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Alternative Sigma Factor σ^E Has an Important Role in Stress Tolerance of *Yersinia pseudotuberculosis* IP32953

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***Yersinia pseudotuberculosis* is an important pathogen that probably survives well in the modern food chain. However, little is known about the mechanisms that allow the growth of this pathogen in foods under stress conditions. The expression of *rpoE* encoding σ^E was defined by quantitative real-time reverse transcription-PCR. Expression of *rpoE* was induced at 3°C, 37°C, and 42°C, under exposure to 3% NaCl, 3% ethanol, or high and low pH, in relation to its expression at the optimum growth temperature of 28°C of *Y. pseudotuberculosis*. Mutation of *rpoE* either impaired or abolished growth under stresses caused by low or high temperature, low pH, and ethanol. In addition, the growth temperature range of the mutant was significantly diminished compared to that of the wild-type strain IP32953. The results were confirmed with complementation of the mutant. Thus, σ^E plays a significant role in the stress tolerance of *Y. pseudotuberculosis* IP32953 and probably contributes to the survival of this pathogen in the food chain.**

Yersinia pseudotuberculosis is an important food-borne Gram-negative pathogen that has caused several outbreaks through raw vegetables or contaminated water (1–5). The average incidence of *Y. pseudotuberculosis* infections between the years 1995 and 2006 in Finland was 1.9/100,000 population (varied between 0.6 and 4.8) (6). *Y. pseudotuberculosis* tolerates stressful conditions in the environment and in the modern food chain. It is able to survive over winter in soil (2) and at least 1 month in food storage facilities (1), and it can grow in pHs ranging from 5.0 to 9.6, at temperatures from 0 to 42°C (7), and in a NaCl concentration of 5% (6).

The mechanisms that allow survival and growth of *Y. pseudotuberculosis* under stressful conditions are poorly understood (7). It is known that the histidine kinase CheA of the CheA/CheY two-component system (8) and the RNA helicase CsdA (9) are needed for optimal growth of *Y. pseudotuberculosis* at low temperature. The role of alternative sigma factors in counteracting environmental stresses has been elucidated for many other food pathogens (10–13), but their role in *Y. pseudotuberculosis* is not known. Understanding the means bacteria use to tolerate stress can provide new ways to control the growth of pathogens in foods.

The envelope of Gram-negative bacteria consists of the cell membrane, periplasm, and the outer membrane. The alternative sigma factor σ^E controls the composition and folding of proteins in the cell envelope (14). Heat (15), increased expression (14), unfolding or misfolding of the outer membrane proteins (16), ethanol (17), and high osmolality (18) induce σ^E-dependent envelope stress response (reviewed in references 19 and 20). The most important regulator of σ^E activity is the inner membrane protein RseA, which is an anti-sigma factor (21, 22). Under nonstress conditions, σ^E is bound by RseA and activity of σ^E is thus inhibited (21, 22). In the σ^E-dependent envelope stress response, σ^E is released from its anti-sigma factor by proteases (16, 23–30). This leads to transcription of several genes involved in protein delivery, assembly, and degradation in order to restore normal protein folding and reset the σ^E-dependent envelope stress response (19).

To our knowledge, there are no studies on the roles of σ^E under stress in *Y. pseudotuberculosis*. In *Escherichia coli*, expression of *rpoE* encoding σ^E is induced by low temperature (31). In this investigation, we studied the expression of *rpoE* and the role of σ^E under different stress conditions in *Y. pseudotuberculosis*. We show that σ^E is needed at both low and high temperatures and under stresses caused by low pH and ethanol in *Y. pseudotuberculosis*.

MATERIALS AND METHODS

Bacterial strains. *Y. pseudotuberculosis* strain IP32953 (gratefully received from Elisabeth Carniel, Institut Pasteur, Paris, France) and an *E. coli* strain comparable to DH5α (Sigma-Aldrich Co., St. Louis, MO), having *recA1* and *endA1* mutations, were grown in Luria-Bertani (LB) broth or on LB agar. *Y. pseudotuberculosis* was incubated at 28°C and *E. coli* at 37°C with shaking.

RNA isolation. RNA isolation was performed as described previously (8). In brief, three biological replicates originating from separate colonies were grown in LB broth at 28°C and 3°C, in LB broth with 5 mM $CaCl_2$ at 28°C, 37°C, and 42°C to inhibit the release of *Yersinia* outer membrane proteins (32), and in LB broth at pH 5.0 (adjusted with 1 M HCl), at pH 9.0 (adjusted with 1 M NaOH), with 3% NaCl, or with 3% ethanol at 28°C. Samples were taken at early (optical density at 600 nm [OD_{600}], 0.5 to 0.8) and late (OD_{600}, 1.1 to 2.2) logarithmic growth phases. During total RNA isolation using the RNeasy kit (Qiagen GmbH, Hilden, Germany), on-column DNase digestion was done by following the manufacturer's instructions. A DNA-*free* kit (Applied Biosystems, Foster City, CA) was used for an additional DNase treatment. The A_{260}/A_{280} ratio was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and the integrity of RNA was investigated with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

Reverse transcription and RT-qPCR. Reverse transcription and quantitative real-time reverse transcription-PCR (RT-qPCR) were done as described previously (8). In short, reverse transcription of the RNAs into cDNAs was done in duplicate (reverse transcription replicates) by

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TABLE 1 Primers used in this study

Primer	Sequence (5′→3′)
rpoE-qPCR-left	GGCGATGTCCCTGATGTT
rpoE-qPCR-right	TCACCACGAAATGACTCCA
16S-qPCR-left	GCTCGTGTTGTGAAATGTTGG
16S-qPCR-right	TATGTGGTCCGCTGGCTCT
rpoE475-IBS	AAAAAAGCTTATAATTATCCTTATAATGCCTGCTAGTGCGCCCAGATAGGGTG
rpoE475-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTGCTATCTAACTTACCTTTCTTTGT
rpoE475-EBS2	TGAACGCAAGTTTCTAATTTCGGTTCATTATCGATAGAGGAAAGTGTCT
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
T7	TAATACGACTCACTATAGGG
rpoE475-flank-left	AGTGATGTGGATGCGAATGA
rpoE475-flank-right	CACGGAAGATACGTGAACGA
N*inv*-left	TAAGGGTACTATCGCGGCGGA
N*inv*-right	CGTGAAATTAACCGTCACACT
K*virF*-left	TCGTGGCAGCTATGCTGTTC
K*virF*-right	ATACGTCGCTCGCTTATCCA
Intron-left	TGGCAATGATAGCGAAACAA
Intron-right	GGTACCGCCTTGTTCACATT
tetR-NotI	GGCGCGCGGCCGCTTCCGCGCACATTTCC
tetR-SacI	GGCGCGAGCTCTGTTCTGCCAAGGGTTGG
*rpoE*NotI	GGCGCGCGGCCGCGAGCGGGCATTGCTGG
*rpoE*XhoI	GGCCGCTCGAGTCATTTTGTCGACTCCAGAACCA
rpoE1	CGACTCCTGCATTAGGAAGC
rpoE2	TAGTGACTGGCGATGCTGTC
rpoE3	TGCTTGCTCAGGAAACAATG
RP	TTTCACACAGGAAACAGCTATGAC

using the Dynamo cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions. The Dynamo Flash SYBR green qPCR kit (Thermo Scientific) was used in RT-qPCR by following the manufacturer's instructions. The total reaction volume of 20 μl contained 4 μl of template cDNA and a 0.5 μM concentration of each primer, designed with the Primer3 software (33, 34) (Table 1). The cycling protocol of Rotor-Gene 3000 (Qiagen) included initial denaturation at 95°C for 7 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s, with a final extension at 60°C for 1 min. After each extension step, fluorescence data were acquired. A melt curve after a temperature upshift from 60°C to 98°C (0.5°C/5 s) was analyzed after each run. The amplification reaction efficiencies for each primer pair were defined with dilution series of pooled cDNA. The Rotor-Gene 3000 software was used to set the threshold fluorescence levels for each primer pair and to calculate the reaction efficiencies as $10^{\frac{1}{M}} - 1$, where *M* is the slope of the straight line from a semilogarithmic plot of the quantification cycle (C_q) as a function of the cDNA concentration. Reaction efficiencies of *rpoE* (locus tag YPTB2897) RT-qPCR primers under different stresses are shown in Table 2. The relative expression levels of *rpoE* under different stresses were normalized to 16S *rrn*, calibrated to samples taken at 28°C, and quantified by calculating the expression ratios (R) using the equation

$$R = \frac{(1 + E_{rpoE})^{\Delta C_{q,rpoE}(\text{calibrator} - \text{sample})}}{(1 + E_{16Srrn})^{\Delta C_{q,16Srrn}(\text{calibrator} - \text{sample})}} \quad (1)$$

(35), where E_{rpoE} is the amplification reaction efficiency of the *rpoE* transcript, E_{16Srrn} is the amplification reaction efficiency of 16S *rrn* transcripts, $\Delta C_{q,rpoE}$ is the C_q deviation between calibrator and sample for the *rpoE* transcript, and $\Delta C_{q,16Srrn}$ is the C_q deviation between calibrator and sample for the 16S *rrn* transcripts. Samples collected at 37°C and 42°C were calibrated to samples grown at 28°C with 5 mM $CaCl_2$. Student's *t* test was used to investigate the significance of the differences between the relative expression levels of *rpoE* under different growth conditions.

Mutagenesis. An *rpoE475-476*::Ltr Kan[r] mutant (hereafter called *rpoE475*) was constructed by using the TargeTron gene knockout system

TABLE 2 Reaction efficiencies, average quantification cycle (C_q) values, average expression ratios calibrated to 28°C, and standard deviations of average expression ratios of *rpoE* under different stress conditions at the early and late logarithmic growth phases[a,c]

		Early logarithmic growth phase				Late logarithmic growth phase			
Condition	E	$\bar{X}(C_q)$[b]	$\bar{X}(R)$[c]	SD (R)	*P* value	$\bar{X}(C_q)$[b]	$\bar{X}(R)$[c]	SD (R)	*P* value
3°C	0.86	14.17	3.2	0.6	0.007	15.02	2.6	1.7	0.184
37°C	0.95	15.28	2.2	0.3	0.011	16.21	2.2	0.4	0.008
42°C	0.99	14.27	3.9	0.4	0.002	16.24	1.8	0.3	0.035
pH 5.0	1.01	18.43	13.0	4.3	0.007	20.74	3.5	3.2	0.235
pH 9.0	0.90	19.17	3.9	0.6	0.005	21.22	0.9	0.5	0.599
3% NaCl	0.97	20.01	7.2	1.6	0.004	22.22	1.1	0.8	0.737
3% ethanol	0.94	16.85	8.5	3.0	0.010	16.65	13.1	13.4	0.102

[a] E, reaction efficiencies; R, average expression ratios.
[b] Average of the reverse transcription replicates, PCR replicates, and biological replicates.
[c] $R = \frac{(1+E_{rpoE})^{\Delta C_{q,rpoE}}}{(1+E_{16Srrn})^{\Delta C_{q,16Srrn}}}$, where $\Delta C_q = \bar{X}(C_{q,28°C}) - \bar{X}(C_{q,stress})$.

(Sigma-Aldrich Co.) according to the manufacturer's instructions as reported earlier (8). Primers (Table 1) were designed with the Primer3 software (33, 34) or generated using the TargeTron algorithm (Sigma-Aldrich Co.). Primers *rpoE475*-IBS, *rpoE475*-EBS1d, *rpoE475*-EBS2, and EBS Universal (Table 1) were used in PCR to retarget the RNA segment of the intron. The PCR product was subsequently ligated into the plasmid pACD4K-C (Sigma-Aldrich Co.) and transformed to *E. coli* by heat shock. Correct retargeting of the intron in an antisense orientation in *rpoE* was verified by sequencing with T7 primer (Table 1). *Y. pseudotuberculosis* IP32953 was made electrocompetent as described previously (36), and plasmid pAR1219 (Sigma-Aldrich Co.), a source of T7 RNA polymerase, was introduced into *Y. pseudotuberculosis* IP32953 by electroporation using 0.1-cm cuvettes with 25 μF, 200 Ω, and 1.8 kV. After electroporation, cells were incubated in superoptimal broth with catabolite repression (SOC) for 3 h and plated on LB agar containing 100 μg/ml of ampicillin. *Y. pseudotuberculosis* IP32953 containing pAR1219 was made competent, and pACD4K-C was introduced in this strain by electroporation. After incubation for 3 h in SOC, incubation was continued overnight in LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of chloramphenicol. The culture was diluted (1:50) in fresh LB broth containing ampicillin and chloramphenicol and grown to an OD_{600} of 0.2. Intron expression and insertion were induced by adding 0.5 mM isopropyl β-D-thiogalactoside and continuing incubation overnight. Cells were centrifuged, resuspended in fresh LB broth, incubated for 3 h, and plated on LB agar containing 50 μg/ml of kanamycin. Insertion was confirmed by PCR using primer pairs *rpoE*-flank-left and *rpoE*-flank-right and *rpoE*-flank-right and EBS Universal (Table 1). To confirm the species *Y. pseudotuberculosis* and the presence of the virulence plasmid pYV, primer pairs N*inv*-left and N*inv*-right (37) and K*virF*-left and K-*virF*-right (38), respectively, were used in PCR (Table 1).

Southern blotting. Single-intron insertion in the mutant genome was confirmed by Southern blotting as reported earlier (8). Briefly, a 199-bp digoxigenin (DIG)-labeled probe was synthesized using a PCR DIG probe synthesis kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions using primers intron-left and intron-right (34) (Table 1). Genomic DNA was isolated from the wild-type and mutant strains. HindIII-digested DNAs and pACD4K-C were transferred to a positively charged nylon membrane and hybridized with the probe as recommended by the manufacturer.

Complementation of the *rpoE* mutant strain. The tetracycline resistance gene in pBR322 (39) was amplified by PCR with primers *tetR*-NotI and *tetR*-SacI (Table 1). The PCR product and pBluescript II KS+ (Stratagene, La Jolla, CA) were digested with NotI and SacI and ligated, yielding pBluescript-*tetR* (hereafter called pBlue-*tetR*). A 2,713-bp region of the chromosome of *Y. pseudotuberculosis* IP32953 (33), containing the coding sequences of *rpoE*, *rseA*, and *rseB*, and 500 bp upstream of the start codon of *rpoE* were amplified by PCR using primers *rpoE*NotI and *rpoE*XhoI (Table 1). The resulting PCR product and pBlue-*tetR* were digested with NotI and XhoI and ligated, producing pBlue-*tetR*-*rpoE*. The correct sequence of pBlue-*tetR*-*rpoE* was confirmed by sequencing with T7, *rpoE*1, *rpoE2*, *rpoE*3, *rpoE475*-flank-left, and RP primers (Table 1). The *rpoE475* mutant was cured of pAR1219 by being subcultured several times in LB broth without ampicillin. The ampicillin-sensitive clone of *rpoE475* was confirmed with primers *rpoE*-flank-left and *rpoE*-flank-right for the presence of the intron and with primers N*inv* (37) and K*virF* (38) for the species *Y. pseudotuberculosis* and the presence of the pYV, respectively (Table 1). pBlue-*tetR* (vector control) and pBlue-*tetR*-*rpoE* were introduced into the *rpoE475* mutant by electroporation and plated on LB containing 100 μg/ml of ampicillin. The presence of pBlue-*tetR*-*rpoE* in the mutant was confirmed by PCR with primers T7 and *rpoE*-flank-right (Table 1). The vector control and the complemented mutant carrying pBlue-*tetR*-*rpoE* were confirmed with primers *rpoE*-flank-left and *rpoE*-flank-right and with primers N*inv* (37) and K*virF* (38) (Table 1).

Growth experiments. Three separate colonies of the *Y. pseudotuberculosis* IP32953 wild-type strain, the *rpoE475* mutant, and the *rpoE475* mutant with pBlue-*tetR*-*rpoE* or pBlue-*tetR* were individually inoculated into fresh LB broth containing 200 μg/ml of ampicillin when appropriate and grown overnight with shaking. To study growth at 3°C, 28°C, 37°C, and 42°C, cultures were diluted (1:100) into fresh LB broth containing ampicillin when appropriate. For experiments at 37°C and 42°C, LB broth was supplemented with 5 mM $CaCl_2$ to inhibit the release of *Yersinia* outer membrane proteins (32). To study growth at 28°C under pH, salt, or ethanol stress, cultures were diluted (1:100) into LB broth adjusted to pH 5.0 (with 1 M HCl) or pH 9.0 (with 1 M NaOH) or containing NaCl 30 g/liter or ethanol 3%, plus ampicillin when appropriate. A quantity of 300 μl of each dilution was pipetted into wells of microtiter plates in triplicate. The plates were placed in the turbidity reader Bioscreen C MBR (Oy Growth Curves Ab, Helsinki, Finland). Turbidity of the cultures was monitored at 15-min intervals, or at 1-h intervals at 3°C, with agitation of the culture before each measurement. Growth curves were constructed by plotting the OD_{600} values against time. Correspondence between OD_{600} values and the number of viable bacteria of the wild type and *rpoE475*, *rpoE475*+pBlue-*tetR*-*rpoE*, and *rpoE475*+pBlue-*tetR* was verified in duplicate by inoculating 1 ml of an overnight culture grown at 28°C into 100 ml of LB or LB supplemented with 5 mM $CaCl_2$ (32), 3% NaCl, 3% ethanol, or ampicillin or with pH adjusted to 5.0 or 9.0. Bacteria were grown at 3°C, 28°C, 37°C, and 42°C with shaking, and OD_{600} and CFU/ml were determined immediately and during the growth by plating on plate count agar.

Minimum and maximum growth temperatures. The minimum and maximum growth temperatures of the wild-type strain, the *rpoE475* mutant, the complemented mutant, and the vector control were defined by using the Gradiplate W10 temperature gradient incubator (BCDE Group, Helsinki, Finland) as described previously (40), with some modifications. In brief, overnight cultures in LB broth, containing ampicillin when appropriate, originating from three separate colonies (biological replicates) were diluted 1:100 in peptone water and transferred by stamping with a piece of glass to cuvettes containing tryptic soy agar with either 1.5% (minimum growth temperature run) or 2.5% (maximum growth temperature run) agar supplemented with ampicillin when appropriate. The cuvettes were placed in the Gradiplate incubator for 10 days for measuring the minimum growth temperatures, and for 2 days for measuring the maximum growth temperatures, under aerobic conditions. The temperature gradient was set to range from 0.8°C to 9.5°C for minimum growth temperature determinations and from 29.7°C to 44.6°C for maximum growth temperature determinations. Limits of growth were detected with a Nikon SMZ-U stereomicroscope (Nikon, Tokyo, Japan) and set to the boundary of growth and no growth. Student's *t* test was used to evaluate the significance of differences between growth temperatures.

RESULTS

RNA isolation and stability of 16S *rrn*. The integrity of RNA was negatively affected by sodium chloride. Other stresses did not have substantial effects on RNA integrity. Stability of the 16S *rrn*, used as a normalization reference in the RT-qPCR analysis of *rpoE*, and the reaction efficiencies of the 16S *rrn* primers are reported in Table 3. The expression of 16S *rrn* was generally stable, and the coefficient of variance as a percentage of the C_q value varied between 1.52 and 6.82.

RT-qPCR analysis of *rpoE*. The relative expression levels of *rpoE* under different stress conditions were investigated by RT-qPCR in *Y. pseudotuberculosis* IP32953. Expression ratios of *rpoE* under different stresses are shown in Table 2. At 3°C in early logarithmic growth phase, the relative expression level of *rpoE* was 3.2-fold higher than that at 28°C in the same growth phase. In late logarithmic growth phase, there was no statistically significant difference between the relative expression levels at 3°C and 28°C. At 37°C the relative expression ratio of *rpoE* was 2.2 in both early and late logarithmic growth phases compared to its expression at 28°C

TABLE 3 Stability of the reference gene 16S *rrn* at different stress conditions at early and late logarithmic growth phases in *Yersinia pseudotuberculosis* IP32953

Parameters	Value at:			
	Early logarithmic growth phase	Late logarithmic growth phase	Both growth phases	E[a]
3°C and 28°C				1.05
Arithmetic mean (C_q)	16.91	17.00	16.96	
Geometric mean (C_q)	16.90	17.00	16.95	
Avg deviation	0.63	0.30	0.46	
CV (% C_q)[b]	3.89	2.00	3.10	
37°C and 28°C				0.94
Arithmetic mean (C_q)	18.40	18.27	18.34	
Geometric mean (C_q)	18.40	18.26	18.33	
Avg deviation	0.25	0.20	0.24	
CV (% C_q)[b]	1.60	1.23	1.48	
42°C and 28°C				0.95
Arithmetic mean (C_q)	17.83	17.64	17.74	
Geometric mean (C_q)	17.83	17.63	17.73	
Avg deviation	0.23	0.31	0.27	
CV (% C_q)[b]	1.52	2.08	1.90	
pH 5.0 and 28°C				1.04
Arithmetic mean (C_q)	13.97	13.84	13.90	
Geometric mean (C_q)	13.96	13.82	13.89	
Avg deviation	0.35	0.53	0.44	
CV (% C_q)[b]	2.84	4.28	3.65	
pH 9.0 and 28°C				0.93
Arithmetic mean (C_q)	18.38	18.13	18.25	
Geometric mean (C_q)	18.36	18.11	18.24	
Avg deviation	0.71	0.72	0.73	
CV (% C_q)[b]	4.07	4.35	4.26	
3% NaCl and 28°C				0.94
Arithmetic mean (C_q)	18.98	18.48	18.73	
Geometric mean (C_q)	18.94	18.45	18.69	
Avg deviation	1.29	1.04	1.16	
CV (% C_q)[b]	6.85	5.78	6.49	
3% ethanol and 28°C				0.95
Arithmetic mean (C_q)	17.91	17.78	17.85	
Geometric mean (C_q)	17.90	17.77	17.84	
Avg deviation	0.46	0.56	0.51	
CV (% C_q)[b]	2.88	3.35	3.14	

[a] E, reaction efficiency.
[b] CV, coefficient of variance as a percentage of the C_q value.

at the respective growth phases. At 42°C the relative expression levels of *rpoE* during early and late logarithmic growth phases were 3.9- and 1.8-fold higher, respectively, than the relative expression levels at 28°C in the corresponding growth phases. Stress caused by pH 5.0 induced the highest relative expression level of *rpoE*, 13.0-fold higher than the relative expression level at 28°C in the early logarithmic growth phase. In addition, stresses caused by pH 9.0, 3% NaCl, and 3% ethanol significantly increased the relative expression levels of *rpoE* in the early logarithmic growth phase, the ratios being 3.9, 7.2, and 8.5, respectively. Exposure to pH 5.0, pH 9.0, 3% NaCl, and 3% ethanol did not affect the relative expression levels of *rpoE* in the late logarithmic growth phase (Table 2).

Growth experiments with the *rpoE475* mutant and the complemented strain. The growth of the *rpoE475* mutant was investigated at 3°C, 28°C, 37°C, and 42°C and at 28°C at pH 5.0, pH 9.0, 3% NaCl, and 3% ethanol. At 3°C, the *rpoE475* mutant showed growth similar to that of the wild type until the late logarithmic growth phase, after which the growth of the mutant ceased (Fig. 1A). Complementation of the *rpoE* mutation by the plasmid pBlue-*tetR*-*rpoE*, containing the coding sequences of *rpoE*, *rseA*, and *rseB* and 500 bp upstream of *rpoE*, restored the wild-type level of growth (Fig. 1A). Growth of the vector control did not differ from growth of the mutant (Fig. 1A). At the optimal growth temperature of 28°C, growth of the *rpoE475* mutant did not differ from that of the wild type (Fig. 1B). At 37°C, the *rpoE475* mutant had a longer lag phase than the wild type. The complemented mutant had a shortened lag phase but slower growth than the wild-type strain (Fig. 1C). Growth of the vector control was impaired (Fig. 1C). A relatively wide variation in the OD_{600} values of the mutant, complemented mutant, and vector control was observed at 37°C (Fig. 1C). At 42°C, the *rpoE475* mutant did not reach as high an OD_{600} as the wild type (Fig. 1D). Growth of the complemented mutant was slower than that of the wild type, but OD_{600} levels similar to those for the wild type were attained, while the vector control did not grow at all (Fig. 1D). The *rpoE475* mutant and the complemented mutant had relatively large variation in their OD_{600} values at 42°C (Fig. 1D).

Under acid stress at pH 5.0, the *rpoE475* mutant had a longer lag phase than the wild type (Fig. 2A). In the complemented mutant the lag phase was restored to the wild-type level, but the growth of the complemented mutant was slower than that of the wild type (Fig. 2A). Moreover, the vector control showed impaired growth (Fig. 2A). Growth of the *rpoE475* mutant under alkali stress at pH 9.0 or under osmotic stress in 3% NaCl did not differ from growth of the wild type (Fig. 2B and C). In contrast, ethanol abolished the growth of the mutant (Fig. 2D). The growth of the complemented mutant was improved, while the vector control did not grow at all (Fig. 2D).

Minimum and maximum growth temperatures. In the minimum-growth-temperature experiment, the wild-type strain IP32953 grew over the temperature gradient (0.8 to 9.5°C) within 10 days, indicating that the minimum growth temperature of IP32953 is lower than 0.8°C. The minimum growth temperature of the *rpoE475* mutant in the time frame of 10 days was 1.2°C and thus statistically significantly higher than that of the wild-type strain ($P < 0.05$). The complemented *rpoE475* mutant had a minimum growth temperature of 1.3°C. The minimum growth temperature of the vector control was 2.5°C. The maximum growth temperature of the *rpoE475* mutant (36.1°C) within 2 days was significantly lower than that of the wild-type strain IP32953 (43.5°C, $P < 0.05$). The maximum growth temperature of the complemented mutant was 41.3°C and that of the vector control was 31.7°C. Thus, the *rpoE475* mutation was successfully complemented, but the plasmids appeared to hinder growth slightly.

DISCUSSION

We studied the role of *rpoE*, the gene encoding the alternative σ factor σ^E, under temperature, pH, osmotic, and ethanol stresses in *Y. pseudotuberculosis* IP32953. We demonstrated that the expression of *rpoE* is induced by several stresses, and a mutation in *rpoE*

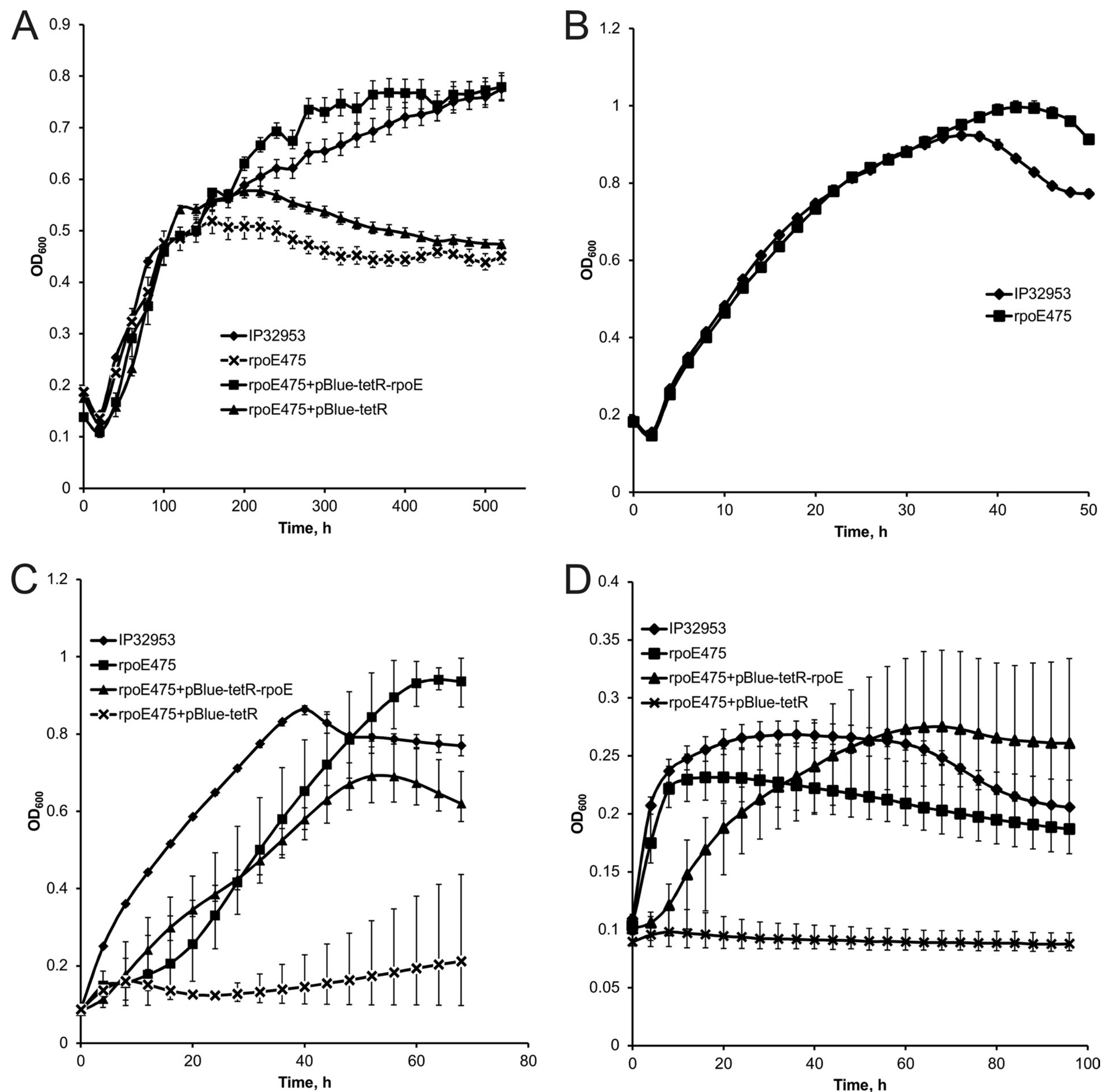

FIG 1 Growth curves of the *Yersinia pseudotuberculosis* IP32953 wild-type strain, *rpoE475* mutant strain, complemented mutant, and vector control at 3°C (A), 28°C (B), 37°C (C), and 42°C (D). Measured OD_{600} values are shown at 20-h intervals (A), at 2-h intervals (B), and at 4-h intervals (C and D). Error bars indicate minimum and maximum values.

impairs the tolerance of *Y. pseudotuberculosis* IP32953 to stresses caused by high or low temperature, low pH, and ethanol and narrows the growth temperature range.

We established by RT-qPCR that the transcription of *rpoE* is induced by low or high temperature, acidic or alkaline pH, increased osmolality, and 3% ethanol in *Y. pseudotuberculosis* IP32953. Most of these expression changes took place in the early logarithmic growth phase, which is not surprising, as many genes belonging to the σ^E regulon are involved in the synthesis of outer membrane components, which is particularly associated with active cell division (41). In *E. coli*, heat (15), increased expression of outer membrane proteins (14), unfolding or misfolding of outer membrane proteins (16), ethanol (17), and high osmolality (18) are known to induce σ^E-dependent envelope stress responses. However, in studies using DNA microarrays or reporter strains in *E. coli* and *Yersinia enterocolitica*, *rpoE* expression was not induced under the same stress response-inducing conditions, or the results were controversial (31, 42–44). Such differences in the *rpoE* expression results are probably explained by the different methods, strains, and test temperatures used. By using RT-qPCR, even small changes in gene expression can be detected (35), provided that the normalization reference gene is stable over the test conditions used. Stability of expression is demonstrated by a low coefficient of variance as a percentage of the C_q value (45). In our study, the expression of the reference gene 16S *rrn* was generally stable (Table 3) and in line with findings with *Listeria monocytogenes*, in

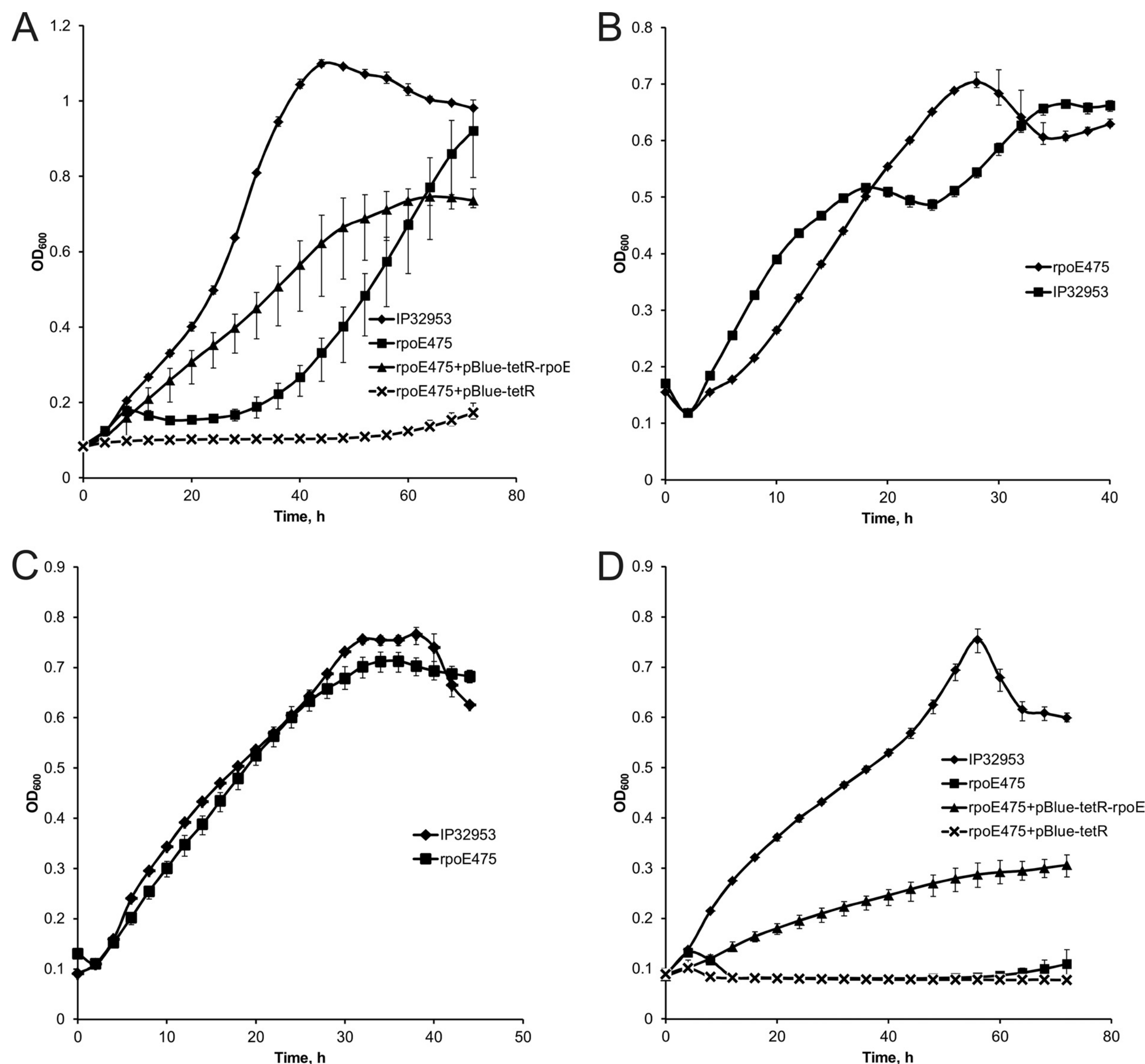

FIG 2 Growth curves of the *Yersinia pseudotuberculosis* IP32953 wild-type strain, *rpoE475* mutant strain, complemented mutant, and vector control at pH 5.0 (A), pH 9.0 (B), 3% NaCl (C), and 3% ethanol (D). Measured OD_{600} values are shown at 4-h intervals (A and D) and at 2-h intervals (B and C). Error bars indicate minimum and maximum values.

which 16S *rrn* was documented to be the most stably expressed reference gene (46). Thus, we expect our gene expression results to be reliable. This is further supported by our finding that in *Y. pseudotuberculosis*, IP32953 *rpoE* expression is increased by the known inducers of σ^E-dependent envelope stress response of *E. coli*.

Apart from transcriptional control, rapid increase in σ^E activity under stress is contributed by the release of σ^E from its anti-sigma factor RseA (19). To gain further information on the role of σ^E, we used mutational analysis and studied growth under different conditions. Growth of the *rpoE475* mutant was impaired at pH 5.0, at 3°C, at 37°C, and at 42°C, suggesting that functional *rpoE* is needed for optimal growth under acid, cold, and heat stress. Moreover, under ethanol stress the *rpoE475* mutant did not grow at all. These findings support the RT-qPCR results suggesting an important role for *rpoE* in stress responses of *Y. pseudotuberculosis*. This role was further confirmed by successful complementation of the *rpoE475* mutation with a plasmid containing the coding sequences of *rpoE*, *rseA*, and *rseB* and 500 bp upstream of the start codon of *rpoE*, and restoration of growth under the indicated stress conditions.

The growth temperature range of the *rpoE475* mutant (1.2°C to 36.1°C) was narrower than the growth temperature range of the wild-type strain, IP32953 (<0.8°C to 43.5°C). The complemented *rpoE475* mutant had a larger (1.3°C to 41.3°C) and the vector control had a narrower (2.5°C to 31.7°C) growth temperature range than the mutant alone. Large plasmids are known burdens for bacterial cells, retarding the lag phase and reducing the overall fitness of cells (47). In addition, a high copy number of a plasmid and the presence of tetracycline resistance gene in a plasmid can inhibit cell growth (48). Thus, the hampered growth of the *rpoE475* mutant carrying the complementation plasmid at stressful conditions is not surprising.

The σ^E regulon of *E. coli* and related organisms contains genes involved in pathogenesis (41). In several Gram-negative pathogens, σ^E promotes survival in the host and is thus related to pathogenesis and needed for full virulence (49). At an early stage of *Y. enterocolitica* infection in mice, *rpoE* is expressed in Peyer's patches (43, 50). In *Y. pseudotuberculosis*, σ^E regulates the Ysc-Yop type III secretion system (51). The observed dramatic effect of the *rpoE* mutation on the maximum growth temperature, which was below the mammalian body temperature in the *rpoE475* mutant, supports the proposed role of σ^E in virulence in *Y. pseudotuberculosis* (51).

In *E. coli*, *rpoE* is essential (52, 53), and its constitutive expression but not stress-related induction is indispensable for viability (54). As σ^E maintains cell envelope integrity (55), *rpoE* mutants are prone to have suppressor mutations to survive (52, 56). In *E. coli*, σ^E-mediated stress response is also needed for stress-induced mutagenesis, which increases genetic diversity and thus enables cell survival in harsh conditions (54). In *Y. enterocolitica*, *rpoE* is assumed to be indispensable because deletion mutants could not be created (43). An essential role for *rpoE* in *Y. pseudotuberculosis* YPIII has been suggested, since an *rpoE* deletion mutant was unstable (51). However, as previously stated, in *E. coli* (57) the successful complementation of the *rpoE475* mutant demonstrates that the observed phenotypic characteristics of *rpoE475* are indeed due to inactivated *rpoE* and less likely due to suppressor mutations.

Y. pseudotuberculosis can grow in wide temperature and pH ranges and thus survives well in the modern food chain. Our study demonstrates that functional σ^E is vital to tolerance of this pathogen to many stresses present during food production and storage.

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