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Acid-Sensitive Mutants of *Salmonella typhimurium* Identified through a Dinitrophenol Lethal Screening Strategy

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Salmonella typhimurium **exhibits a low-pH-inducible acid tolerance response (ATR) that can protect the adapted cell from severe acid challenge (pH 3.3). It is a two-stage system, with some proteins induced at pH 5.8 (pre-acid shock) and others induced below pH 4.5 (acid shock). The genetics of acid resistance was investigated through the use of a new screening medium. The medium contained 200 μM dinitrophenol (DNP) and was adjusted to pH 4.7 to 4.8. This medium will lower the internal pH of cells to a lethal level. However, cells capable of mounting an ATR will survive longer on this medium than acid-intolerant cells. Using this DNP lethal screening strategy, we isolated several acid-sensitive insertion mutants. Some mutants were defective in the pre-acid shock ATR stage but exhibited a normal or nearly normal post-acid shock-induced acid tolerance (*atrB* and *atrC*). Others could not induce acid tolerance by using either pre- or post-acid shock strategies (*atrD*, *atrF*, and *atrG*). The *atrB* locus was found to be part of a regulon under the control of a *trans*-acting regulator, *atbR*. An insertion in *atbR* caused constitutive acid tolerance because of overexpression of the regulon. Mutations in *atrD* and *atrF* affected iron metabolism and, in a manner analogous to ferric uptake regulator (*fur*) mutations, diminished acid resistance. The *atrF* mutation mapped within the *ent* cluster, probably in a *fep* uptake locus. The *atrD* locus mapped near *metC* and may represent an insertion into the *S. typhimurium* homolog of the *Escherichia coli exbB* or *exbD* locus. The mutation in *atrC* caused extreme UV light sensitivity and proved to occur within the *polA* (DNA polymerase I) locus. The results support the concept of overlapping acid protection systems in *S. typhimurium*.**

The acidification tolerance response (ATR) of *Salmonella typhimurium* is a process by which cells exposed to sublethal concentrations of H^+ undergo an adaptation that enables survival under more extreme low-pH conditions (8). One key to mounting a successful ATR is the ability to synthesize a set of acid shock proteins (ASPs). These proteins are synthesized in toto once the external pH falls below 4.5. Some are produced in response to external pH, while others are induced by internal pH changes (6). A subset of ASPs are produced in response to other environmental stresses, such as heat shock, but heat shock alone will not induce acid tolerance.

The period during which synthesis of ASPs occurs is referred to as the post-acid shock phase of the ATR (5). However, the cell does not have an unlimited capacity to synthesize ASPs. If shifted directly from slightly alkaline pH (7.7) to conditions below pH 4.0, protein synthesis capacity is severely compromised by the consequences of a falling internal pH (or a collapsing ΔpH). Consequently, ASPs are not synthesized. However, *S. typhimurium* appears to possess an inducible pH homeostasis system that can be triggered under mild acid conditions (pH 5.8). We refer to this as the pre-acid shock stage of the ATR (9). The inducible pH homeostasis system will enable a cell undergoing a severe acid shock (pH <4.0) to at least temporarily maintain an internal pH (pH_i) suitable for protein synthesis. Thus, preshock adapted cells can undergo post-acid shock adaptation under lower external pH conditions.

Clearly, the ability to isolate mutants defective in acid tolerance is central to initiating molecular approaches to investigating this phenomenon. We have developed a novel lethal screening strategy for ATR-deficient mutants that is based upon two observations. The first was that preshock adapted cells could counteract the equilibrating effect of dinitrophenol (DNP) on pH_i more effectively than could unadapted cells (6). The second observation was that stationary-phase cells, such as might occur within a colony, are significantly acid tolerant (18). This information led to the development of a screening medium that will differentiate between ATR-competent and incompetent *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains and culture media. All bacterial strains used in this study were derived from *S. typhimurium* LT2 (Table 1). Luria-Bertani (LB) complex medium and Vogel and Bonner (27) E minimal medium supplemented with 0.4% glucose were prepared as liquid and solid (1.5% agar) media (22). Antibiotics used included ampicillin (60 μg/ml), kanamycin (100 μg/ml), and tetracycline (10 or 20 μg/ml for minimal and rich medium, respectively). F′ plasmids containing *polA* were provided by R. Maurer (Case Western Reserve University, Cleveland, Ohio) and have been described by Lifsics et al. (19) and Joyce and Grindley (16). The filter-sterilized DNP lethal selection medium used to select *atr* mutants consisted of E salts (pH adjusted to 4.75), 40 mM glycerol, 200 μM DNP, and 3% agar. Enterochelin production was monitored by using chrome azurol S blue agar (CAS) as described by Schwyn and Neilands (25).

DNP lethal screening strategy. Wild-type *S. typhimurium* LT2 was mutagenized with the mini-Mu derivative Mu*dJ* as described by Hughes and Roth (15). Resulting Km^r colonies were patched onto LB plates with kanamycin and grown overnight at 37°C. Patches were then replicated to DNP lethal screening medium and incubated at 37°C. Survivors were

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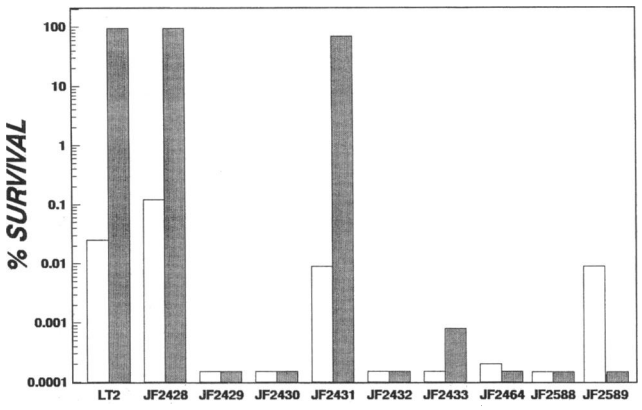

FIG. 1. ATR of DNP-sensitive mutants. The ATR (pre-acid shock adaptation) was assayed as described in Materials and Methods. The value of 100% survival represents 1×10^8 to 3×10^8 cells per ml. Survival for pH 7.7-unadapted (open bars) and pH 5.8-adapted (shaded bars) cultures after 2 h of pH 3.30 treatment are shown.

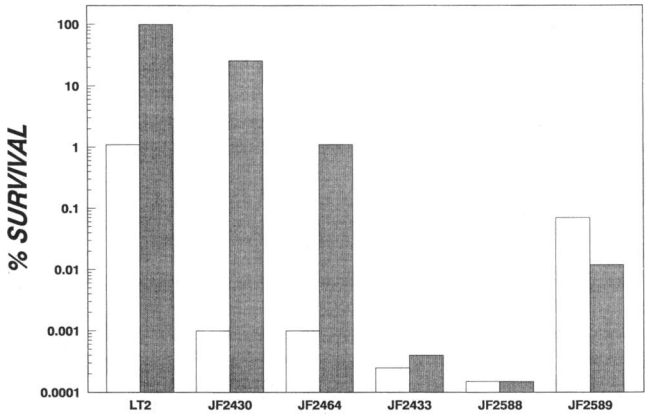

FIG. 2. Post-acid shock ATR. Post-acid shock development of the ATR was tested by adapting cells at pH 4.3 for 20 min before low-pH challenge. Survival parameters are the same as for Fig. 1.

rescued after 4 h by replicating onto LB medium. Mutants that did not survive this treatment were restruck from the original masterplate, and the putative *atr*::Mu*dJ* insertions were transduced to a new LT2 strain to retest the DNP-sensitive phenotype. Once DNP sensitivity was confirmed, these strains were assayed for ATR.

ATR. The adaptive ATR was measured as described earlier (8). Briefly, cultures tested for the pre-acid shock stage were grown in pH 7.7 minimal E glucose medium under semiaerobic conditions to 10^8 cells per ml and then adapted to pH 5.8 for one cell doubling. Unadapted cultures were grown directly to 2×10^8 cells per ml at pH 7.7. Both cultures were then challenged to pH 3.30 and incubated for 120 min. Viable cells were counted at 0 and 120 min. Cultures tested for the post-acid shock stage of the ATR were grown at pH 7.7 to 2×10^8 cells per ml, shifted to pH 4.3 for 15 min, and then challenged at pH 3.3.

TABLE 1. *S. typhimurium*

Strain	Relevant genotype
JF1420	*oxiE4*::Mu*dJ* Δ*nadA100*
JF1436	*oxrG*(*atrE*)*406*::Tn*10*
JF1923	Δ*atp-1*
JF2023	*fur-1 zfi*::Tn*10*
JF2391	Δ*fur-14*
JF2428	*dnp-4*::Mu*dJ*
JF2429	*atrB14*::Mu*dJ*
JF2430	*atrB13*::Mu*dJ* (Lac$^+$)
JF2431	*dnp-2*::Mu*dJ*
JF2432	*atrB15*::Mu*dJ* (Lac$^+$)
JF2441	*zxx-5153*::Tn*10* (50% linked to *atrB*$^+$)
JF2433	*atrD16*::Mu*dJ*
JF2494	*atrC20* (*polA*)::Mu*dJ* *atbR*::Tn*10*(dTc)
JF2464	*atrC20* (*polA*)::Mu*dJ*
JF2471	*atbR*::Tn*10*(dTc) *atrB13*::Mu*dJ*
JF2478	*zif-5155*::Tn*10*(dTc) (73% linked to *atrC*$^+$ [*polA*])
JF2475	*atbR*::Tn*10*(dTc)
JF2568	*galE* Mu*hP1 atrD16*::Mu*dJ*/pEG5183 Cm^r mini-Mu
JF2574	*galE* Mu*hP1*/pFW98 *atrD*::Km (Km^r Cm^r)
JF2588	*atrF22 zbc*::Mu*dJ*
JF2589	*atrG23*::Mu*dJ*
JF2602	*galE* Mu*hP1 atrG23*::Mu*dJ*/pEG5183
JF2654	*zbd-5167*::Tn*10*d(Tc) (77% linked to *atrF*$^+$, 55% linked to *ent*$^+$)
JF2655	*atrF22 zbd-5167*::Tn*10* (77% linked to *atrF*)
JF2688	*zgf-5170*::Tn*10*(dTc) (33% linked to *atrD*$^+$)
JF2695	*zxx-5171*::Tn*10*d(Tc) (5.6% linked to *atrG*$^+$)
SF239	*metC1975*::Tn*10*
SF240	*metC870*::Tn*10*
SF1	LT2 wild type
SF192 (SK811)	*zig-214*::Tn*10* Δ(*dhu-hisF*)*645* (75% linked to *ntrC*)

Genetic and biochemical techniques. Transductions were performed with P22 HT 105/1-*int* as described earlier (1). Tn*10* insertions near many *atr* loci were identified by transduction from among a pool of insertions by their ability to repair *atr*::Mu*dJ*-related phenotypes. Alternatively, a Tn*10* insertion pool was made directly into an *atr*::Mu*dJ* mutant, and Tn*10* insertions located near the Mu*dJ* insertion were selected by cotransduction of the Tc^r and Km^r determinants. Both Tn*10* and its 3-kb derivative Tn*10*Δ16Δ17 [Tn*10*(dTc)] were utilized. General mapping of Tn*10* insertions was done with the Mu*d*-P22 prophage system of Youderian et al. (29) as described by Benson and Goldman (3). In this procedure, approximately 5 ml of Mu*d*P22 phage lysates, each containing approximately 3 min of chromosomal DNA, was spotted onto a lawn of the

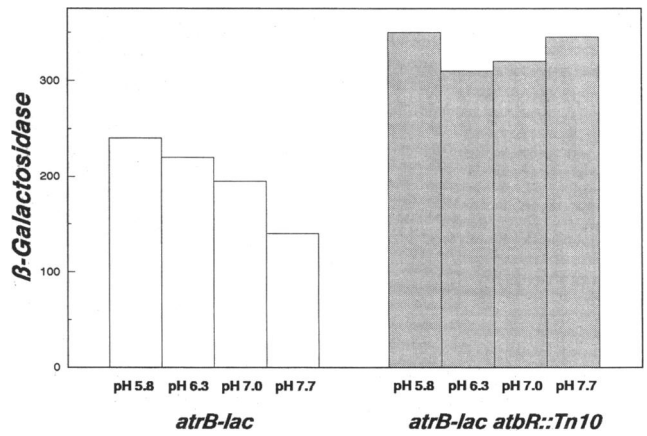

FIG. 3. Expression of *atrB-lacZ*. JF2430 (*atrB13*::Mu*dJ*, open bars) and JF2471 (*atrB*::Mu*dJ* *atbR*::Tn*10*, shaded bars) were grown to mid-log phase in minimal glucose medium at the pHs indicated and assayed for β-galactosidase activity. Values are averages for triplicate assays.

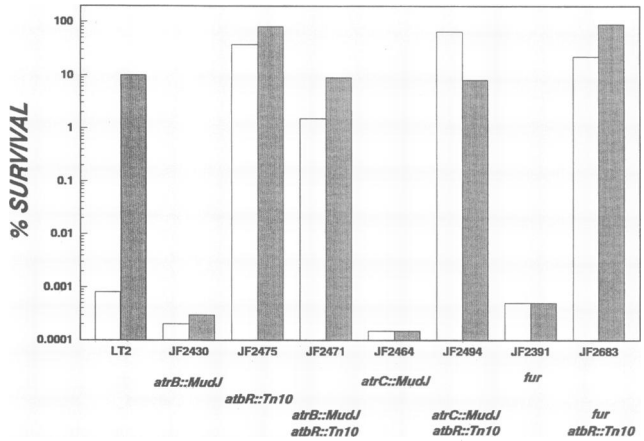

FIG. 4. Increased acid tolerance caused by an *atbR*::Tn*10* mutation. Preshock tolerance and survival parameters are the same as in Fig. 1.

TABLE 2. Proteins overexpressed in an *atbR* mutant

Protein[a]	Coordinates[b] (*x*, *y*)	Alternative regulation[c]
a	42, 92	
b	56, 97	
c	67, 101	IRO-28 (10); Fur, positive regulation
d	33, 67	OXI-4/ATR-3 (8, 26)
e	28, 66	
f	66, 70	
g	89, 69	
h	108, 92 (AtrB)	
i	46, 81	IRO-18 (10); Fur, positive regulation
k	59, 105	

[a] Designations correspond to those in Fig. 5.
[b] Proteins were mapped relative to a standard *S. typhimurium* polypeptide map (26).
[c] Proteins identified as induced by other environmental conditions. OXI, oxygen induced; ATR, pH 5.8 induced; IRO, iron regulated. Other stresses tested included heat shock, anaerobiosis, and acid shock.

Tn*10*-containing strain spread onto fusaric acid medium. Recombinational repair of the Tn*10* resulted in a cluster of fusaric acid-resistant colonies. Assays for β-galactosidase production were performed as described by Miller (22). In vivo mini-Mu cloning was performed as outlined by Groisman and Casadaban (13).

Plasmid pEG5183, containing a Cm-resistant mini-Mu, was used to clone the Km^r locus of the chromosomal Mu*dJ* and the adjacent fusion joint. Sensitivity to UV light was tested by growing cells in LB to approximately 4×10^8 cells per ml, placing 5 ml in an open petri dish, and exposing the culture to UV light while gently rotating the dish. Samples were taken at timed intervals and diluted to determine viable-cell counts. Values are the averages for triplicate samples. The procedure was conducted in subdued light to prevent photoreactivation.

SDS-PAGE. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Spector et al. (26) with cells labeled for 2 min with ^{35}S-Translabel (ICN Biomedical, Inc.). Approximately 5 μg of protein was analyzed for each sample. Basic and acidic proteins are situated to the left and right of the autoradiograph, respectively. The first dimension was a pH 5 to 7 isoelectric focusing gel containing 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholytes (Bio-Rad), and the second dimension was an SDS–11.5% polyacrylamide gel. Coordinates given for individual proteins correspond to a standard two-dimensional map of *S. typhimurium* polypeptides (26).

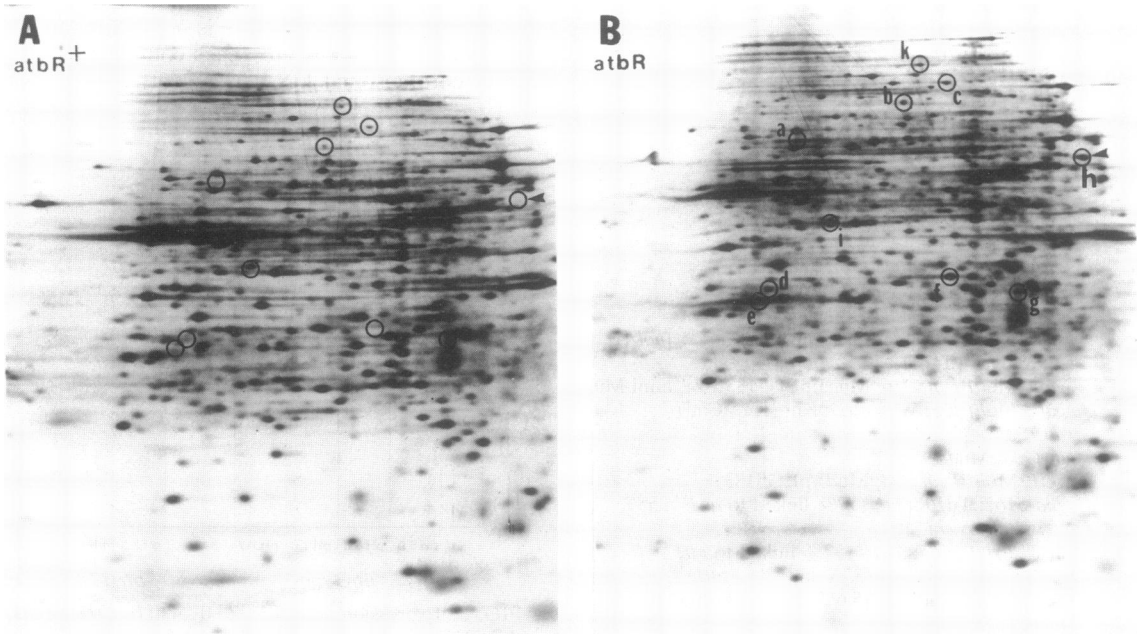

FIG. 5. Two-dimensional SDS-PAGE analysis of AtbR-regulated proteins. Cells were grown to mid-log phase in minimal glucose medium at pH 7.0. (A) LT2, *atbR*$^+$; (B) JF2475, *atbR*::Tn*10*. Cells were labeled for 2 min with 40 μCi of ^{35}S-Translabel (ICN). Proteins overexpressed in the *atbR* mutant are circled and assigned letters (see Table 2). The arrowhead indicates AtrB.

RESULTS

Development of a lethal screening medium for detecting ATR-incompetent mutants. Cells that have undergone pre-acid shock adaptation at pH 5.8 are known to deal more effectively with the deleterious effects of DNP on pH_i (6). We reasoned that cells defective in either the pre-acid shock or post-acid shock stage of the ATR would succumb more quickly to the lethal effects of DNP and low pH than would ATR-competent cells. We further reasoned that if this effect translated onto a solid support medium, we would have a suitable selection medium for ATR-incompetent cells.

This strategy was tested by using wild-type LT2 (SF1) and two proven acid-intolerant mutants (*atp* and *fur* mutations). The basic protocol involved inoculating the surface of an LB plate with a series of 3- to 5-mm patches of the test organisms, allowing them to grow at 37°C for 18 h, and then replicating the patches to test medium for further incubation at 37°C. At timed intervals, survivors were rescued by replication off of the DNP test medium back to an LB plate. Various media were screened for a differential effect on the survival of SF1 versus *atp* and *fur* mutants. The most consistent results were obtained with E glycerol medium at pH 4.75 plus 200 μM DNP.

Patches of SF1 survived for at least 5 h, while the *atp* (JF1923) and *fur* (JF2023) mutants died within 2 h. The time lapse between the death of the acid-intolerant mutants and the death of the acid-tolerant SF1 strain was determined empirically for each batch of medium before use. The results suggested that this medium could be used to screen for acid-intolerant mutants of SF1. Furthermore, it appeared that both preshock and postshock mutants might be selected, since previous results indicated that the *atp* operon is more important for preshock, while the *fur* mutants are defective in both ATR stages (6).

Selection of *atr*::Mu*dJ* insertion mutants. A total of 25,000 random Mu*dJ* insertion mutants were screened on the DNP lethal screening medium. A total of 16 confirmed DNP-sensitive mutants were identified. However, only seven were defective in inducible acid tolerance (Fig. 1). This result indicated that DNP may have more effects on the cell than equilibrating pH_i or that some mutations may increase membrane permeability to this protonophore (12). For this study, only the seven ATR-defective mutants were examined. We subsequently proved that the acid-sensitive phenotypes were due to the Mu*dJ* insertions by transducing each insertion into SF1 and reexamining the ATR. The phenotypes of all but one, *atrF*, were due to the insertion. The acid-sensitive phenotype in JF2588 was not due to the insertion but was caused by a mutation that occurred independently.

Each mutant was tested for inducible acid tolerance after preshock (pH 5.8) and postshock (pH 4.3) adaptation (Fig. 1 and 2, respectively). Previous results with *atp* and *fur* mutants indicated that the stages could be separated through mutation. We discovered that JF2430 (*atrB*) and JF2464 (*atrC*) were unable to induce acid tolerance when adapted at pH 5.8 but did demonstrate ATR when adapted at pH 4.3. This suggests that some facet of the preshock phase is defective but that the postshock phase is still effective in these mutants. The other mutants, JF2433 (*atrD*), JF2588 (*atrF*), and JF2589 (*atrG*), could not induce acid tolerance under either condition, indicating a more general role for their products in protecting the cells from low pH.

Map positions of the *atr* mutations. A variety of strategies were used to map these acid-sensitive mutations. Tn*10* insertions located near the Lac$^+$ *atrB*::Mu*dJ* (JF2430) were selected from a random pool of Tn*10* insertions. Initial selection was based on the loss of the Lac$^+$ and Km^r phenotypes. Cotransduction studies involving crosses between the Tn*10* insertions near *atrB*$^+$ and the other *atr*::Mu*dJ* strains revealed that two additional Mu*d* insertions occurred within the *atrB* region (JF2429 and JF2432). The Tn*10* insertion near *atrB* was mapped between 25 and 29 min by using the set of Mu*d*P22 transducing lysates and selecting for Tet sensitivity. Tn*10* insertions near *atrC*$^+$ were selected by the restoration of DNP resistance on the DNP lethal medium. After transduction of JF2464 with a P22 lysate prepared from a pool of Tn*10* insertion mutants, Tc^r colonies were replicated to DNP plates for 4.5 h, and survivors were rescued to LB with tetracycline. The Tn*10* insertion near *atrC*$^+$ (JF2478) was mapped with Mu*d*P22 lysates to 84 to 93 min. Subsequent cotransductional crosses with a known Tn*10* insertion (SF192) placed *atrC* at 85 min. By a similar strategy, a Tn*10* insertion 77% linked to *atrF*

TABLE 3. Complementation of *atrC* by plasmids containing *polA*

Strain	Relevant genotype[a]	Growth[b] after UV exposure of:			
		0 s	10 s	20 s	30 s
LT2	Wild type	+	+	+	−
JF2464	*atrC20*::Mu*dJ*	+	−	−	−
JF2523	LT2/pCJ100 (F′ *polA*$^+$ Cm^r)	+	+	+	−
JF2524	LT2/pCJ105 (F′)	+	+	+	−
JF2525	*atrC20*::Mu*dJ*/pCJ100 (F′ *polA*$^+$ Cm^r)	+	+	+	−
JF2526	*atrC20*::Mu*dJ*/pCJ102 (F′ *polA* 5′→3′ exo Cm^r)	+	−	−	−
JF2527	*atrC20*::Mu*dJ*/pCJ103 (F′ *polA* Klenow Cm^r)	+	+	+	−
JF2528	*atrC20*::Mu*dJ*/pCJ105 (F′ Cm^r)	+	−	−	−

[a] F′ plasmid transfer was based upon pCJ100, pCJ102, and pCJ103 are F′ factors containing intact *polA*$^+$, a fragment of *polA* containing the 5′→3′ exonuclease activity, or a fragment of *polA* containing the polymerizing Klenow fragment, respectively.

[b] +, growth on an LB plate after exposure to UV light for the indicated time.

TABLE 4. Overexpressed proteins in an *atrD* mutant

Protein[a]	Coordinates (*x*, *y*)	Regulation[b]	
		Iron	Other
a	07, 67	IRO-2*	
b	20, 31		ASP-56
c	38, 36		ASP-6
d	16, 78	IRO-3*	
e	36, 81	IRO-10*	
f	45, 102		
g	46, 81	IRO-18**	
h	44, 76		
i	47, 62	IRO-20*	
j	52, 57		ATR-18
k	52, 102	IRO-17*	ATR-8
l	57, 102	IRO-25*	ATR-10, SIN-12
m	67, 101	IRO-28**	
n	72, 101	IRO-29**	
o	74, 99	IRO-31*	ATR-11, SIN-19
p	89, 51		ASP-34
q	100, 73		
r	108, 92	IRO-38**	
s	93, 50		

[a] Designations correspond to those in Fig. 7.

[b] Regulation by iron (IRO [10]), acid shock (ASP [5]), and pre-acid shock (ATR [8]) or induction by starvation (SIN [26]). *, induced by iron limitation, repressed by ferrated Fur; **, induced by iron limitation, required deferrated Fur for induction (10).

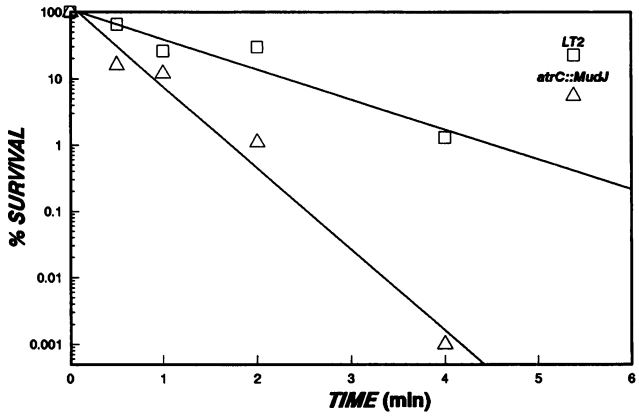

FIG. 6. UV light sensitivity of an *atrC* (*polA*)::Mu*dJ* mutant. Cells were exposed to UV light for the times indicated, and viable cells were counted. A value of 100% represents 10^8 cells per ml. □, LT2; △, JF2464 [*atrC* (*polA*)::Mu*dJ*].

was mapped to the 13-min region of the chromosome, based on a 55% linkage to *ent*.

The strategy used to map the *atrD*::Mu*dJ* insertion involved first cloning the fusion joint by mini-Mu in vivo cloning as described in Materials and Methods. Derivative subclones were radiolabeled and used to probe a Mu*d*P22 library of the *Salmonella* chromosome. The results indicated that *atrD* mapped in the 65-min region. Subsequent cotransductions with Tn*10* insertions in the area revealed that the *atrD* insertion was 97% linked to a Tn*10* insertion in *metC*.

Isolation of a constitutively acid-tolerant *atbR*::Tn*10* regulatory mutant. As noted above, one of the *atrB* insertion mutants proved to be Lac^+ on MacConkey medium and was subsequently tested for pH-regulated expression. As shown in Fig. 3, the *atrB-lac* fusion did exhibit a reproducible 40% induction at pH 5.8 versus expression at pH 7.7. The screen to locate Tn*10* insertions near *atrB* also uncovered a gene involved in regulating *atrB*. Insertions in the locus designated *atbR* caused constitutive overexpression of *atrB-lac* (Fig. 3). The *atbR*::Tn*10* mutation was mapped to the 87- to 90-min region by using the Mu*d*P22 series of mapping strains. Subsequent P22 cotransduction studies revealed that *atbR*::Tn*10* was 85% linked to the oxygen-regulated gene *oxiE* (1). However, the *oxiE*::Mu*dJ* mutant displayed a normal ATR, indicating that it is a locus distinct from *atbR* (data not shown).

***atbR*::Tn*10* mutation causes constitutive acid tolerance.** Since strains lacking AtrB exhibited diminished acid tolerance, we predicted that an *atbR* mutant with increased levels of AtrB should have increased acid resistance. The results shown in Fig. 4 confirmed this prediction. We also discovered that the *atbR* mutation could suppress the acid-sensitive phenotype of other *atr* mutants, including *fur* mutants (Fig. 4, JF2494 and JF2683), and even partially suppressed *atrB* (Fig. 4, JF2471). This suggested that *atbR* controlled several genes that contribute to acid resistance.

The map position of *atbR* relative to that of *atrB* suggested that the *atbR* product was a *trans*-acting regulator. Consequently, two-dimensional SDS-PAGE analysis was used to identify whether other genes were under the control of AtbR. At pH 7.7, JF2475 (*atbR*::Tn*10*) overexpressed 10 proteins compared with SF1 ($atbR^+$) (Fig. 5). One protein, indicated by an arrowhead in Fig. 5, disappeared from extracts of JF2471 (*atrB*::Mu*dJ atbR*::Tn*10*), suggesting that it may be AtrB (data not shown). Table 2 lists the coordinates of these peptides on a standard two-dimensional polypeptide map (25) and indicates whether a given protein appeared when induced by any other environmental stresses that were tested. Surprisingly, only one protein, d, appeared to be induced by low pH 5.8. Most of the other proteins overexpressed in the *atbR* mutant were unique to this regulon and were not observed under other test conditions. Clearly, one facet of the AtbR regulon involves acid tolerance. Why they were not detected earlier is not known. Either the system used to identify low-pH-induced

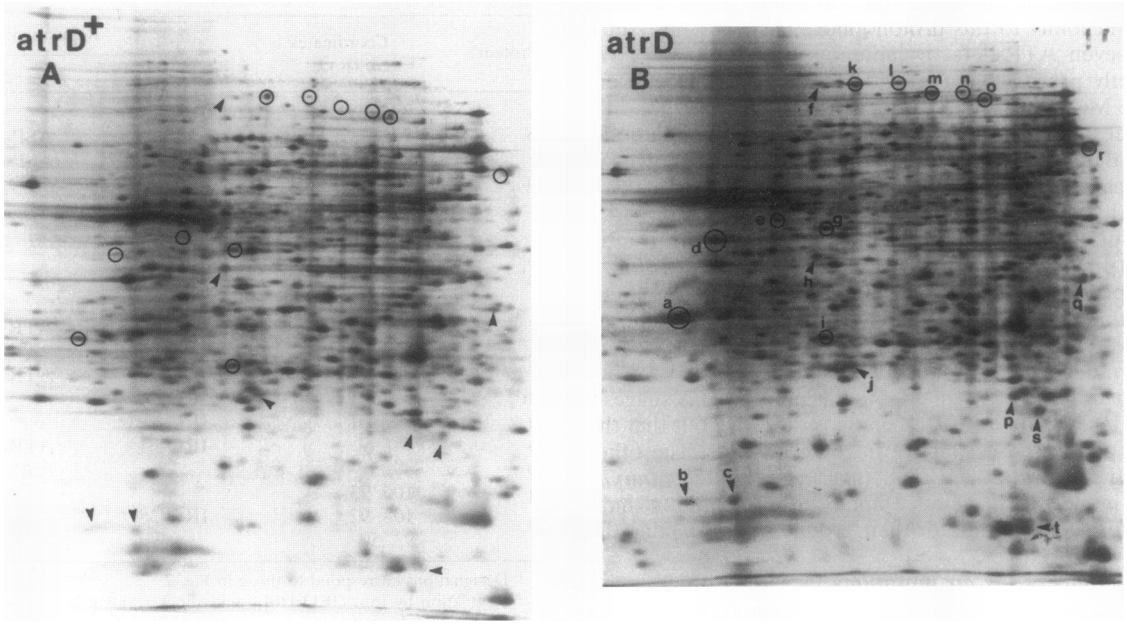

FIG. 7. Two-dimensional SDS-PAGE analysis of an *atrD*::Mu*dJ* mutant. Cells were treated as described in the legend to Fig. 5. (A) LT2, $atrD^+$; (B) JF2433, *atrD*::Mu*dJ*. Circles indicate proteins overproduced by the *atrD* mutant. All proteins were assigned letter designations (see Table 4).

protein expression was not sensitive enough to detect these proteins, or the AtbR regulon is normally triggered in response to a coinducer molecule in addition to acid (6).

The *atrC* locus is *polA*. The map position of *atrC* at 85 min placed it in the region of *polA* (DNA polymerase I). Since *polA* mutants are very sensitive to UV light, we tested JF2464 for this phenotype. As illustrated in Fig. 6, this strain was not only acid sensitive but was also more sensitive to UV light than the control culture. To prove that *atrC* was equivalent to *polA*, a series of *E. coli* F-prime factors containing only *polA* or active fragments of *polA* were moved into JF2464, and the transconjugants were tested for UV sensitivity (Table 3). The results indicate that *atrC* is *polA* and that the UV-sensitive as well as DNP-sensitive phenotype is due to the loss of polymerase rather than exonuclease activity.

***atrD* and *atrF* alleles affect iron metabolism.** Previous studies have revealed that mutants defective in the ferric uptake regulatory locus (*fur*) are extremely acid sensitive (5, 10). One characteristic of *fur* mutants is the overproduction of the iron-scavenging compound enterochelin. Consequently, all of the *atr* insertion mutants were screened for enterochelin production on CAS medium. In this medium, iron is bound to chrome azurol blue, resulting in a blue color. Enterochelin secreted from a colony will chelate iron away from the dye, which then turns yellow. The larger the yellow zone, the more enterochelin was produced. Two of the mutants, *atrD* and *atrF*, greatly overproduced enterochelin, as indicated by the large zone of clearing around colonies on CAS medium (21 to 22 mm, compared with 13 to 15 mm for all others, including LT2). Consequently, the results indicate that both mutants acted as though they were iron starved.

Cotransduction experiments with a Tn*10* insertion near *fur*$^+$ proved that neither mutation mapped to *fur* (15 min). However, a Tn*10* insertion 77% linked to *atrF* was also linked (55%) to the *ent* operon at 13 min. The *atrF* lesion cannot be in an *ent* biosynthetic gene, since enterochelin was produced. The mutation could be within one of the *fep* loci, which map in the same area. The *fep* genes encode the ferric-enterobactin uptake system, so mutants defective in this iron transport system will be iron deficient and thus overexpress iron-repressed genes, including *ent*.

The mechanism by which *atrD* affects iron metabolism is not known. From its map position, the *atrD* locus may be analogous to *exbB* or *exbD* from *E. coli*. ExbB is involved in the transport of several compounds across the membrane, including ferric siderophores (4, 17). Two-dimensional SDS-PAGE analysis of an *atrD* mutant revealed that 19 proteins were overexpressed (Table 4, Fig. 7), 11 of which were previously identified as iron regulated (10). Three of those 11 were also identified as pH 5.8-induced proteins. In addition, eight iron-independent proteins were overexpressed, four of which were acid shock proteins.

DISCUSSION

The DNP lethal screening strategy described herein has been used successfully to identify several genes whose products contribute to acid tolerance in *S. typhimurium*. The six genes reported can be added to those described previously. These include *atp*, *fur*, *ace*, *atr-1*, and *icd* (5, 8, 9). Previous attempts to isolate ATR-defective mutants have met with only modest success. Screening of long-term survivors of acid stress yielded a set of auxotrophic mutants, many of which apparently survived owing to the induction of a starvation-induced rather than pH-induced acid tolerance (9). However, useful information about acid tolerance was obtained from some of the mutants. The *icd* mutants isolated during the long-term survival screen were found to produce a large amount of intracellular citrate that served to better buffer internal pH at a low external pH. This increased buffering afforded increased acid protection and supported a role for improved pH homeostasis as an acid defense mechanism. Likewise, an *atp* mutant lacking the major proton-translocating ATPase was pre-acid shock defective due to interference with the inducible pH homeostasis system. As one would predict from the two-stage model, *atp* mutants were still proficient in the post-acid shock stage of ATR.

The acid-sensitive *fur* mutants were selected by a different protocol in which strains containing pH-regulated *lacZ* fusions were screened for secondary, unlinked regulatory mutations that also affected the ATR. Fur protein is the major regulator of iron metabolism in both *E. coli* and *S. typhimurium*, but it clearly has a very broad impact on cellular physiology (10). The phenotype of *fur* mutants includes an extreme acid sensitivity attributable to defective pre-acid shock and post-acid shock stages of the ATR (5, 6). The discovery of two additional mutations, *atrD* and *atrF*, that affect iron metabolism or regulation supports the role of Fur-regulated genes in acid tolerance and raises the question of what role, if any, iron itself might play in acid tolerance.

The discovery that PolA can influence acid tolerance was not unexpected. Low pH will accelerate the depurination of DNA, thereby increasing the demand for DNA repair (20, 21). As a component of many DNA repair systems, PolA would be of integral importance. It is interesting that in their study of pH-regulated *E. coli* proteins, Hickey and Hirshfield (14) report that PolA is a pH 5.0-induced protein. In addition, Raja et al. (23) have presented data for *E. coli* suggesting specific-acid-induced repair of damaged DNA.

The role of the AtbR regulon in acid tolerance is particularly intriguing for several reasons. It is the first regulon that can produce either acid-sensitive (*atrB*) or acid-resistant (*atbR*) mutant phenotypes. Induction of this system via an *atbR* mutation will suppress the acid-sensitive phenotype of other *atr* mutations. Finally, it is an acid defense system that was not revealed by previous screening for low-pH-regulated polypeptides. As noted earlier, the reason for this is not known. Either our methods were not sensitive enough, or the system requires a coinducer molecule in addition to low pH for induction. A coinducer requirement has been shown for other low-pH-regulated genes, such as *aniG*, which requires mannose (7); the *cadBA* operon, for which lysine is needed (2, 28); and *hyd*, for which formate serves as a coinducer (24). Another interesting aspect of the AtbR regulon is that two members are also under some form of positive control by Fur. It is therefore tempting to postulate that the acid-sensitive nature of *fur* mutants may be due in part to defective expression of the AtbR regulon.

The fact that deregulation of the AtbR regulon will supplant the need for other *atr* genes outside this regulon indicates that overlapping or redundant acid protection mechanisms are employed by *S. typhimurium*. We already have indirect evidence that pH homeostasis, chaperoning, and DNA repair mechanisms may be required for effective acid tolerance. How the overexpression of one system can compensate for the loss of another is a question that cannot be answered until defined functions are assigned to more of these genes.

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REFERENCES

1. **Aliabadi, Z., F. Warren, S. Mya, and J. W. Foster.** 1986. Oxygen-regulated stimulons of *Salmonella typhimurium* identified by Mu*d*(Ap*lac*) operon fusions. J. Bacteriol. **165:**780–786.
2. **Auger, E. A., K. E. Redding, T. Plumb, L. C. Childs, S.-Y. Meng, and G. N. Bennett.** 1989. Construction of *lac* fusions to the inducible arginine- and lysine decarboxylase genes of *Escherichia coli* K-12. Mol. Microbiol. **3:**609–620.
3. **Benson, N. R., and B. S. Goldman.** 1992. Rapid mapping in *Salmonella typhimurium* with Mu*d*-P22 prophages. J. Bacteriol. **174:**1673–1681.
4. **Eick-Helemerich, K., and V. Braun.** 1989. Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of *tolQ* and *tolR* genes, respectively. J. Bacteriol. **171:**5117–5126.
5. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173:**6896–6902.
6. **Foster, J. W.** 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **175:**1981–1987.
7. **Foster, J. W., and Z. Aliabadi.** 1989. pH-regulated gene expression in *Salmonella*: genetic analysis of *aniG* and cloning of the *earA* regulator. Mol. Microbiol. **3:**1605–1615.
8. **Foster, J. W., and H. K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172:**771–778.
9. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129–5135.
10. **Foster, J. W., and H. K. Hall.** 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J. Bacteriol. **174:**4317–4323.
11. **Foster, J. W., Y. K. Park, I. S. Bany, K. Karem, H. Betts, H. K. Hall, and E. Shaw.** Microbiology, in press.
12. **Gage, D. J., and F. C. Neidhardt.** 1993. Adaptation of *Escherichia coli* to the uncoupler of oxidative phosphorylation 2,4-dinitrophenol. J. Bacteriol. **175:**7105–7108.
13. **Groisman, E., and M. Casadaban.** 1986. Mini-Mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing. J. Bacteriol. **168:**357–364.
14. **Hickey, E. W., and I. N. Hirshfield.** 1990. Low-pH-induced effects on patterns of protein synthesis and on internal pH in *Escherichia coli* and *Salmonella typhimurium*. Appl. Environ. Microbiol. **56:** 1038–1045.
15. **Hughes, K., and J. Roth.** 1988. Transitory cis-complementation: a general method for providing transposase to defective transposons. Genetics **119:**9–12.
16. **Joyce, C. M., and N. D. Grindley.** 1984. Methods for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. J. Bacteriol. **158:**636–643.
17. **Kampfenkel, K., and V. Braun.** 1993. Topology of the ExbB protein in the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **268:**6050–6057.
18. **Lee, I., and J. W. Foster.** Unpublished data.
19. **Lifsics, M. R., E. D. Lancy, Jr., and R. Maurer.** 1992. DNA replication defect in *Salmonella typhimurium* mutants lacking the editing (ε) subunit of DNA polymerase III. J. Bacteriol. **174:**6965–6973.
20. **Lindahl, T., and A. Anderson.** 1972. Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid. Biochemistry **11:**3618–3623.
21. **Lindahl, T., and B. Nyberg.** 1972. Rate of depurination of native deoxyribonucleic acid. Biochemistry **11:**3610–3617.
22. **Miller, J. H. (ed.).** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Raja, N., M. Goodson, W. C. M. Chui, D. G. Smith, and R. J. Rowbury.** 1991. Habitation to acid in *Escherichia coli*: conditions for habitation and its effects on plasmid transfer. J. Appl. Bacteriol. **70:**59–65.
24. **Schlensog, V., and A. Böck.** 1990. Identification and sequence analysis of the gene encoding the transcriptional activator of the formate hydrogenlyase system of *Escherichia coli*. Mol. Microbiol. **4:**1319–1326.
25. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
26. **Spector, M. P., Z. Aliabadi, T. Gonzalez, and J. W. Foster.** 1986. Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. J. Bacteriol. **168:**420–424.
27. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
28. **Watson, N., D. S. DunYak, E. L. Rosey, J. L. Slonczewski, and E. R. Olsen.** 1992. Identification of elements involved in transcriptional regulation of the *Escherichia coli* operon by external pH. J. Bacteriol. **174:**530–540.
29. **Youderian, P., P. Sugino, K. L. Brauer, N. P. Higgins, and T. Elliot.** 1988. Packaging specific segments of the *Salmonella* chromosome with locked-in Mu*d*-P22 prophages. Genetics **118:**581–592.