JOURNAL OF BACTERIOLOGY, Apr. 2000, p. 2245–2252 Vol. 182, No. 8
0021-9193/00/$04.00+0
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OmpR Regulates the Stationary-Phase Acid Tolerance Response of *Salmonella enterica* Serovar Typhimurium

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Received 12 November 1999/Accepted 24 January 2000

Tolerance to acidic environments is an important property of free-living and pathogenic enteric bacteria. *Salmonella enterica* serovar Typhimurium possesses two general forms of inducible acid tolerance. One is evident in exponentially growing cells exposed to a sudden acid shock. The other is induced when stationary-phase cells are subjected to a similar shock. These log-phase and stationary-phase acid tolerance responses (ATRs) are distinct in that genes identified as participating in log-phase ATR have little to no effect on the stationary-phase ATR (I. S. Lee, J. L. Slouczewski, and J. W. Foster, J. Bacteriol. 176:1422–1426, 1994). An insertion mutagenesis strategy designed to reveal genes associated with acid-inducible stationary-phase acid tolerance (stationary-phase ATR) yielded two insertions in the response regulator gene *ompR*. The *ompR* mutants were defective in stationary-phase ATR but not log-phase ATR. EnvZ, the known cognate sensor kinase, and the porin genes known to be controlled by OmpR, *ompC* and *ompF*, were not required for stationary-phase ATR. However, the alternate phosphodonor acetyl phosphate appears to play a crucial role in OmpR-mediated stationary-phase ATR and in the OmpR-dependent acid induction of *ompC*. This conclusion was based on finding that a mutant form of OmpR, which is active even though it cannot be phosphorylated, was able to suppress the acid-sensitive phenotype of an *ack pta* mutant lacking acetyl phosphate. The data also revealed that acid shock increases the level of *ompR* message and protein in stationary-phase cells. Thus, it appears that acid shock induces the production of OmpR, which in its phosphorylated state can trigger expression of genes needed for acid-induced stationary-phase acid tolerance.

Bacteria in nature are often exposed to dramatic fluctuations in external pH that threaten viability. Survival, therefore, depends on the presence of adaptive mechanisms that sense an acidifying environment and coordinate an appropriate molecular response (15, 17). *Salmonella enterica* serovar Typhimurium employs several strategies to avoid or repair damage associated with acid stress. Two major low-pH-inducible systems, known as acid tolerance responses (ATR), have been identified. They are classified based on the growth phase at which each becomes induced. Most studies have focused on the log-phase ATR system induced when exponentially growing cells suddenly undergo a rapid transition to low pH (16). Over 50 acid shock proteins (ASPs) are produced during this response (14). The regulatory genes *rpoS*, encoding an alternative sigma factor, and *fur*, encoding the major iron regulator, are required for log-phase acid tolerance and control the production of subsets of the ASPs (15, 17, 19, 33).

The second ATR system, referred to as the stationary-phase ATR, is induced by exposing stationary-phase cells to low pH (34). It is distinct from the general stress response system that is induced by entry into stationary phase regardless of the culture pH. The general stress response system requires stationary-phase induction of the alternative sigma factor σ^S, while the acid-induced stationary-phase ATR does not. The Fur protein, also required for log-phase ATR, is not involved in the stationary-phase ATR, indicating that the two acid-inducible acid tolerance systems are functionally distinct. Consistent with this idea, 10 stationary-phase ASPs have been found that are not log-phase ASPs (34). However, the identities of these proteins and their genetic regulation have not been characterized. In this study, we present the first report of an acid-induced gene required for the stationary-phase ATR of serovar Typhimurium.

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MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or minimal E medium containing 0.4% glucose (EG medium) (55). Buffered LB broth contained 100 mM MOPS (morpholine propanesulfonic acid) buffer for pH 8 medium or MES (morpholineethanesulfonic acid) for pH 5 medium. The pHs of minimal E media used for moderate-acid (pH 4.3) and extreme-acid (pH 3.0) exposures was adjusted with HCl. Antibiotics were used at the concentrations of 30 μg/ml for ampicillin, 20 μg/ml for kanamycin, and 20 (rich medium) and 10 (minimal medium) μg/ml for tetracycline.

Genetic manipulations. General transduction was performed with P22 HT105/int, and nonlysogenic segregants were identified by sensitivity to P22 H5 (37). The Mu*d*J insertion library in UK1 cells (SF530) was generated by the technique of transitory *cis* complementation outlined by Hughes and Roth (25).

Assay of acid-inducible stationary-phase- and log-phase-specific ATR. Acid-induced stationary-phase ATR was measured as previously reported with the following modifications (34). Cells were grown overnight in 3 ml of minimal E medium (pH 8.0; 37°C; shaking). A 500-μl sample of each strain to be tested was harvested by centrifugation, washed in an equal volume of pH 3.0 (EG) broth (for unadapted culture) or pH 4.3 EG broth (for adapted culture), and reharvested, and the pellets were resuspended to 2×10^8 cells/ml in EG broth (3 ml) at the same pH. Viable counts were made to confirm the cell density of each resuspended culture by plating dilutions onto LB agar. Adapted cultures were incubated for 2 h and then washed and resuspended in pH 3.0 EG broth for challenge. Aliquots were collected at timed intervals, and viable counts were measured by serial dilution and plating on LB agar. The results are representative of triplicate experiments with variability observed within 50% of the reported value.

Log-phase ATR assays were conducted using strains grown overnight at 37°C in EG broth containing the appropriate antibiotic. A 1/100 dilution of the overnight broth was inoculated into 3 ml of EG broth, pH 7.7, and incubated at 37°C with shaking. The cells were grown to an optical density at 600 nm of 0.40 (2×10^8 CFU/ml), at which point cultures to be adapted were adjusted with HCl to pH 4.4 and incubated for 60 min. Acid challenge of unadapted and adapted cultures involved readjusting the pH to 3.1 (HCl) for the indicated time. CFUs were calculated following dilution and plating of the cultures on LB agar. Percent

TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Source
E. coli		
EK370	MC4100/pLAN701 (*ompR* of *E. coli* cloned into pACYC177)	31
EK371	MC4100/pLAN702 (*ompRD55E* of *E. coli* cloned into pACYC177)	31
EK372	MC4100/pLAN801 (*ompR* of *E. coli* cloned into pUC19)	31
Serovar Typhimurium		
SF241(CH511)	LT2 *oppBCD250 leu-1151*::Tn*10 ompR*::Tn*5*	18
SF261(TT10287)	LT2 *hisD*::Mu*d*J *his-9941*::Mu*d*1[a]	25
SF463(TT10423)	LT2 *proAB47*/F′ *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10D16D17*Tc^r	K. Sanderson
SF464(TT10427)	LT2/pNK972 (Tn*10* transposase; Ap^r)	K. Sanderson
SF465(TT10604)	LT2 *proAB47*/F′ *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10Δ16Δ17*Cm^r	K. Sanderson
SF500	14028S *rpoS*::pRR10-250V (Ap^r)	F. Fang
SF530(χ3761)	Wild-type UK1	7
SF586(JR501)	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 rpsL120 xyl-404 galE719* H1-6 h2 -e, n, x *nml* (Fels2)$^-$ *fla-66*	5
SF680(TE6153)	*puPA1303*::Km^r *-katE-lac*(op)	4
SF681(JS91)	14028S *ack408*::Tn*10d*Tc	54
SF682(JS105)	14028S *pta209*::Tn*10d*Tc	54
SF790(CH1118)	LT2 *oppBCD*250 *leu3051 envZ*::Mu*d*J	K. Sanderson
SF797	LT2 *supD-zeb-609*::Tn*10 ompF1004*::Mu*d*J	B. Finlay
SF798	SL1344 *ompC396*::Tn*10 ompF1006*::Mu*d*A *tppB83*Mu*d*J	B. Finlay
SF819	SF586/pRDQ55 (*ompRD55Q* of *E. coli* cloned into pBR322)	39
YK3092	UK1 *ompR132*::Mu*d*J	SF261 × SF530
YK3230	UK1 *ompR*::Tn*5*	SF241 × SF530
JF2651	LT2 *ompC9*::Mu*d*A	
JF2757	UK1 *ompR43*::Mu*d*J	SF261 × SF530
JF3063	UK1 *ompR43*::Mu*d*J *rpoS*::*Ap*	SF500 × JF2757
JF3266	UK1 *putPA1303*::Km^r *-katE-lac*(op)	SF680 × SF530
JF3269	UK1 *ack408*::Tn*10d*Tc	SF681 × SF530
JF3271	UK1 *pta209*::Tn*10d*Tc	SF682 × SF530
JF3298	UK1 *ack408*::Tn*10d*Tc *putPA1303*::Km^r *-katE-lac*(op)	SF680 × JF3269
JF4240	UK1 *envZ*::Mu*d*J (Lac$^-$)	SF790 × SF530
JF4289	UK1 *ompC9*::Mu*d*A	JF2651 × SF530
JF4290	UK1 *ompC9*::Mu*d*A *envZ*::Mu*d*J (Lac$^-$)	JF2651 × JF4240
JF4336	UK1 *ompR43*::Mu*d*J *ack408*::Tn*10d*Tc	JF2757 × JF3269
JF4337	UK1 *ompR43*::Mu*d*J *pta209*::Tn*10d*Tc	JF2757 × JF3271
JF4339	UK1 *ack408*::Tn*10d*Tc *putPA1303*::Km^r *-katE-lac*(op)/pNK972 (Tn*10* transposase; Ap^r)	SF464 × JF3298
JF4344	UK1 *ompF1004*::Mu*d*J	SF797 × SF530
JF4352	UK1 *ompF1004*::Mu*d*J *ompC396*::Tn*10*	SF798 × JF4344
JF4369	UK1 *ack408*::Tn*10d*Tet *pta*::Tn*10d*Cm *putPA1303*::Km^r *-katE-lac*(op)	(SF465 × JF4339) × JF3266
JF4370	UK1 *ompR43*::Mu*d*J *ack408*::Tn*10d*Tet *pta*::Tn*10d*Cm	JF4369 × JF2757
JF4371	UK1 *ompR43*::Mu*d*J/pLAN701 [OmpR$_{EC}$]	EK370 × JF2757
JF4372	UK1 *ompR43*::Mu*d*J/pLAN702 [OmpR$_{EC}^{D55E}$]	EK371 × JF2757
JF4373	UK1 *ompR43*::Mu*d*J/pLAN801 [OmpR$_{EC}$]	EK372 × JF2757
JF4414	UK1 *ack-408*::Tn*10d*Tet *pta*::Tn*10d*Cm	JF4369 × SF530
JF4419	UK1 *ompR43*::Mu*d*J *ack-408*::Tn*10d*Tet *pta*::Tn*10d*Cm/pLAN701	EK370 × JF4370
JF4420	UK1 *ompR43*::Mu*d*J *ack408*::Tn*10d*Tet *pta*::Tn*10d*Cm/pLAN702	EK371 × JF4370
JF4450	UK1 *ompR43*::Mu*d*J/pRDQ55	SF819 × JF2757
JF4452	UK1 *ompR43*::Mu*d*J *ack408*::Tn*10d*Tet *pta*::Tn*10d*Cm/pRDQ55	SF819 × JF4370
JF4479	UK1 *ompR43*::Mu*d*J/pAC2005S	pAC2005S × JF2757
JF4481	UK1 *ompC9*::Mu*d*A *ack-109*::Tn*10d*Tc *pta*::Tn*10d*Cm	JF2651 × JF4414
JF4482	UK1*ompC9*::Mu*d*A *ack-109*::Tn*10d*Tc *pta*::Tn*10d*Cm *envZ*::Mu*d*J	JF4240 × JF4481
Plasmids		
pAC2005s	EnvZ$_{EC}$ in pACYC184	52
pLAN701	OmpR$_{EC}$ in pACYC177	31
pLAN702	OmpR$_{EC}^{D55E}$ in pACYC177	31
pLAN801	OmpR$_{EC}$ in pUC19	31
pNK972	pBR333 Ap^r containing Tn*10* transposase	43
pRDQ55	OmpR$_{EC}^{D55Q}$ in pBR322	39

[a] Mu*d*1 and Mu*d*A confer Ap^r; Mu*d*J confers Km^r.

survival was calculated by dividing the CFUs at time points post-acid challenge by the CFUs prior to acid challenge and multiplying by 100.

Cloning of *ompR*-Mu*d*J junctions. The left ends of Mu*d*J junctions were cloned from chromosomal digests of the acid-sensitive mutants JF2757 and YK3092 by first identifying the sizes of *Sal*I restriction fragments containing the kanamycin resistance gene via Southern blot hybridization with a kanamycin gene probe. The appropriate-size fragments were excised and extracted from an agarose gel and ligated to *Sal*I-digested pBluescript SK(+) vector (Stratagene, La Jolla, Calif.). The ligated mixtures were transformed ($CaCl_2$ method) into XL1-Blue (EK112), selecting for ampicillin resistance. Sequencing of the junction sites was

performed using an oligonucleotide on the left end of Mu (Oligo 47, 5′CCAA TGTCCTCCCGGTTTTT).

Western blot analysis. OmpR protein levels were determined through Western blot analysis as previously described by Lee et al. (33). Cells were grown at 37°C for 18 h in 3 ml of EG (pH 8.0) medium (12- by 100-mm test tube with shaking), washed once, and resuspended to 2×10^8 cells/ml in pH 4.4 EG medium for acid shock adaptation. Samples were removed at timed intervals, harvested, and resuspended in 0.01% sodium dodecyl sulfate (SDS) solution. After the total protein in each sample was quantified (Bio-Rad [Hercules, Calif.] protein assay), an equal amount (5 μg) of total protein from each sample was mixed with 2× SDS-polyacrylamide gel electrophoresis loading buffer (125 mM Tris [pH 7.0], 20% glycerol, 10% β-mercaptoethanol, 6% SDS, 0.2% bromophenol blue), boiled for 5 min, and then electrophoresed through an SDS–10% polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes (Millipore Co., Bedford, Mass.) using 3-[cyclohexylamino]-1-propanesulfonic acid transfer buffer (pH 11.0) and a semidry transfer unit (Hoeffer Scientific Instruments) for 1 h at 100 V. Nonspecific protein interactions were blocked with 5% powdered milk in TBST buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.05% [vol/vol] Tween 20) for 2 h at room temperature. The membrane was probed with a 1:5,000 dilution of polyclonal antiserum against OmpR (courtesy of C. Park [27]) in TBST buffer for 1 h at room temperature. The blots were washed several times in TBST buffer and then incubated with a 1:2,000 dilution of secondary anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma Co.) in TBST buffer for 1 h. The proteins were visualized by using a chemiluminescence kit (ECL Western blotting detection reagent; Amersham-Pharmacia Biotech, UK Ltd., Piscataway, N.J.) following the manufacturer's specified protocol. Following autoradiography, densitometry was used to analyze relative levels of OmpR protein and message using Scion Image software (Scion Co., Frederick, Md.).

Northern blot analysis. Cells were grown at 37°C for 18 h in 3 ml of pH 8.0 EG medium, washed once, and resuspended to 2×10^8/ml in pH 4.4 EG medium (for acid-adapted cells) or pH 8.0 EG medium (for unadapted cells). Cell aliquots were removed at various time intervals and immediately frozen until cell lysis. Total RNA was extracted by the method of Laoide and Ullman (32) with modifications (29). Cultured cells (20 ml) were collected and resuspended in a solution of 5 ml of 20 mM sodium acetate (pH 5.5), 1 mM ethylenediaminetetraacetic acid, and 0.5% SDS. Then, 5 ml of acidic phenol saturated with 20 mM Na acetate preheated to 65°C was added to the reaction solution and equilibrated at 65°C for 5 min. The aqueous phase was reextracted with acidic phenol until no visible residue was apparent at the interface. The RNA was precipitated by adding KCl to 0.1 M final concentration and adding 3 volumes of ethanol. RNA was collected by centrifugation after overnight incubation at −20°C and resuspended in 100 μl of RNase-free water. RNA samples were separated by electrophoresis in a denaturing formaldehyde-agarose gel and transferred to nylon membrane (Amersham-Pharmacia). The membranes were probed with a PCR product of *ompR* (5′-CAATCGCCTCATGCTTTAGA for the forward primer and 5′-TTGCGAACCTTTGGGAGTA for the reverse primer) labeled with [α-^{32}P]dCTP (Amersham) using a random-primed-DNA labeling kit (Boehringer-Mannheim Co.) and a 23S ribosomal DNA probe (5′-GGTGTCGACT ATGAACCTGCTTCCCATCGACTAC) end labeled with [γ-^{32}P]dATP.

RESULTS

Isolation of mutants defective in stationary-phase ATR. The method used to isolate stationary-phase ATR mutants involved a brute force screening of a 10,000-member Mu*d*J insertion library constructed in UK1 cells. Microtiter wells containing LB broth (pH 5.0) were inoculated with individual insertion mutants. Overnight culture in this medium produces adapted, acid-tolerant cells. Samples from each well were transferred with a multipronged replicator to pH 2.5 EG broth for acid challenge. The survivors were rescued from acid challenge by replicating them to pH 7 LB plates at 1, 2, and 4 h postchallenge. Mutants that failed to survive compared to controls were taken from the original stock microtiter plate and retested. Although several mutants isolated by this screening procedure showed modest decreases in stationary-phase acid tolerance (5- to 10-fold lower than that of wild-type cells [data not shown]), two atr_{SP} mutants, JF2757 and YK3092, proved to be very acid sensitive (Fig. 1A). Adapted cultures of these mutants exhibited approximately 500- to 1,000-fold less survival than UK1 cells after 4 h at pH 3. Figure 1A also illustrates that the acid-inducible stationary-phase ATR does not require RpoS (Fig. 1A, UK1 versus JF2690). The *rpoS* mutant JF2690 did exhibit a decrease in unadapted acid tolerance (Fig. 1A), as would be expected from the role of RpoS in stationary-phase general stress resistance (<0.0002% for the *rpoS* mutant JF2690 versus 0.25% for UK1 after 4 h at pH 3). However, the *rpoS* mutant was still able to adapt (Fig. 1A, JF2690) to normal levels in response to acid shock (10 to 20% survival after 4 h at pH 3). The results indicate that RpoS is required for the basal levels of acid tolerance provided by entry into stationary phase but is not required for subsequent acid-induced tolerance of low pH. In contrast to the situation with stationary-phase cells, the atr_{SP} mutants possessed a normal log-phase ATR (Fig. 1B, JF2757) (unadapted, 0.08%; adapted, 70%) while the *rpoS* mutant was acid sensitive, as previously described (30) (Fig. 1B, JF2690).

Identification of *ompR* as an atr_{SP} gene. The gene affected in the atr_{SP} mutants was identified by cloning the Mu*d*J junctions from chromosomal digests as described in Materials and Methods. DNA sequence analysis of these clones showed that both mutants contained a Mu*d*J insertion in the *ompR* structural gene. JF2757 and YK3092 insertions occurred 43 and 132 bp, respectively, after the first base of the *ompR* open reading frame. The *ompR* gene is the response regulator of a two-component system known to regulate the osmotically controlled genes *ompC* and *ompF*, which encode two major outer membrane proteins (11, 51). However, neither of these known OmpR-regulated genes, either singly (data not shown) or in combination (Fig. 1A, JF4352 versus UK1), had any effect on stationary-phase ATR, indicating that OmpR-dependent genes other than *ompC* or *ompF* are involved in stationary-phase acid tolerance (Fig. 1A). Figure 2 shows that plasmids expressing *ompR* (pLAN701 and pLAN801) complemented the acid-sensitive phenotype of the *ompR*::Mu*d*J insertions, confirming a role for OmpR in acid-inducible stationary-phase acid tolerance. Plasmids pUC19 and pACYC177, backbone vectors for the pLAN plasmids, did not complement the *ompR* mutation (data not shown).

It should be noted that the low survival levels of the stationary-phase *ompR rpoS* double mutant (Fig. 1A, JF3063 versus UK1) reflect the simultaneous losses of basal acid tolerance afforded by the RpoS system and the acid-induced acid tolerance requiring OmpR. Although this strain exhibited a severely diminished stationary-phase ATR, the mutations did not completely eliminate acid-inducible stationary-phase acid tolerance (Fig. 1A, JF3063). A small yet reproducible acid-inducible stationary-phase ATR was still evident. This result indicates the presence of an OmpR-independent, acid-inducible acid tolerance system that functions in stationary phase.

OmpR is an acid shock-inducible protein. Since the stationary-phase ATR requires de novo protein synthesis, we questioned whether *ompR* might itself be an acid shock protein. Western blot results presented in Fig. 3A revealed that OmpR production increased approximately fourfold within 30 min in stationary-phase cells after acid shock at pH 4.4. No increase was observed over the same time period when cells were resuspended in pH 8 EG broth (data not shown). The protein shown in the figure proved to be OmpR, since it was not detected in *ompR* mutant extracts probed with anti-OmpR antibody. Thus, OmpR is a stationary-phase ASP. However, RpoS, responsible for the stationary-phase induction of many proteins, was not required for this induction (data not shown). Northern blot analysis indicated that the amount of *ompR* message increased dramatically in response to acid shock (Fig. 3B, lanes 1 through 5). This message was not detected in an *ompR* mutant (Fig. 3B, lane 6). Following acid shock, levels of OmpR message in UK1 cells began to increase within 15 min and reached a maximum by 30 min. In contrast, placing stationary-phase cells at pH 8 did not induce an accumulation of OmpR message (Fig. 3B, lane 7). Whether the acid shock-

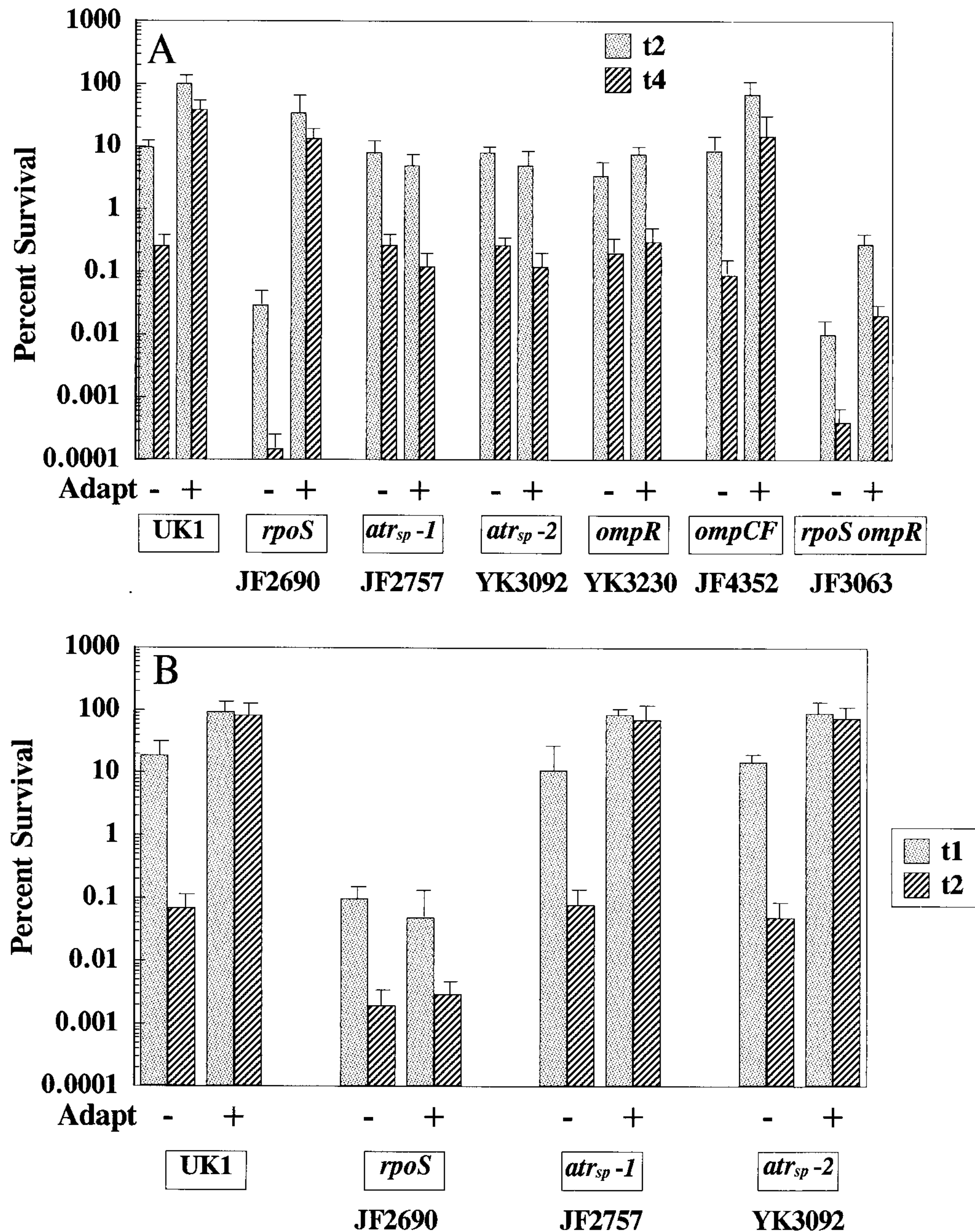

FIG. 1. Mutations in *ompR* result in defective stationary-phase acid tolerance. (A) Cells were grown in minimal EG medium overnight. Unadapted cells (−) were immediately washed and resuspended in fresh pH 3 EG medium to 2×10^8 CFU/ml. Adapted cells (+) were washed and resuspended in pH 4.4 EG medium for adaptation (2 h), after which the cells were washed and resuspended in pH 3 EG medium for challenge. Viable counts were taken at 2 (t2) and 4 (t4) h after challenge. Cell density at t_0 was approximately 2×10^8 CFU/ml. (B) Cells were grown to log phase (2×10^8 CFU/ml) in pH 7.7 EG medium. Unadapted cell cultures were immediately adjusted to pH 3.1. Adapted cultures were adjusted to pH 4.4 for 60 min and then readjusted to pH 3.1. The data represent the means of triplicate experiments with variations ranging less than 50% of the stated percent survival value. Viable counts were taken at 1 (t1) and 2 (t2) h after challenge.

induced increase in OmpR message was the result of increased transcription or decreased message turnover is not known.

Although neither *envZ*, the gene downstream of *ompR* encoding the sensor kinase of the OmpR-EnvZ two-component system, nor acetyl phosphate, known to phosphorylate OmpR in the absence of EnvZ, is known to be involved with the transcription of *ompR*, both were checked to determine if they might be involved in the acid shock increases in OmpR. Mutants lacking EnvZ or acetyl phosphate (*ack pta*) exhibited normal acid shock induction of OmpR (data not shown).

The effect of EnvZ and acetyl phosphate synthesis on acid-induced stationary-phase acid tolerance. OmpR is a well-known and extensively studied transcriptional activator that is part of a bacterial two-component regulatory system (10). Considered to be active only in its phosphorylated form (OmpR-P), OmpR is phosphorylated at residue Asp-55 by its cognate histidine kinase, EnvZ (1, 8), or by other cellular phosphate donors, such as acetyl phosphate (40). Therefore, it is reasonable to predict that increased levels of OmpR might require activation by phosphorylation to regulate other atr_{SP} genes. To

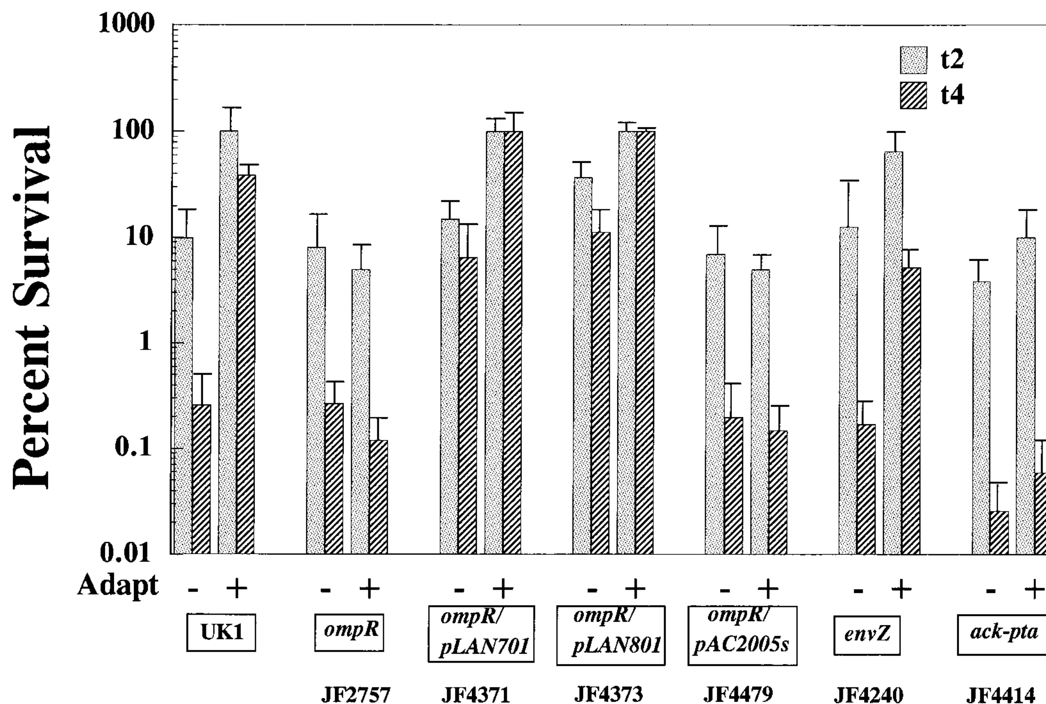

FIG. 2. Effects of *envZ* and acetyl phosphate (*ack-pta*) mutations and cloned $ompR^+$ on stationary-phase ATR. Stationary-phase ATR was measured as described in the legend to Fig. 1A. pLAN701, *ompR* in pACYC177 (medium copy number); pLAN801, *ompR* in pUC19 (high copy number); pAC2005, $envZ_{EC}$. pAC2005 was able to complement a chromosomal *envZ* mutation and regulate serovar Typhimurium *ompC-lacZ* expression. Viable counts were taken at 2 (t2) and 4 (t4) h after challenge.

explore this hypothesis, we tested the stationary-phase acid tolerance of an *envZ* mutant lacking the cognate histidine kinase and of an *ack-pta* double mutant that does not synthesize acetyl phosphate. The *envZ* mutant (Fig. 2, JF4240), which still expresses OmpR, showed a very slight decrease in acid tolerance (<10-fold) relative to UK1, suggesting that EnvZ does not play a major role in signaling activation of the OmpR-dependent, acid-inducible, stationary-phase acid tolerance system (stationary-phase ATR). This also confirmed that the acid-sensitive phenotype of the *ompR* insertion mutation was not due to a polar effect on *envZ* expression. Consistent with this conclusion was the finding that EnvZ expressed from a plasmid did not affect acid tolerance in *ompR* mutant or wild-type backgrounds (Fig. 2, JF4479 versus UK1).

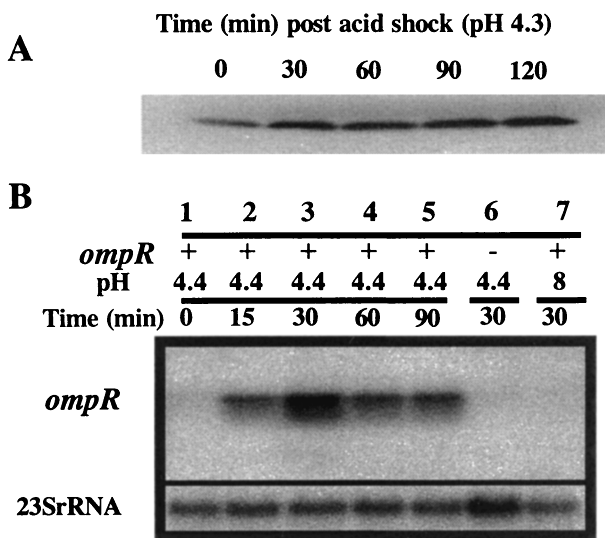

FIG. 3. Acid induction of *ompR*. (A) Western blot analysis. Cells (UK1) were grown for 18 h in EG minimal medium, washed, and resuspended to 2×10^8 in pH 4.4 EG medium. The cells were harvested at the times indicated and processed for Western blot analysis using anti-OmpR antibody as described in Materials and Methods; 5 μg of protein was added per lane. (B) Northern blot analysis. Cells (UK1, $ompR^+$ [lanes 1 to 5 and 7]; JF2757, *ompR*::Mu*dJ* [lane 6]) were grown and acid shocked as for panel A. As a control, stationary-phase UK1 cells were processed as for acid shock but instead of pH 4.4, they were placed in a pH 8 medium (lane 7). At the times indicated, the samples were harvested and processed for Northern blot analysis as described in Materials and Methods; 5 μg of RNA was added per lane. 23S rRNA hybridization was used as a control. +, present; −, absent.

In contrast to *envZ*, the *ack pta* double mutant, which expresses *envZ* normally, proved to be acid sensitive to the same degree as an *ompR* mutant (Fig. 2, JF4414 versus JF2757). However, a mutant lacking OmpR and acetyl phosphate was not any more acid sensitive than mutant lacking OmpR or acetyl phosphate alone (data not shown). This is consistent with acetyl phosphate acting through OmpR, although these results do not exclude the possibility that acetyl phosphate also affects the ATR independently of OmpR (see below). We also examined the acid tolerance of an *ack pta envZ* mutant and found it was no more acid sensitive than the *ack pta* mutant (data not shown). Thus, EnvZ does not play an obvious role in controlling the OmpR-dependent, acid-induced stationary-phase ATR.

To address whether acetyl phosphate acts via OmpR, we tested two mutant forms of *E. coli* OmpR in which aspartate residue 55, the phosphorylation target site, had been changed via site-directed mutagenesis to residues that cannot be phosphorylated (31). When placed in an *ompR ack pta* mutant, a plasmid carrying wild-type $ompR^+$ did not rescue the acid-sensitive phenotype (Fig. 4, JF4419 versus JF2757). However, when the normally acid-sensitive *ompR ack pta* cells contained a plasmid expressing $ompR^{D55E}$, they adapted well to acid stress (Fig. 4, JF4420 versus JF2757). While this mutant form of OmpR cannot be phosphorylated at residue 55, it has been

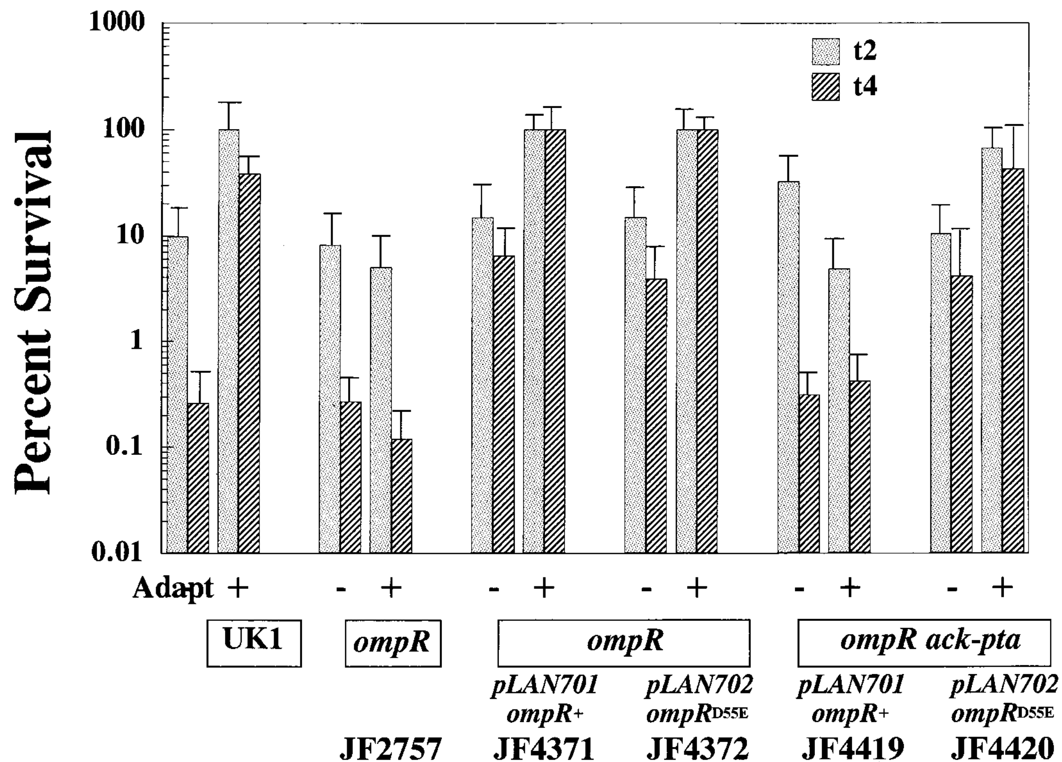

FIG. 4. Phosphorylation-independent *ompR*^{D55E} compensates for the loss of acetyl phosphate in stationary-phase ATR. Stationary-phase ATR was measured as described in the legend to Fig. 1A.

reported to be an active form of OmpR that does not require phosphorylation (31). Another mutant form of *ompR*, *ompR*^{D55Q}, did not act to restore acid tolerance to an *ack pta* mutant (data not shown). The results suggest that the effect of acetyl phosphate on acid tolerance involves OmpR and probably does not occur by a mechanism independent of OmpR. The data support a model in which acetyl phosphate is a phosphodonor for OmpR under acid shock conditions and the hypothesis that phosphorylated OmpR is required for an optimal, acid-inducible, stationary-phase ATR.

Acetyl phosphate is required for acid induction of *ompC*. Expression of the outer membrane porin gene *ompC* is OmpR dependent and is induced by low pH in serovar Typhimurium. We used *ompC-lacZ* as a reporter for OmpR activity to confirm a role for acetyl phosphate as a potential phosphodonor under acid conditions. JF4289 grown to log phase in minimal glucose at pH 5.8 induced three times as much *ompC-lacZ* as at pH 7.7 (Fig. 5). An *envZ* mutation lowered overall expression, but *ompC* was now induced 15-fold by growth at low pH. This result indicates that while EnvZ contributes to *ompC* expression, the acid induction of *ompC* was EnvZ independent, suggesting the presence of an alternative phosphodonor. Acetyl phosphate appears to fill this role, since a mutant lacking both EnvZ and acetyl phosphate failed to express *ompC* under either acid or alkaline conditions. However, an *ack pta* mutant lacking acetyl phosphate but possessing EnvZ managed normal log-phase acid induction of *ompC* at pH 5.8. So, while acetyl phosphate proved to be essential for the pH 4.4-induced stationary-phase ATR, it was not needed for acid induction of *ompC-lacZ* in log-phase cells. Nevertheless, the data using exponential-phase cells grown at pH 5.8 support a model in which acetyl phosphate will serve as an OmpR phosphodonor under acidic conditions. It is not clear why the EnvZ present in the acetyl phosphate mutant enabled acid induction of *ompC-lacZ* in log-phase cells grown at pH 5.8 but participated little in pH 4.4 acid-induced stationary-phase acid tolerance. It may be that EnvZ will not function in stationary-phase cells at the internal pH (ca. 6.5) generated after a pH 4.4 acid shock. In this situation, acetyl phosphate may be the primary phosphodonor.

DISCUSSION

Inducible acid tolerance in serovar Typhimurium is a complex phenomenon involving log-phase and stationary-phase

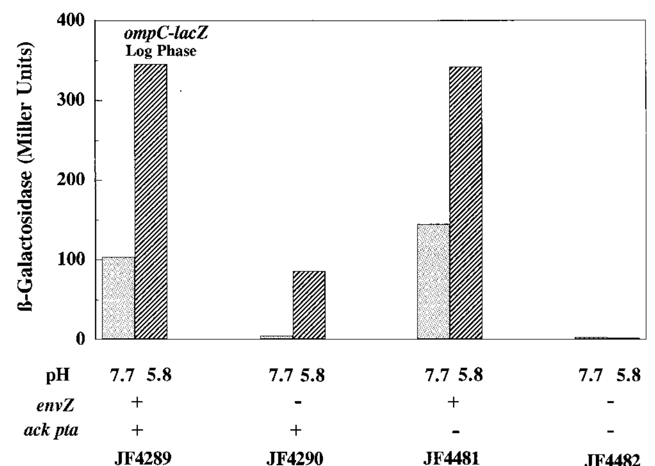

FIG. 5. Effect of *envZ* and acetyl phosphate on acid induction of *ompC-lacZ* in exponential-phase cells. The cells were grown to approximately 10^8 per ml at 37°C in EG medium (pH 7.7 or 5.8 as indicated). β-Galactosidase activity was assayed according to the method of Miller (41). The values are representative of triplicate experiments. +, present; −, absent.

systems. Both growth phase-dependent acid tolerance systems are induced by acid shock regimens in which cells grown at pH 7.7 or 8.0 are subjected to rapid or gradual acidic transitions to pH 4.5. Once induced, the ATR systems will protect cells for extended periods of time against pH 3 stress. Fifty log-phase ASPs have been noted on two-dimensional gels (13, 14), subsets of which are controlled by RpoS, PhoP, or Fur (2, 19, 33). Acid-inducible log-phase and stationary-phase ATR are separate systems based on the fact that mutations having a dramatic effect on log-phase acid tolerance, namely, *rpoS* and *fur*, have little effect on stationary-phase acid-inducible acid tolerance. In addition, 10 unique stationary-phase ASPs have been identified (34). Prior to this report, no gene participating in the stationary-phase ATR had been identified. Our results indicate that *ompR* and genes associated with the synthesis of acetyl phosphate are important for effective acid induction of a stationary-phase ATR.

The EnvZ-OmpR regulatory system is a paradigm of intracellular signal transduction involving two common families of signaling components, sensor histidine kinases and response regulators, that communicate by phosphotransfer mechanisms (12, 42). EnvZ, a transmembrane protein, is thought to sense various environmental signals, such as high osmolarity. Upon sensing a signal, EnvZ phosphorylates itself at histidine residue 243 (28, 48) and then transfers the phosphate to aspartate 55 of OmpR (8). How EnvZ senses environmental change is unclear, since the periplasmic portion of the protein is apparently not required (35). EnvZ also possesses a phosphatase that will remove phosphate from OmpR-P (22, 26, 27).

The conventional model for OmpR regulation holds that the degree of phosphorylation of OmpR governs the expression of *ompC* and *ompF* (1). Phosphorylation induces a conformational change in OmpR (29), but how this change influences OmpR control of *ompC* and *ompF* is not clearly understood (20). Conformational reshaping of OmpR may increase DNA binding affinity to the *ompC* and *ompF* promoters and enable interaction of OmpR with the α subunit of RNA polymerase, thereby activating transcription (29).

A variety of studies indicate that alternative phosphodonors are capable of phosphorylating OmpR in the absence of EnvZ (23, 39, 44, 45). One proven alternative phosphodonor is acetyl phosphate, although a primary role for this compound in the in vivo phosphorylation of OmpR has only been suggested for *flhDC* (40, 50). We have now demonstrated that the regulatory protein OmpR plays an integral role in controlling acid induction of the stationary-phase ATR. Acid shock leads to a significant increase in *ompR* message and OmpR protein. The data suggest that OmpR-P, formed from acetyl phosphate as the phosphodonor, is the form required to induce acid tolerance. EnvZ does not play a primary role in OmpR-dependent induction of acid tolerance. These conclusions are based on several findings. First, the *envZ* mutant exhibited a normal acid-inducible acid tolerance. Second, although the acid-sensitive *ompR*::Mu*dJ* mutant was deficient in both OmpR and EnvZ, plasmids containing only *ompR* were able to complement the acid-sensitive phenotype. Third, in contrast to other systems in which acetyl phosphate plays a role in OmpR phosphorylation only in the absence of EnvZ, the *ack pta* mutant proved to be acid sensitive even in the presence of EnvZ (although it might have been an inactive EnvZ due to low internal pH following acid shock). Finally, a constitutively active OmpR ($\mathrm{OmpR}^{\mathrm{D55E}}$) complemented the acid-sensitive phenotype of an *ack pta* mutant while a wild-type OmpR would not, suggesting that acetyl phosphate acts through OmpR.

Although the molecular details of EnvZ-OmpR signaling have been extensively examined, it has proven difficult to identify a clear physiological consequence associated with the loss of OmpR. The EnvZ-OmpR system, in response to various environmental stresses, appears to influence nutrient availability by changing the ratio of two porins (OmpC and OmpF) that produce pores of different sizes. Studies have also connected OmpR with flagellar expression (50), cell division (47), fatty acid transport (21), microcin synthesis (38), curli fibers (49), and *Salmonella* virulence (3, 6, 9, 36). One mechanism by which OmpR may affect virulence is through its involvement in controlling cytotoxicity toward infected macrophages (36). We can now add a role for OmpR in acid tolerance, although precisely what genes are regulated and how they provide acid tolerance is unknown. Several genes with very different functions (*ompC*, *ompF*, *flhDC*, *fadL*, *tppB*, *csgD*, and the plasmid-encoded *mcb*) are known to be regulated by OmpR, confirming that OmpR has an effect on cell physiology beyond its role in governing porin expression (18, 21, 38, 49, 50).

To our knowledge, this is only the second report that *ompR* itself is regulated by environmental stress and the first indicating control by acid pH (30). Several questions regarding this control remain unanswered. How does acid shock induce *ompR*? In *Escherichia coli*, CRP and cyclic AMP (cAMP) have been shown to affect transcription of *ompR* from four potential start sites (24). Two of the transcripts are negatively regulated by CRP-cAMP, while the other two are positively regulated by this complex. In addition, integration host factor (IHF) has been shown to bind to the promoter region and inhibit transcription (53). Whether acid shock alters cAMP levels or influences IHF interaction with the *ompR* promoter is unknown. However, other acid pH-controlled genes are known to be CRP dependent (46). As an alternative, it is also possible that *ompR* message increases in response to acid shock because of decreased RNA turnover. These various models are currently being tested.

Another question centers on whether acid pH might influence phosphorylation of OmpR or whether phosphorylation occurs at a steady rate. If the phosphorylation level of OmpR is influenced by pH, does the ratio of OmpR to OmpR-P change because acid increases phosphorylation or decreases dephosphorylation? If the effect of acid is to alter dephosphorylation, it is unlikely that the dephosphorylase activity of EnvZ is involved, since *envZ* mutants adapted normally. Finally, it will be important to determine what OmpR-dependent genes are involved in acid tolerance.

The results presented here have refined our knowledge of the intricate regulatory networks associated with inducible acid tolerance and revealed additional physiological relevance for the response regulator OmpR in serovar Typhimurium.

ACKNOWLEDGMENTS

The work presented here was supported by grants from the United States Public Health Service (GM48017) to J.W.F. and from the Korean Science and Engineering Foundation (961-0507-062-2 and 981-0507-037-2) to Y.K.P.

We thank M. Koh and C. Park for their kind gifts of antibody. We are also grateful to M. Igo and T. Mizuno for providing several key plasmids used in this study.

J.W.F. and Y.K.P. contributed equally to this work.

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