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Mu d-Directed *lacZ* Fusions Regulated by Low pH in *Escherichia coli*

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Methods were devised to isolate strains of *Escherichia coli* containing Mu d (*lacZ* Km^r) operon fusions regulated by external pH and by internal pH. External acid-inducible fusions (*exa*) were detected by plating a Mu d fusion pool on Luria broth with 5-bromo-4-chloro-3-indolyl-β-D-galactoside, buffered at pH 7.4, and then replica plating on the same medium buffered at pH 5.5. Two *exa* strains showed induction by external acidification, up to 800-fold and 90-fold. Induction of both fusions was maximal at pH 5.6 and minimal over pH 7.0 to 8.3. There was no induction by membrane-permeable weak acids which depress internal pH at constant external pH. Anaerobiosis increased the steady-state level of transcription of *exa-1* 5-fold and of *exa-2* 2.5-fold at low external pH. Internal acid-inducible fusions (*ina*) were detected by plating a Mu d fusion pool on MacConkey medium, pH 6.8, and then replica plating with 15 mM benzoate. Two *ina* strains showed 10-fold induction by 20 mM benzoate at external pH 7.0. Similar results were obtained with other weak acids; their relative potency (salicylate > benzoate > dimethoxazoledinedione) was consistent with their relative ability to depress internal pH. In the absence of a weak acid, external pH had almost no effect over the pH range 5.5 to 8.0. Anaerobiosis did not affect *ina* induction. To our knowledge, this is the first report of *E. coli* genes induced specifically by internal but not external acidification and the first report of gene fusions induced by external acidification but not by weak acids.

Escherichia coli has a number of genetic response systems that enable it to adapt rapidly and survive periods of suboptimal growth conditions (reviewed in reference 7). These genetic response systems are coordinated in regulons (groups of operons under control of the same regulatory protein) and in stimulons (groups of regulons under control of the same environmental stimulus). Examples include the heat shock regulon (see reference 16 for a review), the SOS regulon (see reference 29 for a review), and the phosphate starvation stimulon (30, 31). The *lacZ* fusion technique (23) which places the conveniently assayable gene product β-galactosidase under control of regulatory elements within regulons, has been applied with great success to the study of these genetic responses, as well as to studies of osmoregulation (8, 14) and anaerobiosis (32, 33).

Bacterial pH homeostasis and pH stress are of interest for a number of reasons. While homeostasis of internal pH is a basic requirement for growth in a wide range of bacterial species, the mechanism of pH homeostasis in *E. coli* remains poorly understood (reviewed in reference 1; S. Schuldiner, J. Membrane Biol., in press). The transmembrane pH difference (ΔpH) is a component of the proton potential (18). *E. coli* maintains its internal pH near 7.6 over a wide range of external pHs (18, 26, 35). Internal pH recovers rapidly in the face of a sudden external acid shift (25) or an alkaline shift (34).

We sought to locate genes whose transcription is increased during *E. coli* response to pH perturbation, possibly genes exhibiting coordinate regulation as a pH regulon or stimulon. These products might either contribute to pH homeostasis or enable the cell to adapt physiologically to pH change. Evidence for the existence of such genes includes the decarboxylases and deaminases induced at low and high external pH, respectively (5) and the alkaline induction of SOS and heat shock genes (22, 27). In *Streptococcus faecalis*, the internal pH-dependent ATPase controls pH homeostasis (12, 13).

We used the technique of *lacZ* operon fusion, with Mu dI1734 (*lacZ*, Km^r) (3), and devised plate screening methods which allowed specific detection of fusions induced by external or internal acidification at constant external pH mediated by membrane-permeable weak acids. We report here the successful isolation of operon fusions specifically induced by external and internal acidity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains used were derivatives of *E. coli* K-12 and are listed in Table 1. Growth media used included Luria broth (LB), lactose-MacConkey agar, and M63 salts with 0.2% glucose (15). The β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was spread on LB plates with 0.1 ml of a 20-mg/ml solution in *N*,*N*-dimethylformamide. Kanamycin sulfate was used at 50 μg/ml in LB and at 100 μg/ml in lactose-MacConkey medium.

The pH of plate and liquid media was maintained by inclusion of sulfonate buffers with appropriate pK_a values, including MES [2(*N*-morpholino)ethanesulfonic acid, pK_a = 6.1], PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pK_a = 6.8], MOPS [3-(*N*-morpholino)propanesulfonic acid, pK_a = 7.2], and TAPS [tris(hydroxymethyl)methylaminopropanesulfonic acid, pK_a = 8.4]. For plate media, the pH was adjusted prior to addition of agar, and then the pH of the solidified plates was measured with a flat-surfaced electrode, TefMark II-FDA (Markson).

The growth temperature used for isolation and characterization of Mu dI1734-*lacZ* fusion strains was 30°C, because such strains reportedly may prove unstable at 37°C (2, 3). Our isolates, however, did appear to retain inducibility and kanamycin resistance at 37°C. Anaerobic growth was performed in tubes layered with sterile mineral oil.

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TABLE 1. Strains of *E. coli* K-12

Strain	Description or genotype	Source or reference
M8820	F⁻ *araD139* Δ(*ara-leu*)*7697* Δ(*proAB-argF-lacIPOZYA*)*XIII rpsL*	M. Casadaban
POI1683 (POI1734)	F⁻ Mu dI1734 (Km^r *lacZYA*) *ara*::Mu *cts3* Δ(*proAB-argF-lacIPOZYA*)*XIII rpsL*	M. Casadaban
JLS821	M8820 Nal^r	This study
JLS8602	JLS821 *exa-1*::Mu dI1734	This study
JLS8605	JLS821 *exa-2*::Mu dI1734	This study
JLS8612	M8820 *ina-1*::Mu dI1734	This study
JLS8615	M8820 *ina-2*::Mu dI1734	This study

Isolation and assay of Mu dI1734-*lacZ* fusion strains. The vector used to generate a pool of operon fusions was Mu dI1734 (*lacZ* Km^r). The procedures were those of Castilho et al. (3) and Silhavy et al. (24). A lysate of Mu dI1734 was produced from a mid-log-phase culture of POI1683 in LB, shifted from 30 to 42°C for 1 h and then to 37°C for 1 h. After the culture was shaken with chloroform, the lysate titer was about 10^{11} PFU/ml, as assayed on strain M8820. The recipient used was either M8820, a strain deleted for the entire *lac* operon, or JLS821, a nalidixic acid-resistant derivative of M8820. Equal volumes of phage lysate and of mid-log-phase culture of the recipient were mixed and incubated at 30°C without shaking for 20 min, diluted 10-fold in LB, and incubated at 30°C with shaking for 1 h. The fusion culture was then suspended in LB and plated on *lacZ* selective medium containing kanamycin, either LB with X-gal or lactose-MacConkey medium (see Results).

In vitro assays of β-galactosidase activity were performed as described (15) and reported in Miller units, which are normalized to cell density. Three Miller units equal approximately one specific activity unit, i.e., the amount of enzyme which will hydrolyze 1 nmol of *o*-nitrophenyl-β-D-galactoside per min per mg of total protein (24). Culture samples in heavily buffered media were washed and suspended in assay buffer to avoid pH effects on the enzyme activity.

RESULTS

Isolation of external-acid-inducible *lacZ* fusions. We devised a screening procedure to detect colonies containing a *lacZ* operon fusion whose transcription was inducible by external acidity. To do this, it was necessary to establish reasonably constant pH conditions in plate media. LB agar medium was supplemented with one of the Good series of biological buffers at the appropriate pK_a, at a concentration of 100 mM (4, 6). Each plate was spread with 0.1 ml of an overnight culture of the strain indicated (see Table 2). After 2 days of growth at 30°C, conditions approximating those of the initial isolation of *lacZ* fusion strains, the bacteria were scraped off with a rubber policeman, and the pH of the plate surface was retested. The pH of various types of plate before and after bacterial growth is shown in Table 2.

Generally, buffered plates showed an increase of 0.3 to 0.6 pH units resulting from bacterial growth, except at pH 9.0, when growth rate was significantly diminished. These effects may be compared with the relatively large pH increases seen in unbuffered LB (2.2 units) and in lactose-MacConkey medium, given a *lacZ*-negative strain (1.5 units).

For initial isolation of *lacZ* operon fusions (see Materials and Methods), the selection medium was LB containing kanamycin and X-gal. The plate pH was 7.4, buffered with 100 mM MOPS to pH 7.4, which rose to 7.9 under bacterial growth (Table 2). This pH range approximates the known optimum range of *E. coli* internal pH (18, 26, 35). Therefore, it was assumed that any genes responsive to external pH stress would be least active within this range.

After 2 days of growth at 30°C, the colonies were replica plated onto LB medium with X-gal and 100 mM MES, pH 5.6 (pH 6.0 under bacterial lawn). Of 82,000 colonies screened, 2 were found (JLS8602 and JLS8605) which appeared white on the original and blue on the pH 5.6 replica and maintained this difference after two restreakings at pH 7.4 and pH 5.6. The result was confirmed on plates buffered with citrate instead of MES (thus ruling out a buffer-specific effect) and quantified by assay in liquid medium (Fig. 1). The external-acid-inducible alleles were provisionally designated *exa-1* (JLS8602) and *exa-2* (JLS8605).

Isolation of internal-acid-inducible *lacZ* fusions. We devised a procedure to preferentially detect *lacZ* fusions induced specifically by perturbation of internal pH as opposed to external pH. Our procedure was based on the effect of membrane-permeable weak acids, which enter the bacterial cell primarily in the protonated form and then dissociate, releasing protons which decrease the internal pH (reviewed in reference 17). Isolation of internal-acid-inducible strains was first attempted by plating the Mu d fusion pool on LB agar with kanamycin, X-gal, and 100 mM PIPES, pH 6.8, and replica plating on the same medium containing 20 mM benzoic acid. Of 49,000 colonies screened, no *lacZ* fusions were found to be reproducibly induced only on the benzoate-containing plate.

We therefore devised an alternate procedure based on the MacConkey indicator, which has a lower range of sensitivity than X-gal (detection threshold at 100 units, as opposed to 1 unit for X-gal [24]). Thus, MacConkey medium would detect genes expressed at a relatively high level under the nonselective condition (absence of benzoate). The fusions were plated initially on lactose-MacConkey medium with kanamycin and then replica plated with 15 mM sodium benzoate (pH 6.8). No buffer was included, because the

TABLE 2. Average pH of plate media[a]

Medium (pH)	Strain	Plate pH			
		Before growth		After 2 days of growth at 30°C	
		No addition	Benzoate	No addition	Benzoate
LB	M8820	5.83	ND[b]	8.04	ND
LB + MES (5.5)	M8820	5.58	ND	5.95	ND
LB + MES (6.0)	M8820	6.05	ND	6.43	ND
LB + PIPES (7.0)	M8820	7.01	ND	7.56	ND
LB + MOPS (7.4)	M8820	7.40	ND	7.87	ND
LB + MOPS (7.6)	M8820	7.57	ND	8.09	ND
LB + TAPS (8.0)	M8820	7.94	ND	8.42	ND
LB + TAPS (8.5)	M8820	8.47	ND	8.76	ND
LB + TAPS (9.0)	M8820	8.96	ND	9.02	ND
MacConkey	M8820	6.77	6.77	8.30	8.20
MacConkey	K-12	6.77	6.77	4.63	5.41
MacConkey	JLS8612	6.77	6.77	7.27	7.15
MacConkey	JLS8615	6.77	6.77	7.26	7.09

[a] Concentration of buffers listed, 100 mM; of benzoate, 15 mM. Each plate was spread with 0.1 ml of an overnight culture of the strain indicated. pH values represent averages for five plates. The standard deviation (SD) was <0.05 unit in all cases, except LB with and without growth (SD = 0.08) and JLS8615 grown without benzoate (SD = 0.06).

[b] ND, Not done.

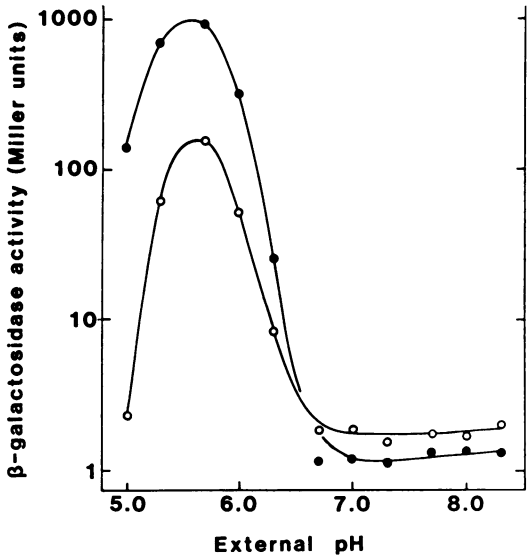

FIG. 1. Steady-state levels of β-galactosidase activity in JLS8602 (●) and in JLS8605 (○) as a function of external pH. The medium used was LB buffered at 100 mM with one of the following: citrate (pH 5.0 to 5.3), MES (pH 5.7 to 6.3), PIPES (pH 6.7 to 7.3), or MOPS (pH 7.7 to 8.3). Cultures were grown overnight in buffered LB and then diluted 1:50 and grown to an OD_{600} of 0.16 to 0.26.

basis of the MacConkey indicator is pH decrease resulting from excretion of fermentation products. Of 3,800 colonies screened, 2 isolates (JLS8612 and JLS8615) showed reproducible β-galactosidase induction by benzoate but not by external acidification in the absence of benzoate (see Fig. 5). The internal acid-inducible loci were provisionally designated *ina-1* (JLS8612) and *ina-2* (JLS8615).

The *exa* and *ina* strains were characterized in rich medium

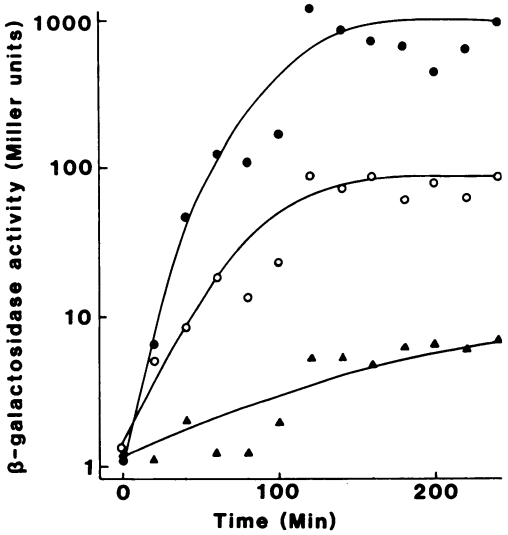

FIG. 2. Time course of induction of β-galactosidase in JLS8602 by external acidification. Cultures were grown overnight in LB with 100 mM MOPS (pH 7.6) and then diluted 1:50 in LB with 10 mM MOPS (pH 7.6). At an OD_{600} of 0.16, the following additions were made: 170 mM MES (pH 4), final pH 5.0 (●); 20 mM MES (pH 4), final pH 5.9 (○); or 170 mM NaCl (▲). Cells were grown to an OD_{600} of 0.69 and then diluted 1:5 (at 100 min) in fresh medium at the same pH and grown to an OD_{600} of 0.80 (at 240 min).

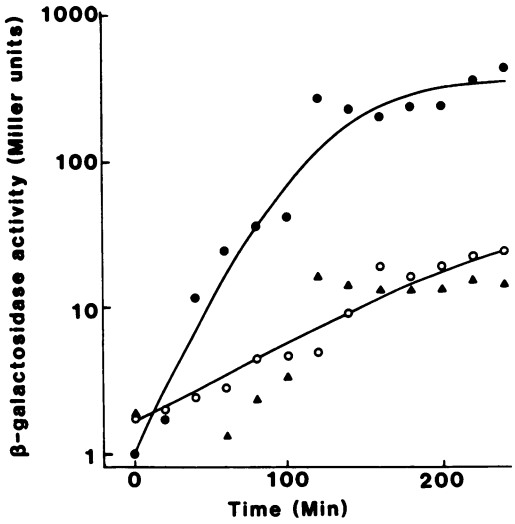

FIG. 3. Time course of induction of β-galactosidase in JLS8605 by external acidification. Cultures were grown overnight in LB with 100 mM MOPS (pH 7.6) and then diluted 1:50 in LB with 10 mM MOPS (pH 7.6). At an OD_{600} of 0.11, the following additions were made: 170 mM MES (pH 4), final pH 5.0 (●); 20 mM MES (pH 4), final pH 5.9 (○); or 170 mM NaCl (▲). Cells were grown to an OD_{600} of 0.51, then diluted 1:5 (at 100 min) in fresh medium at the same pH and grown to an OD_{600} of 0.62 (at 240 min).

because *E. coli* strains were found to grow poorly in minimal medium under conditions of pH stress. The results were essentially confirmed, however, in M63 glucose minimal medium supplemented with 1% tryptone.

External pH dependence of *exa-1* and *exa-2*. Transcription of both *exa-1* and *exa-2* was increased over the external pH range 5.0 to 6.3 (Fig. 1). The curve of enzyme activity was flat over the neutral to alkaline range. The *exa-1* locus was induced more strongly than *exa-2* in the acid range and was also more fully repressed in the alkaline range.

The time course of induction is shown for *exa-1* (Fig. 2) and *exa-2* (Fig. 3). In both cases a steady level of induction was reached after 2 h. A significant degree of induction was also observed to result from the addition of NaCl instead of low-pH buffer. The cause of this effect was unclear, since the steady-state level of induction of both mutants (grown to an OD_{600} of 0.2 from overnight cultures incubated with NaCl) did not differ significantly from induction in the absence of NaCl (data not shown). There may be a transient response to the increase in osmolarity.

Neither strain showed significant induction in the presence of 20 mM benzoate at external pH 7.0, a level of weak acid which strongly induced the *ina* strains (Fig. 4; see below). Both *exa-1* and *exa-2* showed a substantial increase in induction at low external pH under anaerobic conditions (Table 3). Anaerobiosis caused a barely significant increase in enzyme level at pH 7.6.

Internal pH dependence of *ina-1* and *ina-2*. Both *ina-1* and *ina-2* showed essentially the same induction effects. The data shown (Fig. 4 and 5) are for *ina-1*. Figure 4 shows the increase in steady-state level of induction of *ina-1* in the presence of a weak acid (salicylate, benzoate, or dimethoxazoledinedione [DMO]) at concentrations sufficient to depress internal pH (21, 25). The maximum effect observed was a 10-fold increase with 15 mM salicylate. The relative effectiveness of the three weak acids (salicylate > benzoate

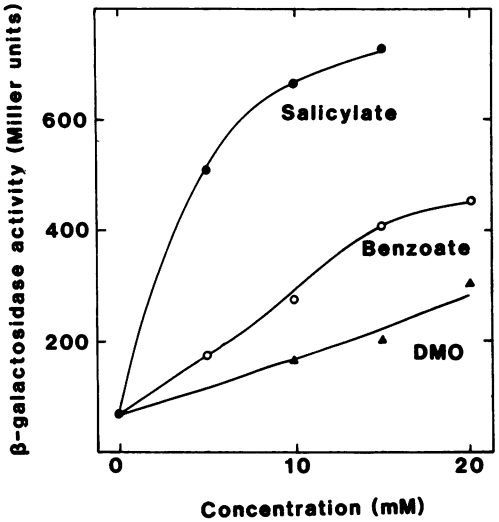

FIG. 4. Induction of β-galactosidase in JLS8612 as a function of weak acid concentration. Cultures were grown to an OD_{600} of 0.20 to 0.38 in LB with 100 mM PIPES (pH 7.0) containing salicylate (●), benzoate (○), or DMO (▲).

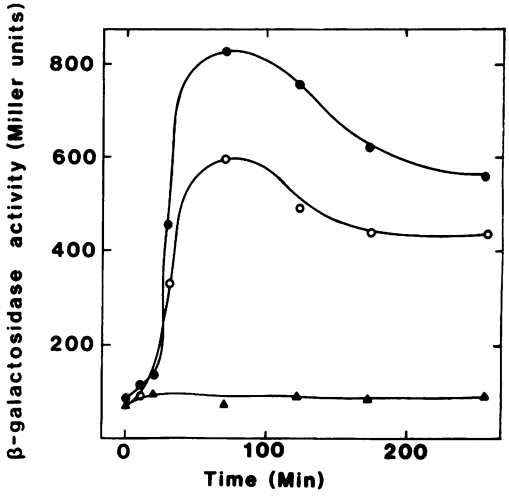

FIG. 5. Time course of induction of β-galactosidase in JLS8612 by addition of benzoate. Cultures were grown to an OD_{600} of 0.05 to 0.86 in LB with 100 mM PIPES (pH 7.0) with the following additions made at time zero: 20 mM sodium benzoate (●), 10 mM sodium benzoate (○), or 20 mM NaCl (▲).

> DMO) paralleled their relative degree of effect on internal pH (25).

The kinetics of induction of *ina-1* showed maximal induction within an hour of addition of benzoate (Fig. 5). Within another 2 h there was partial adaptation, although the levels of enzyme remained higher than those observed in the steady-state experiment (Fig. 4).

Both *ina-1* and *ina-2* showed a barely significant dependence of induction on external pH under aerobic conditions and no significant dependence under anaerobic conditions (Table 3). Anaerobiosis did not significantly affect induction by weak acids at external pH 7.0 (data not shown).

DISCUSSION

The aim of this work was to begin to define the genes of an external-pH stimulon and an internal-pH stimulon in *E. coli*. To our knowledge, we have isolated two novel classes of *lacZ* gene fusions: *exa*, induced by external acidification but not by membrane-permeable weak acids, and *ina*, induced by internal acidification (mediated by weak acids) but not by external acidification. *E. coli* genes with these specific induction characteristics have not been reported previously (for a review, see reference 1; Schuldiner, in press).

The two *exa* strains behaved similarly with respect to pH range and maximum, time course, and increased induction under anaerobic conditions. They differed in that *exa-1* was more strongly induced than *exa-2* at low pH and more strongly repressed at neutral to high pH. It is possible, therefore, that fusions *exa-1* and *exa-2* are contained in different operons.

TABLE 3. Steady-state induction of β-galactosidase in fusion strains[a]

Strain	β-Galactosidase activity (U)			
	Aerobic growth		Anaerobic growth	
	pH 7.6	pH 5.5	pH 7.6	pH 5.5
JLS8602	1.4 ± 0.3	485 ± 62	2.4 ± 0.8	2,580 ± 325
JLS8605	1.9 ± 0.1	155 ± 36	2.4 ± 0.1	375 ± 39
JLS8612	45 ± 2	63 ± 10	54 ± 8	47 ± 10
JLS8615	36 ± 3	61 ± 11	40 ± 12	34 ± 8

[a] Units are Miller units. Each value represents an average of five independent assays. Cells were grown overnight at 30°C in LB buffered with 100 mM MOPS (pH 7.6) or 100 mM MES (pH 5.5). Overnight cultures were diluted 1:50 in the same medium and grown to an OD_{600} of 0.2 to 0.3.

The uniform repression of these genes over the alkaline range is noteworthy. By contrast, there is a report that high pH induces the heat shock genes *dnaK* and *groE* (27).

The two fusion strains induced by internal acidification, *ina-1* and *ina-2* appeared identical with respect to pH range of induction, concentration range of weak acid response, and lack of anaerobic induction. The fact that three structurally unrelated weak acids all caused induction, to degrees consistent with their known abilities to depress internal pH, supports internal acidification as the cause of induction of *ina* fusion strains. The sensitivity of the response should be noted; weak acids were added at external pH 7.0, and major effects were seen at concentrations of benzoate unlikely to completely abolish the ΔpH (21, 25). The response appeared to be highly specific to internal pH; neither anaerobiosis nor osmolarity had any significant effect. The slight increase in induction at low external pH could be explained by the slight dependence of internal pH on external pH (up to 0.1-unit variation with 1.0-unit external pH [26]). Furthermore, the partial adaptation of the response occurring 100 min after addition of benzoate (Fig. 5) could be explained by the partial recovery of internal pH (11).

In interpreting the induction of both *exa* and *ina* loci, a possibility to be considered is that the bacteria respond to changes in ΔpH or in the proton potential $\Delta\tilde{\mu}_{H^+}$, of which ΔpH is a component. Decrease of external pH causes increase of ΔpH and $\Delta\tilde{\mu}_{H^+}$; decrease of internal pH causes decrease of ΔpH and $\Delta\tilde{\mu}_{H^+}$. In the *exa* strains, response to $\Delta\tilde{\mu}_{H^+}$ appears unlikely because the response was increased under anaerobiosis, where the electrical potential $\Delta\psi$ (and hence the total $\Delta\tilde{\mu}_{H^+}$) in fact decreased under acidic conditions (10). In the *ina* alleles, the insensitivity to external pH would not be consistent with response to ΔpH or $\Delta\tilde{\mu}_{H^+}$. A slight degree of induction at low external pH was seen under aerobic conditions (Table 3); this effect could be explained

by a slight decrease of internal pH at low external pH (24, 35), but was not consistent with response to ΔpH or $\Delta\tilde{\mu}_{H^+}$.

There are several possibilities for the identity of the *exa* and *ina* genes. For example, some of the carboxylases known to be induced by external acidification (5) might exhibit the *exa* phenotype. Genes for lysine and arginine decarboxylases show induction at pH 5 to 6 and under anaerobic conditions (E. Auger and G. Bennett, unpublished data). In preliminary studies, all our fusion strains tested positive for lysine and arginine decarboxylases, but we are pursuing this possibility further.

In view of the external acidification which accompanies anaerobic growth and the increased reliance on ΔpH as a source of proton potential, it would not be surprising to find some overlap between genes induced by low external pH and by anaerobiosis. We indeed found that anaerobiosis amplified the maximal induction of *exa-1* and *exa-2* (Table 3). Interestingly, we have preliminary evidence that a gene isolated as anaerobiosis-inducible in *Salmonella typhimurium* is pH dependent (J. Slonczewski and J. Foster, manuscript in preparation).

The *ina* locus (or loci) might govern the formation of neutral fermentation products, a response to acidification documented in *Klebsiella* and *Clostridium* spp. (9, 28). In these species, however, the response appears to be specific to the weak acids accumulated. Alternatively, the internal pH dependence of *ina* induction would be consistent with a role as a regulator of bacterial pH. No such device has yet been demonstrated in *E. coli*, although in *S. faecalis* the proton-translocating ATPase shows internal pH-dependent transcription (12, 13).

It should also be noted that weak acids are chemotactic repellents (11, 19, 25) and effect nonheritable resistance to some antibiotics (20). It will be of interest to test the *ina* strains for alterations in chemotaxis and antibiotic resistance.

We are now attempting to map the *exa* and *ina* loci and also to isolate additional internal- and external-acid-inducible *lacZ* fusions. Furthermore, we are modifying our isolation techniques to screen for internal- and external alkaline-inducible fusions. It is likely that several stimulons of genes responsive to different classes of pH perturbation will ultimately be defined.

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