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Alkaline Stress Response in *Enterococcus faecalis*: Adaptation, Cross-Protection, and Changes in Protein Synthesis

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The alkaline shock response in *Enterococcus faecalis* was studied in this work. Cells adapted to an optimum pH of 10.5 were tolerant to pH 11.9 conditions but acquired sensitivity to acid damage. An analysis of stress proteins revealed that 37 polypeptides were amplified. Two of these are DnaK and GroEL. The combined results show that bile salts and alkaline stress responses are closely related.

The ability to grow at pH 9.6 was one of the resistance tests suggested by Sherman and Stark (19) for the identification of *Enterococcus faecalis*, previously named *Streptococcus faecalis*. The ability of *Enterococcus hirae* ATCC 9790 (cited as *S. faecalis*) to withstand high alkaline pHs is not obligatorily correlated to the maintenance of a neutral cytoplasmic pH (12). Factors such as ion transporter function and fermentation producing protons occur at alkaline pHs and could be required to prevent cytoplasmic alkalinization beyond the medium pH (9, 12).

Bacteria can undergo habituation to alkaline conditions, and this is accompanied by increased resistance against external alkalinization to an optimum pH (pH_o) of 10.0 to 10.5 in *Escherichia coli* (6) or induced thermotolerance at 55°C in *Salmonella enteritidis* (8). On the contrary, exposure of *E. coli* to alkaline habituation conditions sensitizes it to acid stress (16) and vice versa (15).

The ability to grow at or to withstand alkaline pHs could require the acquisition of enzymes capable of remaining active at higher pH values (e.g., the Na^+-ATPase in *E. hirae* [9]) and could be accompanied by modifications in gene expression. Few extended studies concern the latter point. The most important contributions showed the induction of heat shock proteins (HSP) by a mild pH_o upshift (from pH 7.0 to 8.8) (20), induction of the SOS system by alkalinization of intracellular pH (18), and RecA-independent DNA repair in *E. coli* (6). In other specific studies, an unknown alkaline shock protein (23 kDa) in *Staphylococcus aureus* (11) and some genes whose expression changes as a function of alkaline pH (2, 5, 17) have been described.

Bacteria such as *E. faecalis* can be exposed to alkaline environments resulting from pollution of waters by industrial effluents or agricultural wastes. Alkaline pH stress can also affect bacteria in the intestine and in some foods (17). In this article, we present the influence of a pH_o upshift on tolerance and protein synthesis of the gram-positive bacterium *E. faecalis.*

Microorganism and culture conditions. This study was performed with *E. faecalis* ATCC 19433. The culture was grown overnight at 37°C without shaking in brain heart infusion (BHI). A 2% inoculum in BHI was grown to an optical density at 600 nm of 0.6 (about 6×10^7 CFU/ml). This culture was used in the experiments described below.

pH down- or upshift adaptation and antibiotic treatments. Bacteria were harvested by centrifugation and resuspended in BHI adjusted to pH 4.8 with lactic acid or to pH 10.5 with NaOH prior to use. Fresh BHI, pH 7.2, was used for control experiments. Adaptation was conducted for 30 min. Inhibition experiments were conducted with chloramphenicol (50 μg/ml) or rifampin (10 μg/ml) during adaptation. The concentrations used were 5- and 10-fold higher, respectively, than the MICs for this strain.

Challenge conditions. Cellular pellets of control or adapted bacteria were resuspended in BHI and exposed at 37°C (for conditions i to iv) to (i) pH 11.9, (ii) pH 3.2, (iii) 0.3% bile salts (sodium cholate-sodium deoxycholate [1:1]), (iv) 45 mM H_2O_2, or (v) heat (62°C). At intervals, samples were removed, diluted in 0.9% NaCl, poured in 0.5% glucose M17 agar (Difco Laboratories, Detroit, Mich.), and incubated for 48 h at 37°C. CFUs were enumerated.

Pulse-labeling and two-dimensional electrophoresis. Culture conditions were as described before. Labeling, extraction, and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed exactly as described previously (3).

Influence of an alkaline pretreatment on sensitivity at various pHs or against other stresses. Preliminary experiments have shown that *E. faecalis* grows in a wide range of pHs (5.0 to 9.6) with a pH_o between 7.0 and 7.5. Nevertheless, a higher pH (pH 10.5) was necessary to induce maximal tolerance against pH 11.9 (data not shown). In this case, 83.3% of adapted cells survived this treatment (Fig. 1a). The alkaline adaptation did not induce tolerance against acid challenge (Fig. 1b). No significant change was observed for acid-adapted cells exposed to pH 11.9 (Fig. 1a). Thermotolerance was induced in cultures treated for 30 min at pH 10.5, but such tolerance gave a tolerance factor of 10 at 15 min, and this decreased somewhat after 30 min of challenge (tolerance factor of 3) (Table 1). No significant tolerance against the H_2O_2 treatment (45 mM) was exhibited in alkaline-adapted cells (Table 1). Surprisingly, cells incubated at pH 10.5 acquired a very high resistance to bile salts challenge relative to the control (Table 1).

In *E. faecalis*, alkaline shock induces tolerances against only a narrow range of stresses. Cross-responses between alkaline and acid stresses, as observed in *E. coli*, are not induced in *E. faecalis* (15, 16). On the other hand, an up- or downshift of pH induces impressive resistance against homologous challenge. Acid adaptation does not induce significant bile salts tolerance (4). These combined results show that physiological responses

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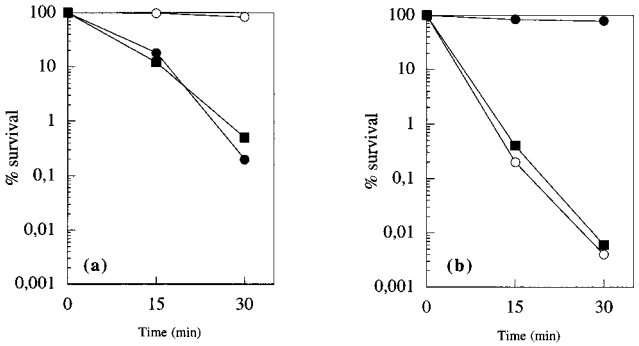

FIG. 1. Effect of exposure to alkaline or acid pH on subsequent pH resistance of *E. faecalis* ATCC 19433. Cells were incubated at pH 10.5 (○) or pH 4.8 (●) for 30 min before challenge at pH 11.9 (a) or pH 3.2 (b). Efficiency of lethal treatments is determined by the amount of challenged, nonadapted cells (■). Each experiment was performed three times with duplicate samples.

induced by alkaline and acid treatment seem to involve different means.

Contribution of protein synthesis on acquisition of alkaline or acid tolerances. Chloramphenicol treatment of a culture at pH 10.5 resulted in a 9% decrease in alkaline tolerance, and the levels of acquisition of acid tolerance in acid-exposed cells in chloramphenicol-treated and untreated cultures were comparable (data not shown). These observations could imply that protein synthesis is not required in growing cells of *E. faecalis* to develop tolerance against various extreme pHs, as observed in *Lactococcus lactis* tolerance against severe acid stress (7). On the contrary, the acid tolerance responses of *E. coli* (14), *Salmonella typhimurium* (5), and *Aeromonas hydrophila* (10) are dependent on the synthesis of protecting proteins. These results demonstrate that pH responses in *E. faecalis* seem to use different means than those used in gram-negative bacteria.

Pretreatment of alkaline-adapted cells with chloramphenicol decreased bile salts tolerance from 87-fold. Rifampin treatment also reduced bile salts tolerance but provoked only a 20% tolerance decrease (data not shown).

Effects of alkaline shock on the protein synthesis pattern. Of the approximately 1,200 spots expressed in control cells, 50% were not present in the alkaline-treated cells (Fig. 2). Thirty-seven proteins are induced more than twofold by alkaline stress. Out of these, nine proteins are amplified more than fivefold. One of them has an impressive induction ratio of 16.6.

The alkaline shift induced a subset of HSP in *E. faecalis* (4). Out of the 10 HSP induced by treatment at pH 10.5, we find that the major HSP DnaK and GroEL were induced 2.9- and 8.5-fold, respectively. The pH of the environment can influence the balance of electric charges on cell surfaces (1) and conse-

TABLE 1. Sensitivity of *E. faecalis* ATCC 19433 to various stresses after shift to alkaline pH

Challenge treatment	Tolerance factor[a] after challenge period	
	15 min	30 min
Heat (62°C)	10	3
H_2O_2 (45 mM)	2	2
Bile salts[b] (0.3%)	1,688	1,450

[a] The tolerance factor is the ratio of percent viability of pH 10.5-adapted cells to percent viability of unshocked cells. In the control, the tolerance factor is equal to 1. Each experiment was performed three times with duplicate samples.
[b] Challenge treatments were performed for 15 and 30 s (3).

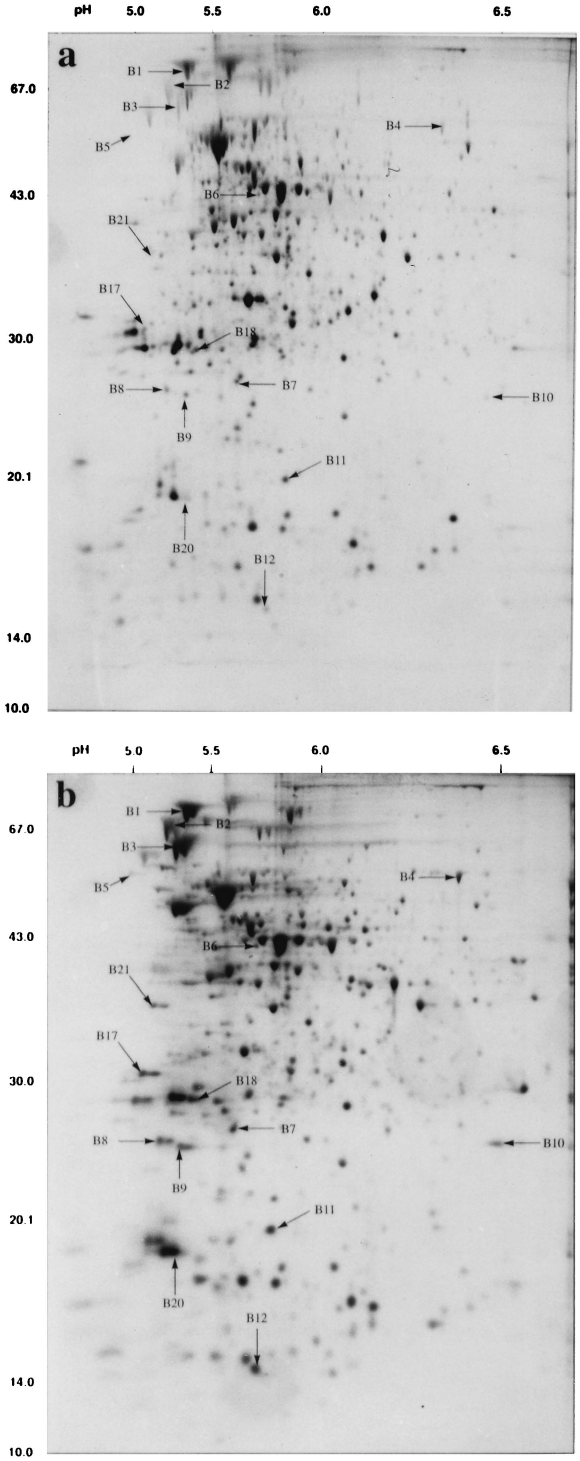

FIG. 2. Two-dimensional autoradiograms of ^{35}S-labeled whole cells from exponential nontreated (a) and pH 10.5-adapted (b) *E. faecalis* ATCC 19433. The proteins correspond to the overlap between alkaline pH and bile salts stress proteins. The DnaK and GroEL homologous proteins are indicated by B2 and B3, respectively. Equal amounts of radioactivity (approximately 250,000 cpm) were loaded onto the first-dimension gels. A uniform 14% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel was used for second-dimension electrophoresis. Labeled proteins were visualized by autoradiography after 7 months of exposure. M_rs (in thousands) are indicated to the left of each gel.

TABLE 2. Stress proteins induced by both alkaline and bile salts treatments in *E. faecalis* ATCC 19433

Protein[a]	Estimated M_r (10^3)	Estimated pI	Synthesis induction ratio[b] at:	
			pH 10.5	0.08% bile salts
B1	81.0	5.30	2.6	2.0
B2	72.0	5.20	5.4	2.9
B3	69.0	5.25	8.5	13.3
B4	54.5	6.35	2.8	2.0
B5	53.0	5.00	4.0	16.5
B6	42.5	5.70	2.1	4.4
B7	26.5	5.15	2.4	2.2
B8	25.5	5.25	2.7	3.8
B9	25.0	5.35	2.3	2.2
B10	25.5	6.45	8.0	3.8
B11	20.0	5.80	2.2	2.1
B12	14.5	5.75	5.5	7.3
B17	30.5	5.10	6.9	6.4
B18	28.0	5.40	2.1	2.3
B20	19.0	5.30	9.4	4.6
B21	36.0	5.20	2.0	2.0

[a] B2 and B3 are proteins immunologically identified as homologous to DnaK and GroEL, respectively (data not shown).

[b] The bacteria were exposed to pH 10.5 or 0.08% bile salts for 30 min. The synthesis induction ratio is the relative intensity of protein spots of the adapted culture divided by the relative intensity of protein spots of the control. Protein patterns were analyzed by visual inspection, and spots were quantified by the computer program 2-D Analyzer (BioImage, B. I. Systems Corp.).

quently can provoke a rapid hyperpolarization of the membrane at alkaline pHs (21). This phenomenon also occurs with temperature upshift (13), but the relationship between alkaline and heat stress response has not yet been elucidated. In *E. faecalis*, an alkaline shift of the external medium is a potent inducer of HSP but did not induce a maximal thermotolerance. Moreover, alkaline tolerance is not entirely correlated to protein synthesis, although this is the case for *E. coli* (17). Therefore, mechanisms by which alkaline shifts trigger HSP synthesis and alkaline tolerance seem not to have a causal relationship in *E. faecalis.*

A significant overlap of alkali-inducible proteins with those induced by bile salts stress is obvious (3). Sixteen proteins are common to both treatments, with five of them showing at least a fivefold amplification over control cultures (Table 2). Because of the physiological and biochemical similarities, it appears that alkali and bile salts stresses can induce common regulatory systems in *E. faecalis*. Surprisingly, stress proteins positively controlled by a pH upshift do not play a major role in alkaline tolerance. However, these stress proteins are necessary for the acquisition of bile salts tolerance. Interestingly, inhibition of transcription has less of an influence on this tolerance response, suggesting that the preexisting mRNAs seem to be sufficiently stable to permit the acquisition of significant bile salts tolerance. Because of its natural enteric environment, *E. faecalis* seems to withstand alkaline and bile salts stresses and to have developed specific pathways to cope with each of them.

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