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Role of RpoS in Survival of *Yersinia enterocolitica* to a Variety of Environmental Stresses

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***rpoS*, a gene that encodes an alternative sigma factor (also known as *katF*), is critical for the ability of *Yersinia enterocolitica* grown at 37°C, but not at 26°C, to survive diverse environmental insults such as high temperature, hydrogen peroxide, osmolarity, and low pH. However, a *Y. enterocolitica rpoS* mutant was not affected in expression of *inv* or *ail*, invasion of tissue culture cells, or virulence in mice.**

Yersinia enterocolitica is an invasive enteropathogen that most commonly causes gastroenteritis in humans (2). Infection with *Y. enterocolitica* is generally due to ingestion of the bacteria via contaminated water or food. After ingestion, the bacteria travel through the gastrointestinal tract, where adherence and invasion occur at the terminal ileum (5). Three invasion genes, *inv*, *ail*, and *yadA*, have been identified in enteropathogenic *Yersinia* species (1, 8, 14, 19, 23). The *inv* gene encodes the primary invasion factor for *Y. enterocolitica*, invasin, a 92-kDa outer membrane protein that has been demonstrated to bind several members of the integrin family and to be important for *Y. enterocolitica* migration through the intestinal epithelium (7, 18, 24). Expression of *inv* is affected by growth phase and a variety of environmental conditions including temperature, low pH, and Na^+ concentration. *inv* expression is maximal in late exponential phase to early stationary phase at 26°C; however, significant expression also occurs at 37°C and pH 5.5 (17).

Bacteria growing in host tissue, as well as in nature, encounter conditions of submaximal growth rate, starvation, and environmental stresses (12). In *Escherichia coli*, optimal growth under these conditions requires the *rpoS* gene, which encodes an alternative sigma factor (also known as KatF or σ^s) (15, 22). Approximately 30 genes in *E. coli* are under control of *rpoS* and are selectively regulated during conditions of nutrient limitation and stationary phase of bacterial growth (6, 11, 13). *E. coli rpoS* mutants are more susceptible than the wild type to several environmental conditions including starvation and oxidative, thermal, irradiative, osmolar, and acid stress (11). In addition, stationary-phase-induced morphological changes in *E. coli* are dependent on *rpoS*; *rpoS* mutant cells appear slightly elongated and remain rod shaped in stationary phase (10).

Recently, it was demonstrated that an *rpoS* homolog is present in *Salmonella typhimurium* and that it is necessary for stationary-phase and starvation-induced expression of *spv* virulence genes (4, 16). In addition, an *rpoS* mutant in *S. typhimurium* is significantly reduced for virulence in mice (4). Recently, *rpoS* was also identified in *Shigella flexneri*, and it was shown that an *rpoS* mutant is more acid sensitive and significantly less infective than the wild-type strain (20, 21).

Because of the similarity in *rpoS*-dependent gene regulation in response to multiple stresses and the pattern of environmental regulation of *inv* expression, we sought to determine if there is an *rpoS* homolog in *Y. enterocolitica*. Furthermore, we wanted to investigate if *Y. enterocolitica rpoS* plays a role in *inv* expression and/or virulence. Preliminary Southern hybridization analysis with a probe from *rpoS* of *S. typhimurium* suggested that *Y. enterocolitica* has an *rpoS* homolog. To clone the *Y. enterocolitica rpoS* gene, oligonucleotide primers flanking an internal 600-bp region of the *E. coli rpoS* gene (both modified with *Bam*HI sites at the 5′ ends; 100B, 5′-CGGGATCCCGG CGCGTCGCGCACTGCGTGG-3′, and 101B, 5′-CGGGAT CCCGGCACGGTCGTGAAGTGCGAC-3′) were used to amplify homologous sequences in *Y. enterocolitica* by PCR. The PCR product (ca. 1,000 bp) was cloned into pCRII (Invitrogen) and subsequently sequenced. The nucleotide sequence of the *Y. enterocolitica rpoS* PCR product was determined on both strands by the dideoxynucleotide-chain termination method with the Sequenase sequencing kit (U.S. Biochemical Corp.). The derived amino acid sequence of the *Y. enterocolitica rpoS* internal fragment showed 92.7% similarity and 87.9% identity to the *E. coli* RpoS (Fig. 1). In addition, alignment to *Salmonella* and *Shigella* RpoS showed 91.9% similarity and 87.2% identity to *Salmonella* RpoS and 91.2% similarity and 85.4% identity to *Shigella* RpoS (Fig. 1).

To investigate the role of *rpoS* in *Y. enterocolitica*, an isogenic *rpoS* mutant of the virulent strain JB580v was constructed. JB580v is a restriction mutant derived from strain $8081v^{Nl}$ (9). The internal fragment of *Y. enterocolitica rpoS* generated by PCR as described above was subcloned into the mobilizable suicide vector pEP185.2 and subsequently transferred into JB580v by conjugation as previously described (9). Transconjugants were selected for resistance to chloramphenicol (20 μg/ml) on minimal plates supplemented with 0.2% glucose. Insertional inactivation of *rpoS* resulted from homologous recombination between the suicide vector and the bacterial chromosome at the *rpoS* locus. Disruption of *rpoS* was confirmed by Southern hybridization analysis with the cloned internal *rpoS* fragment as a probe (data not shown). The *Y. enterocolitica rpoS* mutant was designated JB510v. Inspection of stationary-phase cells grown aerobically in rich medium revealed that both the wild-type strain and the *rpoS* mutant exhibited the classical coccobacillus morphology when grown at 26°C. In contrast, when the strains were grown at 37°C, the *rpoS* mutant appeared slightly elongated and bacterial chains were more filamentous than was observed for the wild-type strain (data not shown).

Survival rates of JB510v and wild-type *Y. enterocolitica* after

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                    1                                                  50
E .coli                       MSQNTLKV HDLNEDAEFD ENG..VEVFD EKALVELEPS
S. typhimurium                MSQNTLKV HDLNEDAEFD ENG..VEAFD EKALSEEEPS
S. flexneri         MFRQGITGRS HLMSQNTLKV HDINEDAEFD ENG..VEVFD EKPLVEEEPS
Y. enterocolitica             MSQNTLKV NELHEDVNFD ENSTETEIFD EKQLVEDEPT

Consensus                     MSQNTLKV ....ED..FD EN....E.FD EK.L.E.EP.


                    51                                                 100
E. coli             DNDLAEEELL SQGATQRVLD ATQLYLGEIG YSPLLTAEEE VYFARRALRG
S. typhimurium      DNDLAEEELL SQGATQRVLD ATQLYLGEIG YSPLLTAEEE VYFARRDLRG
S. flexneri         DNYLAEEELL SQGATQLVLD ATQLYLGKIG YSPLLTAEEE VYFARRALRG
Y. enterocolitica   EASSAEDELL AQGVTQRCW. STQLYLGEIG YSPLLTAEEE VYFARRALRG

Consensus           ....AE.ELL .QG.TQ.... .TQLYLG.IG YSPLLTAEEE VYFARR.LRG


                    101                                                150
E. coli             DVASRRRMIE SNLRLVVKIA RRYGNRGLAL LDLIEEGNLG LIRAVEKFDP
S. typhimurium      DVASRRRMIE SNLRLVVKIA RRYGNRGLAL LDLIEEGNLG LIRAVEKFDP
S. flexneri         DVASRRRMIE SNLRLVVKIA RRYGNRGLAL LDLIEEGNLG LIRAVEKFDP
Y. enterocolitica   DVPSRRRMIE SNLRLVVKIA RRYSNRGLAL LDLIEEGNLC LIRAVEKFDP

Consensus           DV.SRRRMIE SNLRLVVKIA RRY.NRGLAL LDLIEEGNL. LIRAVEKFDP


                    151                                                200
E. coli             ERGFRFSTYA TWWIRQTIER AIMNQTRTIR LPIHIVKELN VYLRTARELS
S. typhimurium      ERGFRFSTYA TWWIRQTIER AIMNQTRTIR LPIHIVKELN VYLRTARELS
S. flexneri         ERGFRFSTYA TWWIRQTIER AIMNQTRTIR LPIHIVKELN VYLRTVRELS
Y. enterocolitica   EPGFRFSTYS HWWIRQTIER AIMNQTRTIR LPIHIVKELN VYFGTARELS

Consensus           E.GFRFSTY. .WWIRQTIER AIMNQTRTIR LPIHIVKELN VY..T.RELS


                    201                                                250
E. coli             HKLDHEPSAE EIAEQLDKPV DDVSRMLRLN ERITSVDTPL GGDSEKALLD
S. typhimurium      HKLDHEPSAE EIAEQLDKPV DDVSRMLRLN ERITSVDTPL GGDSEKALLD
S. flexneri         HKLDYEPSAV EIAEQLDKPV DDVSRMLRLN ERITSVDTPL GGDSEKALLD
Y. enterocolitica   HKLDHEPSAE EIAEQLNKPV DDVSRMLRLN ERITSVDTPL GGDSEKSLLD

Consensus           HKLD.EPSA. EIAEQL.KPV DDVSRMLRLN ERITSVDTPL GGDSEK.LLD


                    251                                                300
E. coli             ILADEKENGP EDTTQDDDMK QSIVKWLFEL NAKQREVLAR RFGLLGYEAA
S. typhimurium      ILADEKENGP EDTTQDDDMK QSIVKWLFEL NAKQREVLAR RFGLLGYEAA
S. flexneri         ILADEKENGP EDTTQDDDMK QSIVKWLFEL NAKQREVIAR RFGLLGYEAA
Y. enterocolitica   ILSDENENGP EDTTQDDDMK QSIVKWLFEL NAKQREVLA. ..........

Consensus           IL.DE.ENGP EDTTQDDDMK QSIVKWLFEL NAKQREVLA
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FIG. 1. Sequence alignment of RpoS homologs. The sequence of *Y. enterocolitica* RpoS beyond amino acid 289 was not determined. The sequences of *E. coli*, *S. typhimurium*, and *S. flexneri* RpoS beyond amino acid 301 are not shown. Protein sequences were aligned by the PILEUP program (Genetics Computer Group). Consensus line shows amino acids that are identical between all four sequences.

exposure to several different environmental stresses known to affect survival of other genera of bacteria harboring an *rpoS* mutation were examined. When bacteria were grown at 26°C, there was no difference in survival of the wild-type strain or the *rpoS* mutant after exposure to hydrogen peroxide, high osmolarity, or high temperature (Table 1). In contrast, when bacteria were grown at 37°C prior to the stress, survival of the *rpoS* mutant was reduced twofold when it was exposed to hydrogen peroxide and sixfold when it was exposed to high osmolarity or high temperature (Table 1). This growth temperature-dependent phenotype was also seen for exposure to low pH. When the bacteria were grown at 26°C, the *rpoS* mutant survived low pH as well as the wild-type strain did. However, when the bacteria were grown at 37°C, the *rpoS* mutant was significantly more sensitive to low-pH exposure than was the wild-type strain (survival was reduced 5,000 fold) (Table 1). Starvation tolerance was also investigated for the *rpoS* mutant. The ability of strain JB510v and the wild type to survive for an extended period in minimal medium was examined. It was demonstrated previously that *Y. enterocolitica* grown at 37°C is auxotrophic for several compounds and amino acids; therefore, the starvation assays were performed only at 26°C (3). The *rpoS* mutant showed an increase in starvation susceptibility compared with the wild type. The ability of the *rpoS* mutant to survive after 6 days of starvation was reduced 13-fold compared with that of the wild type (Table 1). This was the only condition tested in which JB510v grown at 26°C survived less well than the wild type.

The expression of many genes of *E. coli* and *S. typhimurium* is dependent on *rpoS* (6, 11, 13), and *rpoS* is required for full expression of virulence in *S. typhimurium* (4). Because the expression pattern of *rpoS*-regulated genes closely parallels the environmental regulation of *inv*, we wanted to determine if *Y. enterocolitica rpoS* plays a role in *inv* expression. Western im-

TABLE 1. Percent survival upon exposure to a variety of environmental stresses[a]

Stress	% Survival of:			
	JB580v		JB510v	
	26°C	37°C	26°C	37°C
Hydrogen peroxide[b]	92.1	83.3	93.3	41.9
High osmolarity[c]	100	42.4	100	6.7
Low pH[d]	43.7	100	30.0	0.02
High temp[e]	0.2	42.4	0.4	6.7
Starvation[f]	100	ND[g]	7.6	ND

[a] JB580v and JB510v were grown at the indicated temperatures under selection for nalidixic acid (20 μg/ml [Sigma]) and chloramphenicol (25 μg/ml [Sigma]), respectively. For hydrogen peroxide, osmolarity, and pH treatments, overnight stationary-phase cells were collected by centrifugation, resuspended, and exposed to the indicated stress. After exposure to the environmental stresses, the bacteria were collected by centrifugation, resuspended in 0.9% NaCl, and subsequently diluted and plated on LB agar containing appropriate antibiotics.

[b] 15 mM H_2O_2 for 60 min.

[c] 2.4 M NaCl for 60 min.

[d] LB buffered with 100 mM morpholineethanesulfonic acid to pH 3.0 for 5 min.

[e] Overnight stationary-phase cells were transferred to prewarmed 55°C tubes and incubated for 2 min.

[f] Results are presented as percentages of the wild-type result. Bacteria were grown in M63 minimal medium supplemented with 2 mg of Casamino Acids (vitamin free) per ml, 5 μg of thiamine per ml, 1 mM $MgSO_4$, and 0.2% glucose. After 6 days, the cells were diluted and plated on LB agar containing appropriate antibiotics.

[g] ND, not determined.

munoblot analysis with polyclonal antibody to invasin was performed as described previously (17). Wild-type JB580v and the *rpoS* mutant JB510v were grown to stationary phase at 26 or 37°C in buffered media at pH 5.5 or 7.5. The wild-type strain and the *rpoS* mutant exhibited similar invasin protein levels under all conditions tested (Fig. 2). In addition, the effect of *rpoS* on the expression of another invasion gene, *ail*, was analyzed. There was no evident difference between the two strains in the amount of Ail present after growth at either temperature (data not shown). Consistent with the lack of effect of *rpoS* on expression of the invasion factors *inv* and *ail*, the ability of *Y. enterocolitica* JB510c grown at 26 and 37°C to invade tissue culture cells was also unaffected (Table 2). The effect of an

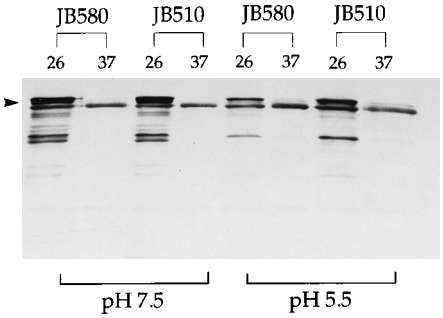

FIG. 2. Western analysis of invasin expression by JB580v and JB510v ("v" denotes that a virulence plasmid is present in that strain). Bacteria were grown to stationary phase in Luria broth buffered at pH 7.5 or 5.5 at either 26 or 37°C as indicated. Equal amounts of whole-cell lysates of the two *Y. enterocolitica* strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and prepared for Western immunoblot analysis with anti-invasin antibody as previously described (17). Binding of the primary antibody was visualized by addition of enhanced chemiluminescence detection agents as specified by the manufacturer (Amersham) followed by exposure to Hyperfilm-MP autoradiography film (Amersham). The arrowhead points to the band representing full-length invasin.

TABLE 2. Invasion phenotype of JB580c and JB510c

Strain	% Invasion[a] at growth temp:	
	26°C	37°C
JB580c[b]	56.3 ± 14.7	12.3 ± 1.7
JB510c	50.0 ± 14.1	15.7 ± 2.3

[a] Data presented are from a representative assay of several assays performed in duplicate on HEp-2 monolayers as described previously (17). Results are presented as percent invasion = 100 × (number of bacteria recovered)/(number of bacteria added).

[b] c denotes virulence plasmid cured from that strain.

rpoS mutation on *Y. enterocolitica* virulence in mice was assayed by using virus-free 6- to 7-week-old female BALB/c mice as previously described (18). Results of oral 50% lethal dose (LD_{50}) assays performed in parallel demonstrated that the *rpoS* mutant JB510v had no reproducible difference compared with the wild-type strain JB580v (LD_{50} by the oral route was 7.5×10^7 for JB580v and 2.4×10^7 for JB510v in a representative experiment with six groups of five mice each). The *rpoS* mutation was found to be stable in vivo (data not shown).

In this study, we demonstrated the presence of an *rpoS* homolog in *Y. enterocolitica* and constructed a mutation in the *Y. enterocolitica rpoS* gene. The *rpoS* mutation did not affect expression of the invasion genes, *inv* or *ail*, and it did not affect invasion of tissue culture cells. In contrast to what has been observed for *S. typhimurium* (4), the *Y. enterocolitica rpoS* mutant demonstrated no change in virulence for orally infected mice when compared with the wild-type strain. This suggests either that *Y. enterocolitica* is not exposed to these stresses in vivo or that *rpoS* is not required in vivo for expression of the necessary survival responses. Although *rpoS* did not appear to play a role in expression of genes involved in the pathogenesis of *Y. enterocolitica* infection, it was required for *Y. enterocolitica* grown at 37°C to survive a variety of environmental stresses. When cultures of *Y. enterocolitica* JB510v were grown at 37°C, cell morphology changed and survival of stress due to hydrogen peroxide, osmolarity, heat shock, and low pH was reduced compared with that of the wild type. However, the ability of JB510v cultures grown at 26°C to survive these stresses was unaffected. Nevertheless, *Y. enterocolitica* RpoS is not completely inactive in cells grown at 26°C, since it is necessary for starvation survival. Together, these data suggest that there may be an additional alternative sigma factor(s), other than *rpoS*, in *Y. enterocolitica* that plays a role in environmental stress survival for cells growing at 26°C. Given the number of alternative sigma factors identified thus far for expression of specific functions such as response to diverse environmental insults, sporulation, flagellum synthesis, and nitrogen fixation, it is not surprising that *Y. enterocolitica* may have an additional alternative sigma factor(s) for the expression of environmental stress response genes at 26°C.

Nucleotide sequence accession number. The nucleotide sequence obtained for *Y. enterocolitica rpoS* was submitted to the GenBank database and assigned accession number U22043.

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