Regulatory circuits involved with pH-regulated gene expression in *Salmonella typhimurium*

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***Salmonella typhimurium* encounters a variety of acid conditions during both its natural and pathogenic existence. The ability of this organism to respond transcriptionally to low pH is an area of active interest but little knowledge. As part of an ongoing investigation of low-pH adaptation, 18 pH-controlled *lacZ* operon fusions in *Salmonella typhimurium* have been identified (15 in this study) and categorized into at least 11 different loci. They include *iroA* (at 57 min), *aciA* (99 min), *aciB* (90–93 min), *aciD* (*ompC*, 45 min), *aciJ*, *aciK* (33–36 min), *aniC* (93 min), *anil* (33–36 min), *hyd* (59 min), *cadA* (54 min) and *aniG* (63 min). All but two were induced by low pH. One of the exceptions, the iron-regulated *iroA* locus, was induced at high pH. The unusual *aciA* locus was induced by low pH under semiaerobic conditions but high pH under aerobic conditions. Most of the other *aci* genes were expressed best under anaerobic conditions. Many of these genes exhibited strict co-inducer requirements for small molecules to be expressed in minimal medium. These included iron for *iroA*, tyrosine for *aniC*, *I* and *aciK*, mannose for *aniG*, formate for *hyd*, lysine for *cadA*, and unknown components of complex medium for *aciA*, *aciB* and *aciD*. Six regulatory circuits were revealed involving at least five regulatory loci (*fur*, *oxrG*, *earAB*, *earC* and *ompR*). As part of the adaptive response to low pH, *S. typhimurium* will induce an acid protection system called the acid tolerance response (ATR). As has been shown for *fur* mutations, the *oxrG* regulatory mutation interfered with the normal induction of this system.**

Keywords: *Salmonella*, pH regulation, gene expression, acid tolerance response, lysine decarboxylase, hydrogenase, *fur*, *omp*

INTRODUCTION

Salmonella typhimurium exhibits remarkable versatility in its molecular responses to diverse environmental stresses (Morgan *et al.*, 1986; Aliabadi *et al.*, 1986; Spector *et al.*, 1986, 1988). One perceived role for these systems is to overcome the impressive array of antimicrobial defences and physical barriers presented by the host (Finlay & Falkow, 1989). The organism is known to sense various conditions in the host environment and respond by coordinately regulating sets of genes that will contribute to survival and, thus, infection (Mekalanos, 1992; Lee & Falkow, 1990; Ernst *et al.*, 1990; Valone *et al.*, 1993; Buchmeier & Heffron, 1990). Consequently, environmental stress management by *Salmonella* is an area of increasing interest.

Abbreviations: ATR, acid tolerance response; LDC, lysine decarboxylase.

Since *S. typhimurium* encounters a variety of life-threatening acid environments in both natural and pathogenic situations, we have been accumulating evidence for a pH-response modulon. Part of this modulon includes a low-pH-inducible acid tolerance response (ATR) that will significantly increase acid resistance (Foster & Hall, 1990). The ATR is a two-stage system that includes mild (pH 5.8) and moderate (pH 4.3) acid adaptations called the preacid shock and postacid shock stages, respectively. An important manifestation of the preshock ATR stage is an inducible pH homeostasis mechanism that helps maintain intracellular pH above the lethal limit during strong acid stress (Foster & Hall, 1991). Acid shock results in the increased synthesis of 43 proteins at least, some of which are required for survival in severe acid conditions (Foster, 1991). In addition to the ATR, there are a variety of amino acid decarboxylases that can serve to neutralize an acidic environment (Gale & Epps, 1942).

Table 1. *Salmonella typhimurium* strains used in this study

Strain	Genotype	Source
JF1023	<i>aniD1047::MudA</i>	Aliabadi <i>et al.</i> (1986)
JF1141	<i>aniI1070::MudA</i>	Aliabadi <i>et al.</i> (1986)
JF1325	<i>aniC1052::MudJ</i>	Aliabadi <i>et al.</i> (1986)
JF1509	<i>aniI1070::MudA oxrG406::Tn10</i>	Aliabadi <i>et al.</i> (1988)
JF1593	<i>aniC1052::MudJ oxrG406::Tn10</i>	Aliabadi <i>et al.</i> (1988)
JF1842	<i>ompR::Tn5</i>	LT2 × ϕ SF241
JF1992	<i>iroA1::MudJ</i>	This paper and Foster & Hall (1992)
JF2023	<i>fur-1 zfi::Tn10</i> (85% linked to <i>iroA</i> ⁺)	JF2021 × ϕ SF221
JF2147	<i>iroA14::MudJ</i>	This paper
JF2148	<i>iroA15::MudJ</i>	This paper
JF2149	<i>aciA1::MudJ</i>	This paper
JF2150	<i>aciA2::MudJ</i>	This paper
JF2151	<i>aciA3::MudJ</i>	This paper
JF2154	<i>iroA18::MudJ</i>	This paper
JF2161	<i>aciA3::MudJ earC1</i>	This paper
JF2170	<i>earC1 zjj-5132::Tn10</i> (95% linked to <i>aciA</i> ⁺)	JF2161 × ϕ Tn10 pool
JF2236	<i>aciB::MudJ</i>	This paper
JF2237	<i>aciD9::MudJ</i>	This paper
JF2238	<i>cadA10::MudJ</i>	This paper
JF2239	<i>aciE11::MudJ</i>	This paper
JF2240	<i>cad12::MudJ</i>	This paper
JF2241	<i>aciB8::MudJ earC1 zjj-5132::Tn10</i> (95% linked to <i>aciA</i> ⁺)	JF2170 × ϕ JF2236
JF2244	<i>aciB8::MudJ earD1</i>	This paper
JF2257	<i>aciD9::MudJ fur-1 zfi::Tn10</i>	JF2023 × ϕ JF2237
JF2264	<i>aciD9::MudJ earC1 zjj-5132::Tn10</i>	JF2170 × ϕ JF2237
JF2265	<i>cadA10::MudJ earC1 zjj-5132::Tn10</i>	JF2170 × ϕ JF2238
JF2303	<i>aciD9::MudJ</i>	LT2 × ϕ JF2237
JF2449	<i>aciA3::MudJ earC::Tn10</i> (dTc)	JF2161 × ϕ Tn10 (dTc) pool
JF2452	<i>aciA3::MudJ earC⁺ zee-5148::Tn10</i> (dTc) (50% linked to <i>earC</i> ⁺)	JF2161 × ϕ Tn10 (dTc) pool
JF2495	<i>cadA12::MudJ</i>	This paper
JF2509	<i>earD1 zjc-5158::Tn10</i> (83% linked to <i>aciB</i> ⁺)	JF2244 × ϕ Tn10 (dTc) pool
JF2563	<i>aciD1(ompC)::Tn10</i> (dTc)	
JF2575	<i>aciD9(ompC)::MudJ earF1 zbf-5164::Tn10</i> (dTc) (80% linked to <i>earF</i>)	JF2303 × ϕ Tn10 (dTc) pool
JF2597	<i>aciJ2005::MudJ</i>	This paper
JF2598	<i>aciK2013::MudJ</i>	This paper
JF2628	<i>aciJ2005::MudJ fur-1 zfi::Tn10</i>	JF2023 × ϕ JF2597
JF2629	<i>aciK2013::MudJ fur-1 zfi::Tn10</i>	JF2023 × ϕ JF2598
JF2643	<i>cadA10::MudJ cadC3::Tn10</i> (dTc)	JF2238 × ϕ Tn10 (dTc) pool
JF2644	<i>cadC3::Tn10</i> (dTc) (LDC ⁻)	This paper
JF2645	<i>cadC3::Tn10</i> (dTc)/pPF106 <i>cadC</i> ⁺ (<i>E. coli</i>) (LDC ⁺)	This paper
JF2651	<i>aciD9(ompC)::MudA</i>	JF2237 × ϕ SF142
JF2652	<i>aciD9(ompC)::MudA ompR::Tn5</i>	JF2651 × ϕ SF241
SF103 (SGSC184)	<i>trpR520 serB80</i>	K. Sanderson
SF142 (TT7692)	<i>bisD9953::MudA</i>	J. Roth, Univ. of Utah, USA
SF184 (TT5371)	<i>zcb-754::Tn10</i> (90% linked to <i>bisW</i>)	J. Roth
SF190 (DB9031)	<i>zcb::Tn10</i> (95% linked to <i>gyrA</i>)	D. Botstein, Massachusetts Inst. Technology, USA
SF213 (TN858)	<i>leuBCD485 pepA1 pepB2 pepD3 zfd-801::Tn10</i> (65% linked to <i>pepB</i>)	C. Miller, Univ. of Illinois, USA
SF221 (KS177)	<i>zfi::Tn10</i> (2% linked to <i>hyd</i>)	K. Sanderson, Univ. of Calgary, Canada
SF241 (CH511)	<i>oppBCA250 leu-1151::Tn10 ompR::Tn5</i>	C. Higgins, Univ. of Oxford, UK
SF261 (TT10287)	<i>bisD9953::MudJ bis-9941::MudJ</i>	J. Roth
SF489 (SA2026)	<i>metG419</i>	K. Sanderson

Determining how *Salmonella typhimurium* regulates its response to low pH is part of understanding how it survives potentially lethal low pH encounters. Toward that goal, we have initiated studies involving direct screening for pH-regulated genes using the technique of *Mud-lac* operon fusion analysis (Hughes & Roth, 1988). We previously identified two *Salmonella* genes, *aniG* and *hyd*, that require an acid environment for maximal induction (Aliabadi *et al.*, 1988). These and several newly identified genes were examined in this current study. Identification and subsequent molecular dissection of these pH-regulated genes will facilitate efforts designed to understand how cells sense and adapt to fluctuations in environmental pH and could reveal genes directly or indirectly involved in acid tolerance.

METHODS

Bacterial strains and culture conditions. The bacterial strains used throughout this study are all derivatives of *Salmonella typhimurium* LT2 and are listed in Table 1. Culture media included E medium (Vogel & Bonner, 1956), Luria-Bertani (LB) medium (Davis *et al.*, 1980) and NCE medium (Malloy & Roth, 1983). LB was buffered to 100 mM with either MES or MOPS as described by Slonczewski *et al.* (1987). Measurement of the adaptive ATR was detailed previously (Foster & Hall, 1990). Briefly, cultures tested for the preacid shock stage were grown in pH 7.7 minimal E glucose under semiaerobic conditions to 10^8 cells ml⁻¹, then adapted to pH 5.8 for one doubling. Unadapted cultures were grown directly to 2×10^8 cells/ml at pH 7.7. Both cultures were then readjusted to pH 3.3 and incubated for 120 min. Viable counts were taken at 0 and 120 min. Cultures tested for the postacid shock stage of the ATR were grown at pH 7.7 to 2×10^8 cells ml⁻¹, shifted to pH 4.3 for 15 min then challenged at pH 3.3. The viable count values presented are the average of three independent experiments. The plasmid pABN203 containing the *E. coli fur*⁺ locus under control of its native promoter was kindly provided by J. B. Neilands (Bagg & Neilands, 1987). Iron depletion was accomplished through the addition of 100 µM diethylene triamine pentacetic acid (DTPA), whilst iron replete conditions were established through supplementation with 60 µM FeSO₄. Lysine decarboxylase (LDC) was assayed in Moeller decarboxylase medium (Difco).

Genetic and other procedures. Transductions were performed using P22 HT 105/1-*int* as described previously (Holley & Foster, 1982; Aliabadi *et al.*, 1988). The procedure to identify random *MudJ* fusions into pH-regulated genes was as described by Slonczewski *et al.* (1987) using the technique of transitory cis-complementation outlined by Hughes & Roth (1988). Specific pH-regulated insertions were identified by transducing LT2 to kanamycin resistance (Km^r) on minimal medium plates containing a pool of amino acid and vitamin supplements excluding histidine. The Km^r colonies were replicated to E medium (containing X-gal and Km) adjusted to pH 6.4 and 8.3, and to buffered LB at pH 5.5 and 8.0. The construction of Tn10 and *lacZ*-directed Hfr strains was as described by Chumley *et al.* (1978) and Spector *et al.* (1988). General mapping of Tn10 insertions using the *Mud*-P22 prophage system of Youderian *et al.* (1988), was performed as described by Benson & Goldman (1992). Lysates from a specific *Mud*-P22 lysogen are enriched for the region of the chromosome starting at the hybrid phage insertion site and proceeding 3 min either clockwise or counter-clockwise, depending on the phage orientation. Lysates pre-

pared from each of the 48 *Mud*-P22 lysogens were spotted on to a lawn of Tet^r cells plated on fusaric acid-containing medium (Malloy & Nunn, 1981). Tetracycline-sensitive, fusaric acid-resistant colonies arose in patches where donor chromosomal DNA spanned the region of the Tn10 insertion. Transformations were performed by electroporation (Tsai *et al.*, 1989). Two-dimensional SDS-PAGE was performed as described by Spector *et al.* (1986). Cells were grown in minimal glucose medium (pH 7.0) to a density of 4×10^8 cells ml⁻¹ and labelled for 10 min with ³⁵S-Translabel from ICN Biomedicals. Assays of β-galactosidase production were performed as described by Miller (1972) and are expressed as µmol per min per OD₆₀₀ unit. Values given are the average of three determinations and reflect a reproducibility of ±10%. Aerobiosis was achieved by growing 5 ml of culture in a 50 ml flask with shaking (240 r.p.m.). Anaerobiosis involved a standing culture with a paraffin oil overlay. A 3 ml culture shaking at 240 r.p.m. in a 10 × 100 mm test tube was used for semiaerobic conditions.

RESULTS and DISCUSSION

Identification of pH-regulated genes

An exhaustive search was made of a random *MudJ* insertion pool for pH-regulated loci. As a result of this study, 15 strains were found to exhibit pH-regulated β-galactosidase production (Table 2). Two other fusion strains, identified previously as pH-regulated, were also studied (*aniG* and *hyd*). Regulation was of two types, acid-inducible and -repressible. Acid-regulated genes were designated *aci* unless something was known about their function or if they were identified previously by a different strategy. Genes designated *iro* and *ani* were regulated by iron and anaerobiosis, respectively.

Some of the fusions under study proved to be inducible in minimal medium (*iroA* and *aciA*), whilst others required a component of complex medium for optimum induction (*aciB*, *aciD*, *aniC*, *aniI*, *aciK*, *cadA*). Efforts to identify these co-inducer requirements included adding combinations of amino acids and vitamins to minimal medium while testing induction. Lysine was required for *cadA* induction in minimal medium (Table 2). The *cadA* locus was subsequently shown to encode LDC as evidenced by the loss of LDC activity in the *cad* insertion mutants. This represents the first report, to our knowledge, identifying *cadA* in *Salmonella*. Partial induction of *aciD* was achieved in minimal medium upon the addition of 3 mM cysteine. The relevance of this is unclear but may be related to osmotic induction of this gene (see below). The *aciK*, *aniC* and *aniI* fusions were all induced in minimal medium by the addition of either L-tyrosine or L-phenylalanine. Induction of these three loci required low pH, anaerobiosis and either one of these aromatic compounds. *Salmonellae* do not possess a tyrosine or phenylalanine decarboxylase, so the reason for this unusual regulation remains a mystery. They may represent an anaerobic equivalent of the aromatic amino acid transport systems which are also induced by tyrosine or phenylalanine (Heatwole & Somerville, 1991; Sarsero *et al.*, 1991). The *aciB* locus also requires a co-inducer molecule but its identity remains unknown. It does not appear to be an amino acid based on the addition of pooled amino acids.

Table 2. pH-regulated operon fusions

Growth was measured under semiaerobic conditions. β -Galactosidase activity values [expressed as $\mu\text{mol min}^{-1}$ ($\text{OD}_{600} \text{ unit}$) $^{-1}$] are means of three determinations $\pm 10\%$. Assays were according to Miller (1972). For all experiments, minimal E medium with glucose was used unless stated otherwise. ND, Not done.

Strain	Genotype	Addition	β -Galactosidase activity			
			Minimal		LB	
			pH 5.8	pH 7.7	pH 5.5	pH 8.0
JF1992	<i>iroA1::MudJ</i>	60 μM FeSO_4	25	430	ND	ND
			23	36	ND	ND
JF2147	<i>iroA14::MudJ</i>		19	190	ND	ND
JF2148	<i>iroA15::MudJ</i>		20	233	ND	ND
JF2154	<i>iroA18::MudJ</i>	3 mM cysteine 300 mM NaCl	21	390	ND	ND
JF2149	<i>aciA1::MudJ</i>		200	16	510	170
JF2150	<i>aciA2::MudJ</i>		210	16	690	243
JF2151	<i>aciA3::MudJ</i>		110	9	663	155
JF2236	<i>aciB8::MudJ</i>		25	1	172	10
JF2237	<i>aciD9::MudJ</i>		0.0	0.0	159	18
			50	4	ND	ND
			21	3	ND	ND
JF2238	<i>cadA10::MudJ</i>	10 mM lysine	0.0	0.0	598	8
			1071	30	ND	ND
JF2495	<i>cadA12::MudJ</i>		0.5	0.5	540	6
JF2597*	<i>aciJ2005::MudJ</i>		190	310	750	3
JF2598*	<i>aciK2013::MudJ</i>	100 μM tyrosine 600 μM phenylalanine	10	2	275	25
			405	0.0	ND	ND
			310	0.0	ND	ND
			0.0	0.0	311	41
JF1325*	<i>aniC::MudJ</i>	Tyrosine	473	2	ND	ND
JF1141*	<i>aniI::MudJ</i>		0.0	0.0	169	42
			301	15	ND	ND

* Expression only observed under anaerobic conditions.

The *aciJ* locus was unusual in that acid induction was clear in LB complex medium but expression was constitutive in minimal glucose (Table 2, JF2597). This could suggest that a corepressor molecule is required for pH 7.7 repression although, again, the addition of amino acid pools to minimal media did not affect expression.

Regulation by external vs internal pH

There are three obvious parameters a cell could measure to trigger the induction of pH-regulated genes. These are external pH (pH_o), internal pH (pH_i) or the difference between the two (ΔpH). Slonczewski *et al.* (1987) have devised a method to differentiate between internal and external inducing signals. The method involves the addition of a weak acid such as benzoate to culture media (pH 7). The protonated form of benzoate will cross the membrane and subsequently lower internal pH. So if internal pH is the signal for *aci* induction, the addition of 20 mM benzoate should lead to *aci* expression. The *aciA*,

aciB, *aciJ*, *aciK* and *cadA* genes did not respond to this treatment. The *aciD-lacZ* fusion was induced in LB 100 mM MOPS (pH 7) containing 20 mM benzoate (402 Miller units) as compared to pH 7 containing no benzoate (27 Miller units) suggesting a role for pH_i in the induction of this gene.

Location and identity of pH-regulated loci

Three of the acid-inducible *lacZ* operon fusions noted above cotransduced to the same position on the *S. typhimurium* chromosome. Tn10 insertions that cotransduced with the insertion of JF2151 (*aciA3::Mud*) were also linked to the insertions in JF2149 and JF2150. Tn10-directed Hfr constructions using *zjj-5132::Tn10* revealed the location of *aciA* to be between 90 and 99 min (data not shown). Later use of the MudPQ set of mapping lysates also indicated a map position between 98 and 0 min. Finally, a cotransductional linkage of 1% was demonstrated between *zjj-5132::Tn10* (JF2170) and *serB* (SF103), localizing *aciA* to 99 min.

The *aciB* locus (JF2236) was mapped to between 90 and 93 min with the MudPQ system. Construction of *lac*-directed Hfrs using the *aciB-lac* operon fusion confirmed this and indicated a clockwise transcriptional orientation. We have discovered that *aciB* is linked (15%) to the anaerobiosis-inducible *aniD* (JF2509 × JF1023) locus (Aliabadi *et al.*, 1986).

Tn10 insertions near *aciD* were used to map this locus with the MudPQ system to between 40 and 50 min. Cotransductional analysis with known markers in the area revealed that *aciD* is approximately 50% linked to *zfb::Tn10* (SF190 × JF2444). This placed *aciD* at 45 min on the linkage map.

The fusions in JF2238, JF2240 and JF2495 all occurred within the *cadA* locus as demonstrated by a deficiency in lysine decarboxylase. A Tn10 insertion near *cadA* was mapped to between 52 and 57 min using the MudPQ system. Cotransductional analysis subsequently placed *cad* at 54 min, 56% linked to *purG::Tn10* (SF256) and 3% to *zfd-801::Tn10* (SF213). This is very different from its position in *Escherichia coli* (93 min). Cloning and preliminary sequencing results (Park & Foster, unpublished) indicate that, other than for map position, the *S. typhimurium cadBA* operon is very similar to its counterpart in *E. coli* (Auger *et al.*, 1989; Watson *et al.*, 1992; Meng & Bennett, 1992a, b).

The tyrosine-inducible *aniC* and *aniI* loci were previously mapped to 93 and between 33 and 36 min, respectively. Based on a similar tyrosine dependence for expression, *aciK* was tested for linkage to these two loci. Cotransduction results indicated *aciK* was 60% linked to *aniI*, (JF1141) placing it between 33 and 36 min.

The alkaline induced *iroA* locus was mapped to 57 min based upon cotransduction (85%) with *zfi::Tn10* (SF 221 × JF1992). This locus has an undefined role in iron transport. Its regulation by pH is due, at least in part, to the reduced solubility of Fe(OH)₃ under alkaline pH since the addition of 60 µM FeSO₄ to pH 7.7 cultures fully repressed *iroA* (JF1992, Table 2). All of the *iroA* insertions have been mapped to the 57 min region and may represent insertions into the same gene. It should be noted that none of the other pH-regulated genes identified were controlled by iron (data not shown).

Identification of *trans*-acting regulators

The *earC* regulator. Strains containing mutations in a *trans*-acting regulatory locus controlling *aciA* were isolated by streaking bacteria containing *aciA-lacZ* fusions onto MacConkey lactose plates. These fusion strains have a Lac⁻ phenotype toward the heavy streak area of the plate. However, red papillae representing spontaneous mutants arose within this area. All of the mutants selected were derepressed and deregulated with respect to pH-regulated control of *aciA* (e.g. JF2161, Table 3). Five were subsequently tested for linkage between the regulatory mutation and the *aciA::MudJ* insertion. One regulatory mutation was linked; however, four were

unlinked to the insertion and appear to represent mutations in one or more *trans*-acting regulatory loci. We have previously used the designation external acid regulator (*ear*) to refer to these genes and favour retaining this nomenclature until the specific function of the gene is determined (Foster & Aliabadi, 1989). Consequently, we have designated the new *trans*-acting regulator as *earC*. Tn10 insertions were identified near the *earC* locus and used to map the gene to between 40 and 44 min. Subsequent cotransduction with known markers indicated that the gene resides at 44 min and is 60% linked to *metG* (JF2452 × SF489). The *earC* mutation caused deregulation of all three *aciA-lacZ* fusion strains. We also identified a Tn10dTc insertion into JF2161 (*aciA::Mud earC1*) which eliminated *aciA-lacZ* expression resulting in a Lac⁻ phenotype. The transposon also cotransduced with *metG* (JF2449 × SF489, 90%) and appears to be an insertion into *earC*. Lack of *aciA* expression in the *earC::Tn10* background suggests *earC* is a positive regulator of *aciA* (JF2449, Table 3).

The *earF* regulator. An *aciD-lacZ* culture mutagenized with mini Tn10 gave rise to a regulatory mutant that overexpressed *aciD* (JF2575). The regulatory mutation, *earF1*, was not caused by the Tn10 insertion itself but cotransduced with it (80% linkage). The effect of *earF* on *aciD* expression is shown in Table 3. It is interesting that this mutation removed the co-inducer requirement for expression but did not affect pH-regulated expression as shown in minimal media. This suggests the existence of a second, unknown, regulator that controls pH regulation of *aciD*. MudPQ mapping experiments placed *earF* between 72 and 79 min. Subsequent cotransductional tests revealed that the Tn10dTc insertion near *earF* cotransduced with *ompR::Tn5* (JF2575 × JF1842, 80%). This raised the possibility that *earF* is in fact a mutation in *ompR* or *envZ* (see below).

The *cadC* regulator. Several investigators have demonstrated that *cadA* in *E. coli* occurs within an operon (*cadBA*). A gene upstream from *cadBA*, called *cadC*, produces a positive regulatory protein essential for *cadBA* expression. The *cadC* regulatory locus of *S. typhimurium* was discovered in our work by screening the various Tn10 insertions located near *cadA*. Several of these were LDC⁻ indicating a defect in *cadA* expression. A few were insertions into the *cadBA* operon and were not complemented by a cloned *cadC* locus from *E. coli*. However, three LDC⁻ Tn10 insertions were complemented by this plasmid, indicating that the positive regulator of *cadA* expression was inactivated by the insertion (e.g. JF2643, Table 3; JF2645, Table 1).

The *oxrG* regulator. A mutation in a previously identified regulator (*oxrG::Tn10*) of *aniC* and *aniI* was tested for its effect on low-pH induction of these loci and of *aciK*. The data presented in Table 3 illustrate that *oxrG*⁺ is required for low pH expression of all three loci. Whilst a minor amount of low-pH induction remained, the level appears insignificant. The *oxrG* locus was mapped previously to 88 min, near the oxidative stress regulator *oxyR*, but is distinct from *oxyR* (Aliabadi *et al.*, 1988). We

Table 3. Effect of regulatory mutations on pH-regulated operon fusions

β -Galactosidase activity [expressed as $\mu\text{mol min}^{-1} (\text{OD}_{600} \text{ unit})^{-1}$] was measured according to Miller (1972). All strains were grown throughout the experiment at the pH indicated under semiaerobic conditions. Minimal medium was unsupplemented except for experiments involving the *aniC*, *aniI* and *aciK lac* fusion strains in which tyrosine (100 μM) was added. LB contained either 100 mM MES (pH 5.5) or MOPS (pH 8.0) buffers.

Strain	Genotype	β -Galactosidase activity			
		Minimal glucose		LB	
		pH 5.8	pH 7.7	pH 5.5	pH 8.0
JF2151	<i>aciA::MudJ</i>	88	6	600	210
JF2161	<i>aciA::MudJ earC1</i>	856	329	920	650
JF2449	<i>aciA::MudJ earC::Tn10</i> (dTc)	2	1	5	2
JF2236	<i>aciB8::MudJ</i>	11	9	118	8
JF2244	<i>aciB8::MudJ earD1</i>	150	1.0	94	125
JF2241	<i>aciB8::MudJ earC</i>			110	0
JF2238	<i>cadA::MudJ</i>			440	4
JF2265	<i>cadA::MudJ earC</i>			382	4
JF2643	<i>cadA::MudJ cadC::Tn10</i>			11	13
JF2237	<i>aciD9::MudJ</i>	0	0	190	25
JF2264	<i>aciD9::MudJ earC</i>			211	31
JF2575	<i>aciD9::MudJ earF1(ompR)</i>	260	15	638	239
JF2651	<i>aciD9::MudJ</i>	22	7	190	17
JF2652	<i>aciD9::MudJ ompR::Tn5</i>	83	1.5	0	9
JF1325	<i>aniC1052::MudJ</i>	532	4		
JF1593	<i>aniC1052::MudJ oxrG::Tn10</i>	19	4		
JF1141	<i>aniI1070::MudJ</i>	321	11		
JF1509	<i>aniI1070::MudJ oxrG::Tn10</i>	17	1		
JF2598	<i>aciK2013::MudJ</i>	415	1		
JF2647	<i>aciK2013::MudJ oxrG::Tn10</i>	7	1		

originally categorized *oxrG* as an oxygen sensitive regulator. However, with its observed effect on pH-regulated gene expression, it is now unclear whether it is involved with O_2 and/or pH sensing. Nevertheless, it is essential for the expression of this pH-controlled regulon.

The effect of osmolarity and anaerobiosis on *aci* expression

It is recognized that many environmentally responsive genes respond to more than one environmental condition. We sought to test the *aci* fusions for similar multiple controls by screening them for regulation by osmolarity and anaerobiosis. For studies of osmotic control, LB adjusted to a pH of 7 was formulated to contain 0, 200 or 400 mM NaCl. A pH of 7 does not normally trigger induction of most *aci* loci. Cells were grown to exponential phase in these media and β -galactosidase assayed. The results (Table 4) reveal that three loci were clearly affected by osmolarity, *cadA*, *aciB* and *aciD*. The magnitude of control by osmotic conditions was less than by low pH

but there was a definite effect upon induction. The *aciD* locus was induced by high osmolarity, whilst the other two genes were induced by low osmolarity. The map positions of *aciD* and its regulator *earF* suggest they are *ompC*, encoding an outer-membrane porin, and *ompR* or *envZ*, the regulators of *ompC*, respectively (Forst *et al.*, 1990). Subsequent two-dimensional PAGE gels showed *OmpC* is missing in JF2337, indicating *aciD* is *ompC* (data not shown). *E. coli ompC* is induced by low pH and high salt which is consistent with our results (Hyde & Portalier, 1987). Hyde & Portalier (1987) also found that *envZ* mutations within *E. coli* eliminate most of the pH control for *ompF* and *ompC*. Whilst this also proved true for *earF aciD* double mutants in LB, the *earF* mutation now allowed significant pH control (15–20-fold) in minimal medium (JF2575, Table 3). Introduction of a known *ompR::Tn5* insertion into an *aciD(ompC)::MudA* derivative strain also exposed significant pH regulation in minimal medium. But instead of enhancing expression in LB, as was the case for *earF* mutations, *ompR::Tn5* prevented expression of *aciD(ompC)* (JF2652, Table 3). The results suggest that environmental sensing by the

Table 4. The effect of oxygen and osmolarity on *aci-lacZ* expression

β -Galactosidase activity [expressed as $\mu\text{mol min}^{-1} (\text{OD}_{600} \text{ unit})^{-1}$] was measured according to Miller (1972). LB medium was used throughout unless otherwise indicated.

Strain	Genotype	pH	β -Galactosidase activity				
			+O ₂	-O ₂	NaCl concn (mM)*		
					0	200	400
JF2236	<i>aciB8::MudJ</i>	5.5	38	29			
		7	24	30	34	6	0
		8.0	6	12			
JF2237	<i>aciD9::MudJ</i>	5.5	165	117			
		7	8	30	5	15	42
		8.0	13	10			
JF2238	<i>cadA10::MudJ</i>	5.5	443	3352			
		7	37	600	251	11	6
		8.0	15	0			
JF2495	<i>cadA12::MudJ</i>	5.5	1018	6940			
		7	9	225	200	2	0
		8.0	9	6			
JF2150	<i>aciA2::MudJ</i>	5.5	10 (90†)	1062 (92†)			
		7	263	1491	177	195	205
		8.0	385 (42†)	1202 (430†)			
JF2597	<i>aciJ2005::MudJ</i>	5.5	4	1261			
		7	2	430	8	10	12
		8.0	4	8			
JF2598	<i>aciK2013::MudJ</i>	5.5	2	669			
		7	1	76	21	15	9
		8.0	2	7			

* Cultures were grown under partial aerobic conditions. Base LB contained no salt in this series.

† Results in minimal media.

EnvZ/OmpR two-component system is even more complicated than the current models suggest. The finding that mutations within EnvZ/OmpR allow pH-regulated expression of *aciD(ompC)-lac* in minimal medium indicate the existence of an additional pH-dependent regulatory locus.

We also examined the effect of anaerobiosis on the induction of this set of low-pH-regulated genes (Table 4). Of the 10 acid-inducible genes identified, seven were also induced under anaerobic conditions. These were *aciA*, *cadA*, *aciJ*, *aciK*, *aniC*, *aniI* and *hyd*. The *cadA* locus was very sensitive to oxygen levels and was clearly anaerobically induced, as was found for the analogous locus in *E. coli* (Auger *et al.*, 1989). As with *hyd* and *cadA*, both the *aciJ* and *aciK* loci were strictly induced by low pH and anaerobiosis. This pattern was maintained in minimal media. The reason why these genes are optimally expressed anaerobically is probably linked to the fermentative production of acid. Certainly, the *hyd* locus will facilitate the dissimilation of formic acid, directly preventing a decrease in pH_o. LDC, in contrast, will produce an

alkaline product that will titrate fermentative acid end-products.

Interestingly, *aciA* was subject to a very complex set of environmental controls when grown in LB. Anaerobically, *aciA* was fully induced and exhibited no pH control. Under semiaerobic conditions it was acid-induced (threefold, Table 3), but under aerobic conditions it was acid-repressed (30-fold, Table 4). Conversely, under acid conditions it was anaerobically induced 100-fold, whilst in alkaline conditions only fourfold anaerobic induction was observed. In addition, there must be an enhancing co-inducer molecule for *aciA* since expression was much greater in LB than in minimal media. Expression in minimal medium was also greatest under anaerobic, alkaline conditions, but, as seen in Table 2, it became acid-induced under semiaerobic conditions (12-fold). The reason(s) for the intricate controls placed on *aciA* are unknown. It appears that anaerobiosis represents the primary control for this gene. However, a demand for the *aciA* product also occurs aerobically if the cells are subjected to high pH. The effects seen under semiaerobic

Table 5. Effect of *fur* mutations on pH regulated operon fusions

β -Galactosidase activity was measured according to Miller (1972). All strains were grown throughout the experiment at the pH indicated. LB contained either 100 mM MES (pH 5.5) or MOPS (pH 8.0) buffers.

Strain	Genotype	β -Galactosidase activity			
		Minimal glucose		LB	
		pH 5.8	pH 7.7	pH 5.5	pH 8.0
JF1992	<i>iroA::MudJ</i>	7	569		
JF2021	<i>iroA::MudJ fur</i>	306	411		
JF2151	<i>aciA::MudJ</i>	130	20	245	61
JF2212	<i>aciA::MudJ fur</i>	40	15	272	2
JF2236	<i>aciB8::MudJ</i>	11	9	118	8
JF2242	<i>aciB8::MudJ fur</i>			124	6
JF2238	<i>cadA::MudJ</i>			440	4
JF2266	<i>cadA::MudJ fur</i>			403	4
JF2237	<i>aciD-9::MudJ</i>			179	13
JF2257	<i>aciD-9::MudJ fur</i>			223	54
JF2597	<i>aciJ::MudJ</i>			645*	2*
JF2628	<i>aciJ::MudJ fur</i>			730*	0*
JF2598	<i>aciK::MudJ</i>	383†	5†		
JF2629	<i>aciK::MudJ fur</i>	222†	0†		

* Grown anaerobically.

† Growth anaerobically with 100 μ M tyrosine for JF2598 and JF2629.

conditions must reflect the competition between oxygen and pH controls.

The effect of *fur* mutations on pH-regulated gene expression

The *fur* (ferric uptake regulator) locus encodes a major iron-regulatory protein in both *E. coli* and *S. typhimurium*, although its role in cellular physiology is thought to extend beyond iron metabolism (Hantke, 1981, 1987; Foster & Hall, 1992). We have reported previously that *fur* mutations in *Salmonella* severely compromise the ability of this organism to mount an effective ATR (Foster & Hall, 1992). One explanation for why *fur* mutations may prevent adaptive acid tolerance is that the Fur regulator might be required for optimal expression of specific acid-inducible genes, some of which may be involved with acid tolerance. Consequently, the various *aci-lac* strains identified in our laboratory were used to construct *aci-lac fur* derivatives and *aci-lacZ* expression subsequently measured under acid and alkaline conditions. The results presented in Tables 5 and 6 reveal that *fur*⁺ is clearly required for regulated expression of *iroA* and maximum expression of *hyd* in minimal medium. Of the remaining *aci* fusions, only *aciA* appeared to be influenced by *fur*. However, the effect was not dramatic and thus not pursued further. The role of *fur* in iron-regulated control of *iroA* was shown previously (Foster & Hall, 1992). The

data in Table 5 illustrates a similar role for *fur* in the pH control of *iroA*. It is unclear at this point whether *iroA* responds directly to both Fe³⁺ and pH or whether the decreased solubility of Fe(OH)₃ at alkaline pH is sufficient to explain the pH effect. However, if that were the case one might expect all iron-regulated genes should be subject to significant pH control. Evidence to the contrary was found in that at least one iron-regulated locus in *Salmonella* displays very little control by pH, indicating pH has a varied effect on iron-regulated genes (N. Buchmeier & J. Foster, personal communication).

Maximal induction of the *hyd* locus, which encodes a component of hydrogenase, has been shown to require an acid pH, anaerobiosis and formate (Pecher *et al.*, 1983; Schlensog & Böck, 1990). The hydrogenase system may aid in the maintenance of pH_i through the evolution of H₂ gas and the dissimilation of formic acid. Schlensog & Böck (1990) proposed that pH control of the *E. coli* hydrogenase loci was due to an effect on uptake of the inducer, formate. However, we found in *S. typhimurium* that mutations in *fur* severely limited induction of *hyd* (JF2235 and 2392, Table 6) both in terms of anaerobiosis and pH. Thus, the *hyd* locus requires the *fur* product for complete induction. Regulation of other oxygen- and pH-regulated (*aniC*, *aniG*, *aciD*, *cadA*) genes remained undisturbed by the *fur* mutations, indicating that Fur specifically affects the expression of some, but not all,

Table 6. The effect of *fur* mutations on *hyd-lacZ* expression

β -Galactosidase activity [expressed as $\mu\text{mol min}^{-1} (\text{OD}_{600} \text{ unit})^{-1}$] was measured according to Miller (1972). ND, Not determined.

Strain	Genotype	Addition	β -Galactosidase activity					
			LB $-\text{O}_2$		Minimal pH 5.8		Minimal $-\text{O}_2$	
			pH 5.5	pH 8.0	$-\text{O}_2$	$+\text{O}_2$	pH 5.8	pH 7.8
JF927	<i>hyd::MudA</i>		371	1.3	510	2	600	70
JF1065	<i>hyd::MudA oxrA(fur)</i>		102	1.0	183	2	176	16
		30 mM formate	350	10.0	521	5	550	110
JF2235	<i>hyd::MudA fur-1</i>		11	0	11	2	0	0
JF2292	<i>hyd::MudA fur-1/pABN203fur⁺</i>		310	12	ND	ND	375	66
JF1534	<i>hyd::MudJ</i>						790	42
		120 μM FeSO_4					750	150
JF2392	<i>hyd::MudJ fur-2</i>						0	0
		30 mM formate					0	0
JF900	<i>aniC::MudA</i>				220*	20*		
JF2234	<i>aniC::MudA fur</i>				195*	5*		
JF1295	<i>aniG::MudJ</i>		470	30				
JF2233	<i>aniG::MudJ fur-1</i>		500	45				
JF1537	<i>aniG::MudJ earA</i>		1900	2510				

* *aniC* was done in LB + glucose.

acid-inducible genes (Tables 5 and 6). It should be pointed out that there was no obvious difference between the growth rates of *fur⁺* or *fur* derivatives. Extended growth of the *fur* mutants still did not result in any *hyd-lacZ* expression which argues against *fur* mutants simply delaying expression. Introduction of pABN203, which carries the *fur⁺* locus of *E. coli*, completely restored regulated expression of the *hyd-lac* fusion (Table 6, JF2292).

The results indicate that either directly or indirectly, Fur influences the expression of at least two pH-regulated genes, *iroA* and *hyd*. One question we wanted to address was whether or not a variation in iron availability due to a pH effect on $\text{Fe}(\text{OH})_3$ solubility could be solely responsible for the pH-regulated expression of these loci. As noted above, the case for *iroA* was unclear since we could not unequivocally separate pH from iron regulation. The *hyd* locus, however, was more amenable to study in this regard. As shown in Table 6 (JF1534), the shift from pH 7.8 to 5.8 induced *hyd* 19-fold. Adding 60–240 μM iron to pH 7.8 media did result in a modest threefold induction when compared to pH 7.8 without excess iron but did not fully compensate for the pH effect as was seen with *iroA*. This suggests two important features for *hyd* expression. Firstly, holo-Fur (Fur + Fe^{2+}) is required for the induction of this locus and secondly, iron availability alone does not account for pH-regulated gene expression.

The oxygen-regulatory *fur* product is known to indirectly affect *hyd* expression by diminishing the production of formate, a requirement for *hyd* induction (Birkman *et al.*, 1987; Sawers & Böck, 1989; Aliabadi *et al.*, 1986). The

possibility that *fur* mutants might prevent *hyd* expression in a similar manner was eliminated since the addition of excess formate to *hyd::MudJ fur* mutants did not increase *hyd* expression beyond background levels whereas formate addition did reverse the effect of an *fur* mutation (Table 6). The fact that holo-Fur, normally considered a repressor, may act as a positive regulator of gene expression confirms earlier studies involving two-dimensional SDS-PAGE analysis from *Salmonella* (Foster & Hall, 1992) and *lacZ* fusion studies from *E. coli* involving the oxygen-regulated genes *dmsA* (Cotter *et al.*, 1992) and *sodA* (Beaumont & Hassan, 1992). It is not clear whether Fur acts directly as an activator or indirectly in the form of a regulatory cascade. The question of how Fur may affect some pH-regulated genes in an apparently iron-independent manner is enigmatic. We have shown through two-dimensional polypeptide analysis that *fur* mutations affect the production of several ATR gene products (Foster & Hall, 1992). Some of these gene products are not produced in response to iron limitation under the conditions tested. Consequently, the mechanism by which Fur affects the expression of these loci is intriguing.

OxrG is involved in acid tolerance

As noted above, one reason for conducting these studies was to identify genes associated with acid tolerance in *S. typhimurium*. Two of the genes tested, *fur* and *oxrG* have clear effects upon the ATR. The *fur* locus and its involvement in acid tolerance have been presented previously (Foster, 1991; Foster & Hall, 1992). Acid tolerance of the *oxrG::Tn10* mutant is shown in Table 7.

Table 7. The effect of *oxrG* on the ATR

Values are the means of three experiments. Reproducibility was within 30% of the viable counts given (e.g. $1.5 \times 10^8 \pm 0.04 \times 10^8$ cells ml⁻¹).

Strain	Genotype	Time (h)	No. of cells surviving			
			Pre-acid shock*		Post-acid shock†	
			Unadapted	Adapted	Unadapted	Adapted
LT2	<i>oxrG</i> ⁺	0	1.5×10^8	2×10^8	2.3×10^8	1.9×10^8
		1	6×10^7	2×10^8	2.3×10^7	1.5×10^8
		2	3.7×10^6	2×10^8	2.11×10^4	9.5×10^7
JF1436	<i>oxrG::Tn10</i>	0	1.7×10^8	1×10^8	1×10^8	1.3×10^8
		1	4.5×10^5	$< 2 \times 10^2$	8×10^5	9.1×10^7
		2	5.1×10^4	$< 2 \times 10^2$	1×10^4	3.9×10^7

* Pre-acid shock adaptation. Unadapted cells pH 7.7, adapted cells pH 5.8 for 1 generation. Survival shown at 0, 1 and 2 h after adjusting pH to 3.3.

† Post-acid shock adaptation. Unadapted cells at pH 7.7, adapted cells at pH 4.3 for 15 min. Survival shown at 0, 1 and 2 h after adjusting pH to 3.3.

There are two stages involved in the low-pH-inducible ATR system. They include the pre-acid shock phase induced at pH 5.8 and a post-acid shock phase induced at levels below pH 4.5. A main function of the pre-acid shock stage is to improve pH homeostasis (Foster & Hall, 1991) and allow synthesis of post-acid shock proteins (ASPs) to occur below pH 4.0. Among the ASPs are key acid protection proteins essential for acid tolerance (Foster, 1993). As shown in Table 7, the *oxrG* mutant was defective in the early pre-acid shock phase of ATR in which cells were adapted to pH 5.8 prior to challenge at pH 3.3. These cells exhibited a 5 log decrease in cell number, even when adapted, as compared to a less than 1 log decrease for adapted LT2. However, this mutant displayed a normal ATR if the post-acid shock stage was engaged at pH 4.3 prior to pH 3.3 challenge. In this case, viability of adapted (pH 4.3) *oxrG* cells decreased by a factor less than 1 log after acid challenge (pH 3.3).

In conclusion, the growing interest in pH-regulated gene expression in *E. coli* and *S. typhimurium* has resulted in the identification of many genetic loci demonstrably regulated by pH. They include *aniG* (Aliabadi *et al.*, 1988), *adi*, *cadA* (Auger *et al.*, 1989), *hyd* (Aliabadi *et al.*, 1988; Pecher *et al.*, 1983), *lysU* (Hirshfield *et al.*, 1984), *ompF*, *ompC*, *lamB* (Hyde & Portalier, 1987; Foster & Hall, 1990) and *ldb* (Mat-Jan *et al.*, 1989). Cytochrome *o* and *d* oxidases are recent additions to this list of pH-regulated genes. Cotter *et al.* (1990) have shown that in *E. coli*, *cyo* is repressed by acid, whilst *cyd*, in a Δfur background, is distinctly acid-inducible. We can now add *aciA*, *B*, *D*, *J* and *K*, as well as *aniC* and *I* to this growing list.

We have also discovered several regulatory loci, in addition to *fur* and *earAB* (Foster & Aliabadi, 1989), that appear to mediate control of specific subsets of pH-regulated genes. These include the *earC* and *oxrG* loci which regulate *aciA* and the *aniC*, *I*, *aciK* regulon,

respectively. In all, our studies have revealed six pH-sensitive control circuits in *S. typhimurium*. The sensory transduction mechanism(s) which must sense a change in external pH and subsequently transmit a signal to the transcriptional apparatus is not understood. However, it is interesting to note that most, if not all, of the target pH-regulated genes require a second component for induction; mannose for *aniG*, lysine for *cadA*, formate for *hyd*, tyrosine for *aniCI* and *aciK*, and still unknown components of complex media for *aciB*, *D*, and even *aciA*. The significance of this trend is not readily apparent but could suggest the existence of surface sensory proteins with coinducer binding sites that are conformationally triggered at low or high pH. Studies are now underway to examine these molecular aspects of pH-controlled gene expression in *Salmonella*.

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REFERENCES

- Aliabadi, Z., Warren, F., Mya, S. & Foster, J. W. (1986). Oxygen-regulated stimulons of *Salmonella typhimurium* identified by Mud(Aplac) operon fusions. *J Bacteriol* **165**, 780–786.
- Aliabadi, Z., Park, Y. K., Slonczewski, J. L. & Foster, J. W. (1988). Novel regulatory loci controlling oxygen and pH-regulated gene expression in *Salmonella typhimurium*. *J Bacteriol* **170**, 842–851.
- Auger, E. A., Redding, K. E., Plumb, T., Childs, L. C., Meng, S.-Y. & Bennett, G. N. (1989). Construction of *lac* fusions to the inducible arginine- and lysine decarboxylase genes of *Escherichia coli* K-12. *Mol Microbiol* **3**, 609–620.

- Bagg, A. & Neilands, J. B. (1987). Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**, 5471–5477.
- Benson, N. R. & Goldman, B. S. (1992). Rapid mapping in *Salmonella typhimurium* with Mud-P22 prophages. *J Bacteriol* **174**, 1673–1681.
- Beaumont, M. D. & Hassan, H. M. (1992). Characterization of *trans*-acting regulatory elements affecting the expression of Mn-superoxide dismutase (*sodA*) in *Escherichia coli*. *Curr Microbiol* **25**, 135–141.
- Birkman, A., Zinoni, F., Sawers, G. & Böck, A. (1987). Factors affecting transcriptional regulation of the formate-hydrogen-lyase pathway of *E. coli*. *Arch Microbiol* **148**, 44–51.
- Buchmeier, N. A. & Heffron, F. (1990). Induction of *Salmonella* stress proteins upon infection of macrophages. *Science* **248**, 730–732.
- Chumley, F. G., Menzel, R. & Roth, J. R. (1978). Hfr-formation directed by Tn10. *Genetics* **91**, 639–655.
- Cotter, P. A., Darie, S. & Gunsalas, R. P. (1992). The effect of iron limitation of the aerobic and anaerobic electron transport pathway genes in *Escherichia coli*. *FEMS Microbiol Lett* **100**, 227–232.
- Davis, R. W., Botstein, D. & Roth, J. R. (1980). *A Manual for Genetic Engineering*. Advanced Bacterial Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Ernst, R. K., Dombroski, D. M. & Merrick, J. (1990). Anaerobiosis, type 1 fimbriae and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. *Infect Immun* **58**, 2014–2016.
- Finlay, B. B. & Falkow, S. (1989). *Salmonella* as an intracellular parasite. *Mol Microbiol* **3**, 1833–1841.
- Forst, S., Douglas, J., Rampersaud, A. & Inouye, M. (1990). *In vivo* phosphorylation of OmpR, the transcriptional activator of *ompF* and *ompC* genes in *Escherichia coli*. *J Bacteriol* **172**, 3473–3477.
- Foster, J. W. (1991). *Salmonella* acid shock proteins are required for the acid tolerance response. *J Bacteriol* **173**, 6896–6902.
- Foster, J. W. (1993). The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. *J Bacteriol* **175**, 1981–1987.
- Foster, J. & Aliabadi, Z. (1989). pH-regulated gene expression in *Salmonella*: genetic analysis of *aniG* and cloning of the *earA* regulator. *Mol Microbiol* **3**, 1605–1615.
- Foster, J. W. & Hall, H. K. (1990). Adaptive acidification tolerance response of *Salmonella typhimurium*. *J Bacteriol* **172**, 771–778.
- Foster, J. W. & Hall, H. K. (1991). Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. *J Bacteriol* **173**, 5129–5135.
- Foster, J. W. & Hall, H. K. (1992). Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron and pH-regulated protein synthesis. *J Bacteriol* **174**, 4317–4323.
- Gale, E. F. & Epps, H. M. R. (1942). The effect of the pH of the medium during growth on the enzymic activities of bacteria (*E. coli* and *M. lysodieticus*) and the biological significance of the changes produced. *Biochem J* **36**, 600–619.
- Hantke, K. (1981). Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol & Gen Genet* **182**, 288–292.
- Hantke, K. (1987). Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12: *fur* not only affects iron metabolism. *Mol & Gen Genet* **210**, 135–139.
- Heatwole, V. M. & Somerville, R. L. (1991). The tryptophan-specific permease gene, *mtr*, is differentially regulated by the tryptophan and tyrosine repressor in *Escherichia coli* K12. *J Bacteriol* **173**, 3601–3604.
- Hirshfield, I. N., Tenreiro, R., Vanbogelen, R. A. & Neidhardt, F. C. (1984). *Escherichia coli* K-12 lysyl-tRNA synthetase mutant with a novel reversion pattern. *J Bacteriol* **158**, 615–620.
- Holley, E. A. & Foster, J. W. (1982). Bacteriophage P22 as a vector for Mu mutagenesis in *Salmonella typhimurium*: isolation of *nad-lac* and *pnc-lac* gene fusions. *J Bacteriol* **152**, 959–962.
- Hughes, K. & Roth, J. (1988). Transitory *cis*-complementation: a general method for providing transposase to defective transposons. *Genetics* **119**, 9–12.
- Hyde, M. & Portulier, R. (1987). Regulation of major outer membrane porin proteins of *E. coli* K-12 by pH. *Mol & Gen Genet* **208**, 511–517.
- Lee, C. A. & Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc Natl Acad Sci USA* **87**, 4304–4308.
- Malloy, S. R. & Nunn, W. D. (1981). Selection for loss of tetracycline resistance by *Escherichia coli*. *J Bacteriol* **145**, 1110–1112.
- Malloy, S. R. & Roth, J. R. (1983). Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put::Mud(Aplac)* operon fusions. *J Bacteriol* **154**, 561–568.
- Mat-Jan, F., Alam, K. Y. & Clark, D. P. (1989). Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase. *J Bacteriol* **171**, 342–348.
- Mekalanos, J. J. (1992). Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol* **174**, 1–7.
- Meng, S.-Y. & Bennett, G. (1992a). Nucleotide sequence of the *Escherichia coli* *cad* operon: a system for neutralization of low extracellular pH. *J Bacteriol* **174**, 2659–2669.
- Meng, S.-Y. & Bennett, G. (1992b). Regulation of the *Escherichia coli* *cad* operon: location of a site required for acid induction. *J Bacteriol* **174**, 2670–2678.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Morgan, R. W., Christman, M. F., Jacobson, F. S., Sturz, G. & Ames, B. N. (1986). Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proc Natl Acad Sci USA* **83**, 8059–8063.
- Pecher, A., Zinoni, F., Jatisatienr, C., Wirth, R., Hennecke, H. & Böck, A. (1983). On the redox control of synthesis of anaerobically induced enzymes in enterobacteriaceae. *Arch Microbiol* **136**, 131–136.
- Sarsero, J. P., Wookey, P. J. & Rottard, A. J. (1991). Regulation of expression of the *Escherichia coli* K-12 *mtr* gene by TyrR protein and Trp repressor. *J Bacteriol* **173**, 4133–4143.
- Sawers, G. & Böck, A. (1989). Novel transcriptional control of pyruvate formate-lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. *J Bacteriol* **171**, 2485–2498.
- Schlenz, V. & Böck, A. (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.
- Slonczewski, J. L., Gonzalez, T. N., Bartholomew, M. & Holt, N. J. (1987). Mud-directed *lacZ* fusions regulated by acid pH in *Escherichia coli*. *J Bacteriol* **169**, 3001–3006.
- Spector, M. P., Aliabadi, Z., Gonzalez, T. & Foster, J. W. (1986). Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis- and heat shock-inducible proteins. *J Bacteriol* **168**, 420–424.
- Spector, M. P., Park, Y. K., Tirgari, S., Gonzalez, T. & Foster, J. W. (1988). Identification and characterization of starvation-regulated

genetic loci in *Salmonella typhimurium* by using Mud-directed *lacZ* operon fusions. *J Bacteriol* **170**, 345–351.

Tsai, S. P., Hartin, R. J. & Ryu, J. (1989). Transformation in restriction *Salmonella typhimurium* LT2. *J Gen Microbiol* **135**, 2561–2567.

Valone, S. E., Chikami, G. K. & Miller, V. L. (1993). Stress induction of the virulence proteins (SpvA, -B and -C) from native plasmid pSDL2 of *Salmonella dublin*. *Infect Immun* **61**, 705–713.

Vogel, H. J. & Bonner, D. M. (1956). Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J Biol Chem* **218**, 97–106.

Watson, N., DunYak, D. S., Rosey, E. L., Slonczewski, J. L. & Olsen, E. R. (1992). Identification of elements involved in transcriptional regulation of the *Escherichia coli cad* operon by external pH. *J Bacteriol* **174**, 530–540.

Youderian, P., Sugino, P., Brewer, K. L., Higgins, N. P. & Elliot, T. (1988). Packaging specific segments of the *Salmonella* chromosome with locked-in Mud-P22 prophages. *Genetics* **118**, 581–592.

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