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The PhoE porin and transmission of the chemical stimulus for induction of acid resistance (acid habituation) in *Escherichia coli*

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R.J. ROWBURY, M. GOODSON AND A.D. WALLACE. 1992. *Escherichia coli* K12 becomes resistant to killing by acid (habituates to acid) in a few minutes at pH 5·0. Habituation involves protein synthesis-dependent and -independent stages; both must occur at an habituating pH. The habituation sensor does not detect increased ΔpH (or decreased $\Delta\psi$) nor an increased difference between pH_o and periplasmic pH but probably detects a fall in either external or periplasmic pH. Phosphate ions inhibit habituation, at any stage, probably by interfering with outer membrane passage of hydrogen ions. Most outer membrane components tested are not required for habituation but *phoE* deletion mutants habituated poorly and are acid-resistant. Strains derepressed for *phoE*, in contrast, showed increased acid sensitivity. These and other results suggest that habituation involves hydrogen ions or protonated carriers crossing the outer membrane preferentially via the PhoE pore, a process inhibited by phosphate and other anions. Stimulation by phosphate of the poor growth of *E. coli* at pH 5·0 is in accord with the above. Acetate did not enhance acid killing of pH 5·0 cells, suggesting that their resistance does not depend on maintaining pH_i near to neutrality at an acidic pH_o level.

INTRODUCTION

Polluting enterobacteria, such as *Escherichia coli*, can be exposed to a wide range of acidifying conditions in the natural aquatic environment and such exposures often involve a gradual increase in acidity (Rowbury *et al.* 1989) which can lead to induced resistance (habituation) to acid (Goodson & Rowbury 1989a). This involves a phenotypic change such that the habituated organisms can survive a level of acidity which is lethal to non-habituated ones although growth at acid pH values is not affected by habituation. Habituation may be an important factor in the survival of polluting bacteria in acidified waters, of contaminating organisms in acid foods and of putative pathogens at acidic locations in the animal body (Goodson & Rowbury 1989b; Foster & Hall 1990). Habituation is dependent on protein synthesis and at least 14 or 15 proteins are produced in increased amounts at pH 5·0 (Raja *et al.* 1991a) including, perhaps, the LysU gene product (Hickey & Hirshfield 1990).

Habituation was first observed in *E. coli* (Goodson & Rowbury 1989a, b) but it may be widespread in bacteria as it has been described in *Salmonella typhimurium* (Foster & Hall 1990).

Habituation to acid is one of a number of inducible stress resistance processes, each of which appears to involve the interaction of the chemical (or physical) stimulus with a sensor molecule which generates a signal. The signal produces a response, often *via* transducing molecules (Neidhardt *et al.* 1990). For a Gram-negative bacterium, a chemical stimulus might affect an outer membrane sensor, or need to penetrate the outer membrane to interact with a cytoplasmic membrane or periplasmic sensor molecule or cross both membranes to activate a cytoplasmic component. Generally, it is believed that molecules which sense altered environmental conditions are on the outer face of the cytoplasmic membrane, i.e. penetrating into the periplasm (Stock *et al.* 1989; Igo *et al.* 1989; Aiba & Mizuno 1990) but, in some cases, the agent causing the stimulus must enter the cytoplasm to activate a molecule on the inner face of the cytoplasmic membrane (Repaske & Adler 1981). The involvement of acidification of the cytoplasm as a stage in acid habituation has already been ruled out (Raja *et al.* 1991a) and, accordingly, the possibility has been investigated here that the process results from hydrogen ions interacting with an outer membrane component or crossing this membrane to modify a molecule which has a sensory region free in the periplasm.

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Phosphate pollution of natural waters is an important factor in eutrophication. Detergents are still a major source of phosphates because the low phosphate and phosphate-free products developed to reduce such pollution (Hammond 1971; Boughton *et al.* 1971) have not fully replaced high phosphate products. Additionally, phosphates enter natural waters from human wastes, industrial processes, run-offs from fertilized land and dumping of agricultural waste slurries. These sources of phosphate also contribute acid pollutants (Rowbury *et al.* 1989) and, therefore, phosphate might influence the response of polluting enterobacteria to acid pH. A study of how phosphate affects habituation of *E. coli* to acid is described here.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used were all derivatives of *E. coli* K12. They are listed, with their known genetic characteristics, in Table 1. Strains 1157-2, 1157-4 and W2961-1 were produced by transduction of strains 1157 and W2961 to *proA*$^+$ *phoE*$^+$ with phage Plvir as described below.

Growth media

Organisms were generally grown in Nutrient Broth (Oxoid No. 2) at 37°C in shaken culture and used in the exponen-

Table 1 Strains of *Escherichia coli* used in the present study

Strain	Genotype	Source
1829	*trp*	This laboratory
1829 ColV, I-K94	1829 ColV$^+$	This laboratory
P678-54	*thr, leu, thi, lacY, ara, xyl, rpsL, lamB*	H. Adler
RJR200	P678-54*ompA2101*	This laboratory
1157	*thr, leu, proA2 (ΔproA, phoE, gpt), his, thi, argE, lacY, galK, xyl, rpsL*	N.E. Gillies
1157-2	1157 *proA*$^+$ *phoE*$^+$	This study
1157-4	1157 *proA*$^+$ *phoE*$^+$	This study
CE 1194	*thr, leu, proA2(ΔproA, phoE, gpt), phoS21, bgl, his, thi, argE, lacY, galK, xyl, rpsL*	H. Tristram
CE 1195	CE1194 *proA*$^+$ *phoE*$^+$	H. Tristram
CE 1237	*thi, pyrF, thyA, argG, ilvA, his, mal, tonA, phx, rspL, deoC, supE, susuvrB, vtr, glpR, ompR, phoR69, phoA8*	F. van Asma
CE 1238	CE 1237 Δ*phoE, proA*	F. van Asma
PCO479	*thr, leu, thi, pyrF, codA, thyA, argG, ilvA, his, lacY, tonA, tsx, phx, rpsL, deoC, susuvrB, vtr, supE, glpR*	B. Lugtenberg
CE 1062	PCO479*ompC*	B. Lugtenberg
CE 1107	PCO479*ompR*	F. van Asma
HfrK10	*tonA22, garB10, ompF627, fadL701, relA1, pit-10, spoT1, rrnB2, mcrB1, phoM510*	B.J. Bachmann
C75a	HfrK10*phoR64*	B.J. Bachmann
C75b	HfrK10*phoS164*	B.J. Bachmann
C112a	HfrK10*phoT34*	B.J. Bachmann
E15	HfrK10Δ*phoA8*	B.J. Bachmann
W2961	*thr-1, ara-14, leuB6, proA62 (ΔproA, phoE, gpt), lacY, supE, galK, rac, rfbD1, mgl-51, rpsL20, xyl-5, mtl-1, thi-1*	B.J. Bachmann
W2961-1	W2961*proA*$^+$*phoE*$^+$	This study
BW3414	*lac-169*	B. Wanner
BW4975	*lac-169*, Δ*(phoT, pstB, pstC, phoU, bgl)*	B. Wanner

tial phase of growth. This broth was solidified where necessary with 20 g/l Difco Bacto Agar to produce nutrient agar (NA).

For transduction, TNA broth was used. This contained (g/l): Difco Bacto Tryptone, 10; NaCl, 8; glucose, 1; plus $CaCl_2$, 5 mmol/l. The minimal medium employed was prepared as described previously (Rossouw & Rowbury 1984) with glucose 2 g/l and any required amino acids (L-form 20 μg/ml) added as required. This medium was solidified with Difco Bacto Agar (20 g/l) to give minimal agar (MA).

Transduction

The transducing lysate was prepared by infecting a culture of strain 1829 grown in TNA broth at 37°C with 10^8 phage Plvir/ml with incubation for 10 min at 37°C; following 10-fold dilution in TNA broth, the mixture was then shaken at 37°C for 2 h before chloroform treatment. The resulting suspension contained *ca* 10^{12} infective phage/ml.

For transduction, exponential cultures in TNA broth at 37°C were harvested, resuspended in dilution buffer (NaCl, 8 g/l; $CaCl_2$, 0·1 mmol/l; TNA broth, 4 ml/l) with the above phage P1 lysate (300 μl/ml) and incubated for 1 h static at 37°C. After centrifugation for 5 min at 12 000 rev/min in an Eppendorf microcentrifuge, the pellet was resuspended in 1·5 ml of dilution buffer and diluted volumes plated on NA plates and on plates of suitably supplemented MA. Approximately 1 in 10^7 of the organisms was transduced to *proA*$^+$*phoE*$^+$.

Habituation conditions

Incubation was in broth with shaking at 37°C and at the stated pH. Organisms of the previously tested *E. coli* strains gained resistance to acid (habituated) in 7·5–10 min at 37°C (Raja *et al.* 1991a). In the present study, in most experiments, 15 or 20 min incubation times were used to ensure that all capable organisms had habituated. Where it was desired to compare results directly, the same habituation times were used. In some cases where strains showed poor habituation in 15 or 20 min at 37°C, incubation at the habituating pH was also tested for 10 min and/or 75 min.

The effects of phosphate and other anions were tested on habituation. For these studies dipotassium hydrogen phosphate, disodium hydrogen phosphate, sodium polyphosphate ($Na_7P_5O_{16}H_5$), trisodium citrate, potassium sulphate, potassium chloride, magnesium sulphate or other salts were added to the habituation broth at the appropriate molar concentrations and the pH readjusted appropriately, if necessary.

Acid challenge

The sensitivity of organisms to killing by acid was tested by exposure to the appropriate pH (generally 3·0 but, in a few experiments, 3·5) in shaken broth at 37°C with any required additions (e.g. acetate or phosphate). Exposed organisms were subsequently plated on NA with incubation for 24–36 h at 37°C.

Most of the results of survival after acid challenge shown in the tables and in the text are for single experiments but each experiment was performed at least three times with consistent results. Statistical analysis was performed for some critical experiments and, for these, standard errors and significance were ascertained as described previously (Hicks & Rowbury 1986).

Gel electrophoresis of proteins and autoradiography

To test for proteins newly synthesized at acid pH, organisms were grown at pH 7·0 to exponential phase in broth at 37°C, then shifted to fresh broth at pH 7·0, 4·3 or 4·3 plus 10 mmol/l phosphate. After 2·5 min, 15 μC/ml of [^{35}S]-L-methionine (1 C/μmol) was added and incubation continued for 30 min. The organisms were then harvested, broken by sonication and unbroken cells and large fragments removed by centrifugation at 3000 rev/min for 20 min in a MSE Centaur 2 centrifuge. Samples of the resulting supernatant fluids were then run on SDS-polyacrylamide gels prepared, operated and stained as described by Rossouw & Rowbury (1984). Autoradiography was performed according to Moores & Rowbury (1982).

RESULTS

Habituation depends on an acidic pH_o throughout the induction period

Habituation can take place in about 10 min at 37°C in broth, although a few organisms gain acid resistance more rapidly (Raja *et al.* 1991a). Essential protein synthesis occurs in the first 2·5–3·0 min at acid pH and inhibitors of this process added at the shift to pH 5·0 prevent habituation; addition of, e.g. chloramphenicol at 2·5–3·0 min or subsequently, however, has much less effect (Raja *et al.* 1991a). Presumably, proteins formed in the first 2·5–3·0 min at pH 5·0 need to function or be modified or secreted before resistance to acid killing appears. It was possible that these protein synthesis-independent stages could occur at pH 7·0; transfer to this pH after 2·5 or 3·0 min at pH 5·0, however, completely prevented habituation (Table 2).

Table 2 Effects of phosphate and chloramphenicol on habituation

Conditions during the habituation period 0–3 min	3–15 min	Percentage survival after acid challenge
pH 5·0	pH 5·0	70·8
pH 5·0	pH 7·0	0·1
pH 5·0	—*	1·8
pH 5·0 plus phosphate	pH 5·0 plus phosphate	4·0
pH 5·0	pH 5·0 plus phosphate	0·8
pH 5·0 plus chloramphenicol	pH 5·0 plus chloramphenicol	0·1
pH 5·0	pH 5·0 plus chloramphenicol	26·0
pH 5·0 plus phosphate	pH 5·0†	21·6
pH 5·0 plus phosphate	pH 5·0 plus chloramphenicol‡	0·2

Habituation was for 15 min at 37°C (using organisms of strain 1829 ColV, I-K94 previously grown at pH 7·0) except for one sample (*) which was incubated at pH 5·0 for only 3 min. Phosphate was at 2 mmol/l and chloramphenicol at 200 μg/ml.

† Cells were filtered rapidly at 3 min, washed with pH 5·0 broth and further incubated until 15 min at pH 5·0 in fresh broth.

‡ Cells were filtered rapidly at 3 min, washed with pH 5·0 broth plus chloramphenicol and further incubated (until 15 min) in fresh pH 5·0 broth plus chloramphenicol. Acid challenge was at pH 3·0 for 7 min.

Habituation does not involve detection of increased ΔpH

Although acidification of a bacterial growth medium leads to a slight fall in cytoplasmic pH (Respaske & Adler 1981), it also causes a large increase in ΔpH, and a concomitant decrease in $\Delta\psi$. Accordingly, the habituation process might involve the detection of, and response to, such changes in ΔpH or $\Delta\psi$. This is not so, because addition of acetate at 15 mmol/l, which would completely collapse ΔpH and increase $\Delta\psi$ (Repaske & Adler 1981; Salmond *et al.* 1984), did not prevent habituation. Organisms grown at pH 7·0 and then habituated at pH 5·0 for 20 min gave 81·1% survival after 7 min at pH 3·0 whereas with acetate added during habituation, survival after challenge was 31·1%; non-habituated cells gave 0·7% survival after acid exposure. Accordingly, it is probable that the absolute level of acidity must be detected by a sensor in the outer membrane, in the periplasm or on the periplasmic face of the cytoplasmic membrane.

The addition of acetate during acid challenge was also tested; 7·5 mmol/l had little effect on the killing at pH 3·0 of organisms grown at pH 5·0 or 7·0. Minus acetate, pH 5·0 cells gave 60·1% survival after 7 min challenge, whereas with acetate present during challenge, survival was 25·1%.

Outer membrane components and habituation

Habituation appeared to be normal in strains with lesions affecting a range of outer membrane proteins. Mutation of PC0479 to produce a strain (CE 1062) which completely

Table 3 Outer membrane proteins and habituation

Strain	Relevant genotype	Habituation pH	Percentage survival after acid exposure at pH 3·0	pH 3·5
PCO479	*ompF*$^+$ *ompC*$^+$	7	0·1	NT
		5	32·5	NT
CE 1062	*ompF*$^+$ *ompC*	7	0·1	NT
		5	52·5	NT
CE 1107	*ompR* (phenotypically	7	0·1	NT
	ompFompC)	5	82·0	NT
P678-54	*ompA*$^+$	7	0·2	NT
		5	85·7	NT
RJR200	*ompA*	7	NT	0·1
		5	NT	31·4

Acid challenge was at pH 3·0 for 5 min (strain PCO479 and its *ompC* and *ompR* mutants) or 7 min (strain P678-54). Because of its extreme acid sensitivity, strain RJR200 was exposed to pH 3·5 for 8 min. The *ompR* lesion in strain CE 1107 leads to absence of both OmpF and OmpC porins.

NT, Not tested.

lacks the OmpC protein did not prevent habituation (Table 3) and although a strain which had lost the OmpA protein was very acid-sensitive, it gained resistance to acid killing normally at pH 5·0. Additionally, mutations in *lamB*, *tsx*, *tonA*, *ompF* and *ompR* do not prevent habituation because strains CE 1107 (Table 3) and CE 1237 are *ompR*, PCO479 is *tsx*, strain P678-54 is *lamB* and HfrK10 and the other *pit* mutants (see below) carry *tonA* and *ompF* lesions but all can habituate normally.

Habituation was also unaffected in the absence of the outer membrane proteins involved in uptake of vitamin B12 and iron since repression of the former (BtuB component) by growth with cobalamin (10 mg/l) or of the latter with iron salts (0·1 mmol Fe^{2+}/l) did not prevent organisms of strain 1829 ColV, I-K94 from gaining resistance to acid killing at pH 5·0.

Phosphate and habituation

Potassium phosphate prevents habituation. Initially, organisms of strain 1829 Col V, I-K94 were grown with this salt (phosphate 65 mmol/l), this being present both during growth at pH 7·0 and during exposure to pH 5·0; there was complete inhibition of habituation. Subsequent experiments demonstrated that organisms grown at pH 7·0 with this salt would habituate at pH 5·0 if the phosphate were removed (Table 4) but that addition during the habituation period only was inhibitory.

For organisms exposed to pH 5·0, as little as 2 mmol/l phosphate almost completely prevented habituation but there was no effect at 0·5 mmol/l (Table 5). The difference, with respect to acid resistance, between cells habituated at pH 5·0 without potassium phosphate and those habituated with 4 mmol/l of this salt was significant at the 99% confidence level (Table 5). Other potassium salts were also tested; the chloride was inhibitory at high concentration (10 or 65 mmol/l) but the sulphate was less effective. Sodium phosphate was an active inhibitor, having a similar effect to that of the potassium salt and sodium polyphosphate was even more effective, reducing habituation markedly even at 0·25 mmol/l (Table 6). Organisms also failed to habituate with low concentrations of sodium citrate whereas magnesium sulphate was ineffective on habituation and neither did it reverse the inhibitory effect of potassium phosphate.

Potassium phosphate also prevented habituation at pH 4·5 and 5·8; at the lower pH, between 10 and 20 mmol/l was needed to fully inhibit the process whilst at the higher pH less than 1 mmol/l sufficed (Table 5).

Phosphate was inhibitory at any stage of the habituation process; when it was added at 3 min with a further 12 min at pH 5·0, the organisms did not gain resistance to acid killing whilst if present only for the first 3 min at pH 5·0 with phosphate removed and chloramphenicol added for the rest of the period at pH 5·0, habituation was also prevented (Table 2).

Phosphate also prevented habituation in P678-54 but had no effect on its *ompA* mutant.

Table 4 Phosphate and habituation

Prior growth conditions	Habituation conditions	Percentage survival after acid challenge
pH 7·0	pH 7·0	1·5
pH 7·0	pH 7·0 + phosphate	1·1
pH 7·0 + phosphate	pH 7·0 + phosphate	1·1
pH 7·0 + phosphate	pH 7·0	1·6
pH 7·0	pH 5·0	57·8
pH 7·0	pH 5·0 + phosphate	2·0
pH 7·0	pH 5·0 + phosphate*	0·2
pH 7·0 + phosphate	pH 5·0 + phosphate	0·7
pH 7·0 + phosphate	pH 5·0	84·6

Habituation was for 20 min. Acid challenge was for 7 min at pH 3·0. Potassium phosphate was added at 65 mmol/l where appropriate.
* Phosphate added at 3 min after the shift to pH 5·0. Strain used was 1829 ColV, I-K94.

Table 5 Phosphate and habituation at different pH values

Phosphate present during habituation (mmol/l)	Percentage survival after acid challenge for organisms habituated at		
	pH 4·5	pH 5·0	pH 5·8
0	80·1	89·5	64·2
0·05	NT	NT	60·1
0·1	NT	NT	44·8
0·3	NT	NT	25·1
0·5	91·6	82·3	NT
1·0	45·4	7·2	1·1
2·0	27·6	0·8	NT
4·0	10·6	1·0	0·5
10·0	7·6	0·7	NT
20·0	0·3	NT	NT

Organisms grown at pH 7·0 were habituated for 15 min at the appropriate pH (4·5, 5·0 or 5·8) and then challenged at pH 3·0 for 7 min. The above results are for a single experiment. Statistical analysis was conducted on the results of five experiments where habituation was at pH 5·0. After acid challenge (7 min at pH 3·0), the mean survival of organisms habituated at pH 5·0 without phosphate was 80·7% ± S.E. of 7·5%. For organisms habituated at pH 5·0 plus 4 mmol/l potassium phosphate, mean survival after acid challenge was 0·9 ± 0·13%. The difference between these means is significant at the 99% confidence level.
NT, Not tested.

Table 6 Polyphosphate and habituation

Additions to habituation medium (mmol/l)	Habituation pH	Percentage survival after acid challenge
None	7·0	0·4
None	5·0	64·1
Potassium phosphate, 0·5	5·0	73·6
Potassium phosphate, 1·0	5·0	6·8
Sodium phosphate, 0·5	5·0	96·1
Sodium phosphate, 1·0	5·0	58·3
Sodium phosphate, 2·0	5·0	7·6
Sodium phosphate, 4·0	5·0	0·5
Sodium polyphosphate, 0·1	5·0	68·2
Sodium polyphosphate, 0·25	5·0	18·5
Sodium polyphosphate, 0·5	5·0	2·1
Sodium polyphosphate, 1·0	5·0	2·7

Habituation was for 15 min. Acid challenge was for 7 min at pH 3·0.

Habituation and the high affinity phosphate-uptake system

A group of strains derived from a *pit* parent were used to investigate the role of the high affinity phosphate-uptake system in the phosphate effect on habituation. The parent, HfrK10, and its *phoA* derivative showed acid sensitivity for pH 7·0-grown cells similar to that of strain 1829 ColV, I-K94 (cf. Tables 6 and 7) and both gained increased acid resistance on incubation at pH 5·0. Organisms of the *phoR*, *phoS* and *phoT* derivatives of K10, grown at pH 7·0, were all markedly more acid-sensitive than their parent but each habituated at pH 5·0 (Table 7); additionally, a strain deleted for *phoT*, *pstB*, *pstC* and *phoU* (strain BW4975) habituated very similarly to its parent (strain BW 3414). Phosphate inhibits habituation in strain HfrK10; its response at pH 5·0 was rather similar to that of strain 1829 ColV, I-K94 (cf. Tables 5 and 8). Phosphate also inhibited habituation in the *phoT* mutant but its effect on the *phoS* mutant (which has only poor penetration of phosphate into the periplasm at low concentrations of this anion due to absence of the phosphate binding protein), was somewhat less marked (Table 8).

Strain 1157, which is deleted for *phoE*, was after growth at pH 7·0 found to be very resistant to acid compared with strains 1829 ColV, I-K94 and HfrK10; it also appeared to habituate very poorly, if at all, at pH 5·0 (Table 9). Accordingly, two other strains deleted for *phoE* (W2961 and CE1238) and one deleted for this gene and also mutated in *phoS* (strain CE1194) were examined for acid sensitivity and for ability to habituate. The *phoE phoS* mutant and one of the other *phoE* isolates (strain CE1238) were more acid-

Table 7 Habituation in strain HfrK10 and its *pho* mutants

Strain	Relevant genotype	Habituation pH	Percentage survival after acid challenge for 5 min	7 min
HfrK10	*pit*	7·0	NT	0·3
		5·0	NT	70·5
E15	*pit phoA*	7·0	NT	0·1
		5·0	NT	28·3
C75a	*pit phoR*	7·0	0·01	0
		5·0	5·5	2·7
C75b	*pit phoS*	7·0	0·02	0
		5·0	31·5	6·1
C112a	*pit phoT*	7·0	0·004	0·002
		5·0	21·1	4·8

Habituation was for 20 min. Acid challenge was at pH 3·0. A percentage survival shown as 0 is actually less than 0·001%.
NT, Not tested.

Table 8 Phosphate and habituation of the *phoS* mutant

Strain	Habituation pH	Phosphate added during habituation (mmol/l)	Percentage survival after acid challenge
HfrK10	7·0	No	0·3
	5·0	No	70·5
	5·0	Yes (0·5)	26·1
	5·0	Yes (1·0)	14·2
	5·0	Yes (2·0)	2·4
C75b(*phoS*)	7·0	No	0·01
	5·0	No	52·3
	5·0	Yes (1·0)	63·6
	5·0	Yes (2·0)	10·0
	5·0	Yes (4·0)	3·0

Habituation was for 20 min. Acid challenge was at pH 3·0 for 7 min (HfrK10) or 5 min (C75b).

resistant than HfrK10 or 1829 ColV, I-K94, and were also aberrant in habituation. Whereas other tested strains showed essentially complete acid resistance after 15 or 20 min at pH 5·0, this was not true for the *phoE* mutants. One *phoE* mutant, W2961, resembled the others in habituation characteristics but was very sensitive to acid killing. The kinetics of habituation were further examined in strain 1157. Although the organisms showed poor habituation after 10, 15 or 20 min at pH 5·0, full acid resistance was acquired in 75 min (Table 10) but phosphate (10 mmol/l) had essentially no effect on the habituation acquired in this longer period. Strain 1194 also showed some habituation in 75 min at pH 5·0, and phosphate had little effect (Table 10).

There were no $phoE^+$ parents available for the deletion mutants but a $phoE^+$ transductant of strain CE 1194 (the *phoE phoS* mutant) was obtained and strain 1157 was transduced to $phoE^+$ (see Materials and Methods). Two $phoE^+$ transductants of 1157 and one of CE 1194 showed increased acid sensitivity (of pH 7·0 cells) compared with their *phoE* parents but were able to habituate after incubation for 15 or 20 min at pH 5·0 (Table 9). Statistical analysis of five experiments showed that pH 7·0 organisms of strain 1157-4($phoE^+$) were significantly more sensitive (99% confidence) to acid than were pH 7·0 organisms of strain 1157 (Table 9), but that after 20 min at pH 5·0, organisms of strain 1157-4 had fully habituated and were

Table 9 Mutants with *phoE* deletions and habituation

Strain	Relevant genotype	Growth pH	Percentage survival after acid challenge
1157	Δ*phoE*	7·0	4·2
		5·0	8·4
1157-4	$phoE^+$	7·0	0·1
		5·0	61·5
CE 1194	*phoS*, Δ*phoE*	7·0	14·0
		5·0	16·0
CE 1195	*phoS*, $phoE^+$	7·0	0·02
		5·0	2·1

Habituation was for 20 min. Acid challenge was for 7 min (CE 1194 and CE 1195) or 12 min (1157 and derivative) at pH 3·0. The above results are for a single experiment but statistical analysis of five experiments with strains 1157 and 1157-4 ($phoE^+$) revealed the following. Firstly, the mean survival for pH 7·0-grown organisms after acid challenge was 5·8% ± S.E. of 1·2% for strain 1157, and 0·29 ± 0·13% for strain 1157-4. The difference is significant at the 99% confidence level. After habituation for 20 min at pH 5·0, however, strain 1157-4 became significantly more acid-resistant (99% confidence) than strain 1157, the respective mean survival percentages after challenge being 62·9 ± 4·6 for strain 1157-4 and 6·0 ± 1·3 for strain 1157. Additionally, there was no significant difference between the mean percentage survival values after challenge for pH 5·0 and 7·0 organisms of strain 1157.

Table 10 Habituation in 75 min at pH 5·0 in *phoE* mutants

Strain	Habituation pH	Habituation time (min)	Phosphate (10 mmol/l) added	Percentage survival after challenge for 10 min	12 min
1157	7·0	10	No	68·7	7·5
(*phoE*)	5·0	10	No	63·3	12·5
	5·0	75	No	100·0	70·0
	5·0	75	Yes	83·6	56·3
1194	7·0	10	No	10·6	0·1
(*phoE*	5·0	10	No	14·1	0·9
phoS)	5·0	75	No	22·8	15·0
	5·0	75	Yes	43·4	9·1

Incubation was in broth at 37°C. Acid challenge was at pH 3·0.

significantly more resistant to acid killing (99% confidence) than those of strain 1157 incubated at pH 5·0 for the same time (Table 9).

One *phoE* deletion mutant (W2961) was more acid-sensitive than 1829 ColV, I-K94; no parent was available but transduction to $phoE^+$ produced an even more sensitive derivative. Like strain 1157 and the other *phoE* mutants, strain W2961 habituated poorly in 15 or 20 min at pH 5·0.

Effect of phosphate on the synthesis of proteins induced at acid pH

Habituation requires protein synthesis with several proteins being induced at low pH_o (Raja *et al.* 1991a). Preliminary autoradiographic studies have now shown that several proteins are produced in relatively much larger amounts in the first few minutes after a shift to pH 4·3 than at pH 7·0 prior to the shift. Most of these induced proteins are also formed at pH 4·3 plus phosphate (10 mmol/l) but in reduced amounts. Also many proteins were synthesized actively at pH 7·0 and at pH 4·3 plus phosphate but less actively, or not at all, at pH 4·3.

Phosphate and growth inhibition at acid pH

Escherichia coli grows poorly at pH 5·0 compared with pH 7·0 and this applies even if organisms have been habituated by previous growth at pH 5·0 (Table 11). Potassium phosphate (10 mmol/l) greatly enhanced the growth rate at pH 5·0 but had no effect at pH 7·0. The results are shown for strain E15 but the effects were similar with other *pit* mutants. Strain 1829 ColV, I-K94 was less affected by phosphate at pH 5·0 but this anion markedly stimulated growth of P678-54 at acid pH but not its *ompA* mutant.

DISCUSSION

Previous studies (Raja *et al.* 1991a) ruled out the possibility that the acid habituation sensor detects a fall in pH_i. The present work shows, furthermore, that collapsing the ΔpH (difference between pH_i and periplasmic pH) at pH_o 5·0 also did not stop habituation, establishing that the process is not triggered by the increase in ΔpH, or decrease in $\Delta\psi$, which results from a low pH_o. This finding, that weak acids which collapse the ΔpH do not prevent habituation, is of considerable applied importance. It suggests that polluting enterobacteria, e.g. in acid foods, could habituate even when those foods contain weak acid.

The outer membrane plays a key role in protecting enterobacteria from extreme acidity and factors which enhance its penetrability, such as *lps* mutations and the presence of certain plasmids, increase acid sensitivity (Bielecki *et al.* 1982; Cooper & Rowbury 1986); *ompA* lesions have a similar effect (Table 3). Accordingly, at low pH_o, organisms with normal outer membranes must maintain a pH differential between the periplasm and the outside which could be detected and trigger the habituation response. Such a mechanism of control is unlikely; the acid sensitivity of the *ompA*, *phoS*, *phoR* and *phoT* mutants, which is due to increased permeability of the outer membrane to hydrogen ions and which would reduce the pH difference between the outside and the periplasm, did not prevent habituation (Tables 3 and 7).

Habituation, which takes *ca* 10 min at 37°C, requires a protein synthesis-dependent stage (of *ca* 2·5–3·0 min) plus a further essential period of incubation which is protein synthesis-independent. The experiments where organisms were shifted from pH 5·0 back to pH 7·0 indicate that both these stages of habituation must occur at an habituating pH. Phosphate also prevented the process at any stage (Table 2).

Table 11 Phosphate and growth at pH 5·0

	Growth pH	Phosphate (10 mmol/l) added	Optical density at time (min)							
			0	30	60	90	120	150	180	210
(A) Prior growth at pH 7·0	7·0	No	0·05	0·05	0·09	0·15	0·26	0·43	0·52	0·60
	7·0	Yes	0·05	0·05	0·08	0·12	0·21	0·34	0·45	0·58
	5·0	No	0·05	0·05	0·05	0·05	0·05	0·07	0·10	0·14
	5·0	Yes	0·05	0·05	0·06	0·08	0·17	0·33	0·49	0·52
(B) Prior growth at pH 5·0	7·0	No	0·05	0·05	0·11	0·14	0·26	0·45	0·52	0·58
	7·0	Yes	0·05	0·05	0·11	0·17	0·25	0·40	0·52	0·54
	5·0	No	0·05	0·05	0·05	0·05	0·07	0·09	0·11	0·15
	5·0	Yes	0·05	0·05	0·09	0·13	0·23	0·38	0·54	0·60

Organisms of strain E15 were grown in shaken broth to exponential phase at 37°C at the stated pH and then diluted into fresh broth such that the new pH was as stated. Incubation was continued with shaking at 37°C. Optical density was followed using a Hilger photo electric colorimeter.

If hydrogen ions must cross the outer membrane to interact with a sensor in the periplasmic space, a cation pore would seem to offer the most likely penetration route. In fact, habituation was unaffected by loss of either the OmpF or OmpC proteins since the *ompF* mutant used here has no trace of the OmpF protein whilst the OmpC protein is completely absent from CE1062; even an *ompR* mutant, which produces no trace of either protein, showed normal habituation. The process was also normal in the absence of other pores (the *tsx* and *lamB* mutants used here show a complete absence of the corresponding pore proteins), or of a number of other outer membrane proteins.

Phosphate inhibits habituation at low pH_o; it was possible that it interfered with the activation by H^+ of an outer membrane acidity sensor or crossed into the periplasm and either buffered this compartment (i.e. maintaining its pH near to neutrality at acid pH_o) or interfered with the activation by H^+ of a periplasmic sensor. The finding that phosphate does not act on habituation in the *ompA* mutant is not in accord with any of the above possibilities. The *ompA* mutant clearly has a normal habituation sensor (since it can habituate similarly to other strains) and, accordingly, phosphate would have prevented habituation if it interferes with activation of an outer membrane or periplasmic sensor. Alternatively, if phosphate enters the periplasm merely to produce a buffering effect, it would have affected the *ompA* mutant which has normal phosphate entry *via* the PhoE porin. The findings that phosphate prevents habituation at pH 5·0 (Tables 5 and 8) in strains 1829 ColV, I-K94 and HfrK10 but not in the *ompA* mutant (in which H^+ can penetrate by some other means, in addition to the PhoE pore) and that this anion also stimulates growth at pH 5·0 (Table 11) are in accord with phosphate preventing normal hydrogen ion passage across the outer membrane.

Phosphate can cross the outer membrane of *E. coli* efficiently using the PhoE porin and much less efficiently *via* the non-specific OmpF and OmpC pores. Passage into the periplasm at low phosphate concentrations is aided by the PhoS gene product, the phosphate-binding protein. Transport across the cytoplasmic membrane is then ensured by the presence of the Pst components which include the Pst B and C gene products and the PhoT protein. The PhoE porin, the PhoS protein and the Pst components are all constituents of the high affinity phosphate (Pho) uptake system; the PhoA gene product, alkaline phosphatase, the PhoU gene product, and the Ugp transporter for glycerol-3-phosphate are also components of this system. The Pho components are formed even at high phosphate concentrations, but induction, controlled by the PhoB and PhoR proteins, occurs on phosphate starvation (Wanner & Chang 1987; Makino *et al.* 1989). Lesions in *phoR* cause derepression of the Pho system as do *phoS* and *phoT* mutations. At high phosphate concentrations, a second entry system operates. It is dependent on the PhoE porin, and to a lesser extent on the other porins, for phosphate penetration of the outer membrane, but uses the Pit system for passage across the cytoplasmic membrane (Elvin *et al.* 1987).

Several of the findings suggest that the PhoE pore, paradoxically since it is predominantly an anion pore, is involved in the entry of hydrogen ions. All the *phoE* and *phoE phoS* mutants except one were acid-resistant and transduction of strains 1157 and CE 1194 (both *phoE* deletion mutants) to $phoE^+$ caused increased acid sensitivity (Table 9). Additionally, derepression of the PhoE porin in *phoR*, *phoS* and *phoT* mutants led to increased acid sensitivity compared with the repressed parental strain (Table 7). A role for the PhoE protein in hydrogen ion entry is also in accord with the *phoE* and *phoE phoS* mutants failing to habituate appreciably in short periods at 37°C (Table 9). This could have indicated that the PhoE protein forms the sensor detecting acidity but since these mutants will habituate if incubated for longer periods at pH 5·0 (Table 10), it is likely that poor hydrogen ion penetration into *phoE* mutants merely delays habituation at this pH.

Since hydrogen ions appear to cross the outer membrane mainly *via* the PhoE pore and possibly associated with a polyanion such as a polyphosphate, it was feasible that detection of acidity in the periplasm might depend on the presence of a normal unmutated component of the phosphate detection, regulation or uptake system(s). In fact, mutation to *phoR* or *pit* did not prevent habituation and the process also occurred normally in the *phoS* mutant, strain C75b, which makes no PhoS gene product, in the deletion mutant E15 which has no PhoA gene product, and in the deletion mutant BW4975 which contains no Pst B or C components or PhoT or PhoU gene products. Accordingly, it seems unlikely that a component of the phosphate detection, regulation or transport systems is involved in sensing acidity.

Although the PhoE pore is considered to be relatively specific to anions (Overbeeke & Lugtenberg 1982; Bauer *et al.* 1989), it seems highly likely that hydrogen ions use the PhoE porin as the major outer membrane penetration route, with anions competing with hydrogen ions at the PhoE pore and preventing their reaching the habituation sensor on the cytoplasmic membrane or in the periplasm. The concentration dependence of the phosphate effect when the habituation pH was varied (Table 5) first suggested such competition but a competitive mechanism is also in accord with polyphosphate being the most effective inhibitor of habituation (Table 6) with phosphates and citrate also being highly effective because it is such anions which strongly inhibit the passage of other compounds through the PhoE pore (Overbeeke & Lugtenberg 1982). The failure of phosphate to reverse habituation of the

ompA mutant also agrees with such a mechanism of action for anions, and rules out the possibility of phosphate acting directly on the sensor, since in this strain the PhoE pore is not the major route of hydrogen ion penetration. Hydrogen ions presumably also cross the outer membrane weakly by some route other than the PhoE pore in *phoE* mutants; this would explain why habituation of such mutants in 75 min at pH 5·0 is refractory to phosphate (Table 10). It may be that hydrogen ions, as such, can actually penetrate the PhoE pore. Alternatively, since this is believed to be predominantly an anion pore, hydrogen ions might, at acid pH_o, associate with organic polyanions from broth, and these protonated polyanions penetrate the PhoE pore releasing H^+ in the periplasm. This seems a feasible mechanism since organic polyanions are believed to use the PhoE porin for entry (Wanner 1990). A role of phosphate in preventing H^+ penetration through the outer membrane is also in accord with the finding that this anion is effective added at any stage of habituation (Tables 2 and 4) since periplasmic pH must be maintained acidic throughout the process (Table 2).

One *phoE* mutant (strain W2961) was exceptional in that it was at least as sensitive to acid as 1829 ColV, I-K94 and HfrK10 although it resembled the other *phoE* mutants in failing to show appreciable habituation in short periods at pH 5·0. However, transduction to $phoE^+$ produced a derivative which was even more acid-sensitive. Strain W2961 may carry another mutation which, like *polA* and *recA* lesions (Raja *et al.* 1991a), leads to greater sensitivity to the cytoplasmic damage caused by even the reduced influx of hydrogen ions which occurs in *phoE* mutants.

It is not yet clear how habituated cells are protected from acid although they show less DNA damage when exposed to pH 3·0 than non-habituated ones (Raja *et al.* 1991b). One possibility was that habituated cells maintain pH_i at a more favourable (less acidic) level at an extreme acidic pH_o. The finding that acetate (7·5 mmol/l), which collapses ΔpH, did not sensitize pH 5·0 cells to killing by pH 3·0 rules this out. Another possibility is that the PhoE porin is repressed, unstable or modified in structure and properties at pH 5·0, so that H^+ penetration of the outer membrane is reduced. In fact the PhoE porin is present and active at pH 5·0, since strains derepressed for the *pho* genes, if grown at pH 5·0, are more acid-sensitive than those with repressed *pho* genes, e.g. compare acid sensitivity of pH 5·0-grown HfrK10 and E15 with that of the *pho*-derepressed strains C75a, C75b and C112a (Table 7) and also compare acid sensitivity (Table 9) of pH 5·0-grown strains 1157-2 and 1157-4 (repressed) with strain 1195 (derepressed).

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