagocytic cells. Resistance to nutrient deprivation, oxidative stress, acid stress, and DNA damage is relevant to conditions anticipated within macrophage phagosomes. Furthermore, the ability to survive conditions of prolonged nutrient deprivation may pertain directly to the characteristic ability of virulent *Salmonella* to persist for long periods in the host. The *spv* genes appear to be involved in the ability of *Salmonella* to grow within the cells of the reticuloendothelial system (36, ||). Although the *spv* genes are clearly implicated in virulence, a plasmid-cured derivative of 14028s had an intermediate lethal dose between the wild-type strain and the *katF* mutant, suggesting that *katF* regulates chromosomally encoded genes in addition to the plasmid-encoded *spv* genes which contribute to *Salmonella* virulence. Further investigation of gene loci regulated by

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

Strain	Dose, no. of bacteria	Mortality, no. dead/total (%)	log LD ₅₀
14028s	3×10^5	1/4 (25)	5.9
	3×10^6	7/8 (88)	
	3×10^7	4/4 (100)	
	3×10^8	4/4 (100)	
SF1005 (<i>katF</i>)	3×10^6	0/8 (0)	8.8
	3×10^7	0/8 (0)	
	3×10^8	3/8 (38)	
	3×10^9	4/8 (50)	
14028s (plasmid-cured)	3×10^6	0/4 (0)	7.8
	3×10^7	3/7 (43)	
	3×10^8	4/7 (57)	
	3×10^9	2/4 (50)	
LB broth control	0	0/7 (0)	

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).

katF should provide significant insights into the molecular basis of *Salmonella* virulence.

||Gulig, P. & Doyle, T., Abstracts of the 92nd General Meeting of the American Society for Microbiology, May 26–30, 1992, New Orleans, LA, #B163.

We are grateful to Rick Roberts and Martin Krause for the generous gifts of strains and plasmids. This work was supported by Program Project Grant AM35108, Physician–Scientist Award DK01408-05, and Training Grant 5 T32 AI07036 from the National Institutes of Health and by Grant R91SD057 from the University of California Universitywide AIDS Research Program. Additional support was provided by a grant from the Natural Sciences and Engineering Research Council and from the University of Manitoba.

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