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Comparison of the Bile Salts and Sodium Dodecyl Sulfate Stress Responses in *Enterococcus faecalis*

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The resistance to detergents and detergent-induced tolerance of a gastrointestinal organism, *Enterococcus faecalis* ATCC 19433, were examined. The most remarkable observation was the rapid response of cells in contact with bile salts and sodium dodecyl sulfate (SDS). The killing by high concentrations of detergents was nearly instantaneous. A 5-s adaptation with moderate sublethal concentrations of bile salts or SDS (0.08 or 0.01%, respectively) was sufficient to induce significant adaptation against homologous lethal conditions (0.3% bile salts or 0.017% SDS). However, resistance to a subsequent lethal challenge progressively increased further to a maximum reached after 30 min of adaptation. Furthermore, extremely strong cross-resistances were observed with bile salts- and SDS-adapted cells. However, no relationship seems to exist between levels of tolerance and de novo-synthesized proteins, since blockage of protein synthesis during adaptation had no effect on induction of resistance to bile salts and SDS. We conclude that this induced tolerance to detergent stress is independent of protein synthesis. Nevertheless, the stress-induced protein patterns of *E. faecalis* ATCC 19433 showed significant modifications. The rates of synthesis of 45 and 34 proteins were enhanced after treatments with bile salts and SDS, respectively. In spite of the overlap of 12 polypeptides, the protein profiles induced by the two detergents were different, suggesting that these detergents trigger different responses in *E. faecalis*. Therefore, bile salts cannot be substituted for SDS in biochemical detergent shock experiments with bacteria.

Enterococcus faecalis is a natural member of the human and animal intestinal flora. Indigenous bacteria of the intestinal tract are inherently resistant to components of this selective environment. Among these, enteric bacteria implement various mechanisms to thwart bactericidal effects of bile salts, detergent-like biological substances. Since the mammalian gastrointestinal tract contains high concentrations of the bile salt detergents, sodium dodecyl sulfate (SDS) resistance is often considered to be a physiologically relevant shock system. Extreme detergent resistance is a frequent property of enteric gram-negative bacteria, which grow in ≤10% SDS, but gram-positive bacteria are completely inhibited by SDS at ≥0.1% (6).

The outer membrane of gram-negative bacteria would be the major permeability barrier for hydrophobic inhibitors (10). Furthermore, recent studies have shown that various drug efflux pumps may play an important role in the protection of gram-negative bacteria (8), i.e., the pump AcrAB of *Escherichia coli*, whose physiological function is mainly to efflux fatty acids and bile salts (7). A pump model (e.g., an active SDS efflux system located in the cytoplasmic membrane) has also been investigated to explain the extreme energy-dependent resistance against SDS in *Enterobacter* sp. (3, 9).

The protein pattern of *E. coli* cells grown in the presence of 5% SDS had a dramatically altered composition. Indeed among 19 detergent stress proteins, 4 proteins were turned on and 15 proteins increased their rate of synthesis from 3.0- to 11.8-fold (1). Some of the SDS stress proteins were also induced by osmotic stress, since the high (5%) level of SDS used may cause osmotic shock (2). Nevertheless, these authors emphasized that the detergent stress stimulon seems not to overlap other stress stimulons (i.e., heat shock, oxidative, and anaerobic stress). Although bile salts and SDS are commonly related to similar surfactant stresses, no information seems to be available concerning bile salt stress proteins compared with the SDS stress stimulon other than a comparison between the physiological responses to various detergents. Moreover, there is very little information available concerning gram-positive bacteria, which may encounter not only industrial detergents during hygienic treatments but also bile salts within the gastrointestinal tract.

In this paper, we report the susceptibility of *E. faecalis* ATCC 19433 to the detergents bile salts and SDS and examine the induction of stress tolerances. Finally, comparison of the detergent-induced protein patterns shows differences between the stress responses to bile salts and SDS.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The *E. faecalis* strain used in this study was the type strain, ATCC 19433 (NCTC 775). The cells were grown on brain heart infusion medium (BHI) (Merck-Clévenot S.A., Nogent-sur-Marne, France) without shaking at 37°C.

Adaptation conditions and challenge treatments. Cultures were grown to an optical density of 0.6 at 600 nm. At this point, the cells were either treated with 0.08% (wt/vol) bile salts (sodium cholate, sodium deoxycholate [1:1]) or with 0.01% (wt/vol) SDS in BHI for 5 s, 30 s, 1 min, 5 min, or 30 min. Bile salts and SDS were solubilized in water to obtain concentrations of 20% (wt/vol) and 5% (wt/vol), respectively. When necessary, chloramphenicol (50 μg/ml) was added to the detergent during adaptation. (This concentration is fivefold higher than the MIC for *E. faecalis* ATCC 19433.) Preliminary experiments with 1 ml of culture and 200 μCi of [^{35}S]methionine/cysteine protein labelling mix (1,175 Ci/mmol [Dupont, NEN Research Products, Les Ulis, France]) showed that no protein bands were visible after a 3-week exposure (separation on a one-dimensional Phast gel [Phast System; Pharmacia]) (data not shown). Chemical products were obtained from Sigma Chemical Co. (St. Louis, Mo.). After the 1-, 5-, and 30-min-adapted cells were centrifuged at 3,000 × *g* for 4 min, they then were challenged either with 0.3% (wt/vol) bile salts or with 0.017% (wt/vol) SDS in BHI for 30 s. After the 5- or 30-s adaptation period, the challenge concentration was obtained by addition of the remaining bile salts or SDS to reach final concentrations of 0.3 and 0.017%, respectively.

Viable counts were determined by pouring appropriate dilutions in 0.5% glucose–M17 (12) agar (1.0% [wt/vol] agar) (Difco Laboratories, Detroit, Mich.) at 37°C. Three independent experiments were performed, and each point is the

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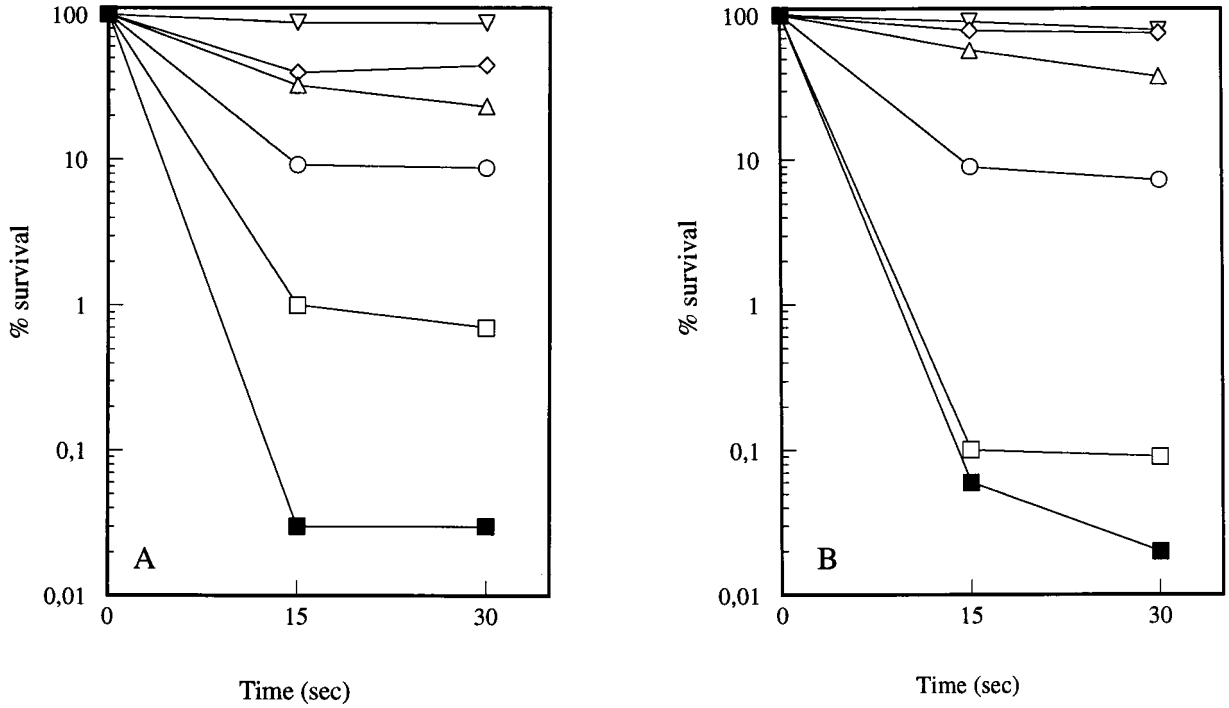


FIG. 1. Kinetics of induction of detergent tolerance in *E. faecalis* ATCC 19433. Mid-exponential-growth-phase cells were cultivated at 37°C with 0.08% bile salts (A) or 0.01% SDS (B) for 5 s (□), 30 s (○), 1 min (△), 5 min (◇), or 30 min (▽) before being challenged with 0.3% bile salts (A) or 0.017% SDS (B). The lethality of nonadapted cells during challenges with bile salts and SDS was controlled (■).

average of duplicate platings. The ability to develop tolerance toward challenge treatment was expressed by the tolerance factor (i.e., the ratio of percent survival of adapted cells to percent survival of control cells).

Radioactive pulse-labelling and protein extraction. Cultures of adapted and nonadapted cells were dispensed as described above. Each 1-ml portion of bacterial suspension was mixed with 200 μCi of [^{35}S]methionine/cysteine protein labelling mix. After a 30-min labelling period, the cells were pelleted, washed twice in chilled saline solution (0.9% [wt/vol] NaCl), and suspended in 500 μl of protoplast buffer (25 mM Tris [pH 7.0], 0.1 mg of lysozyme per ml, 1.5 mM phenylmethylsulfonyl fluoride, 50 μg of chloramphenicol per ml, 0.5 M sucrose) for 10 min at 37°C. The bacterial suspension was harvested by centrifugation, and 200 μl of lysing solution (0.3% SDS, 170 mM dithiothreitol, 28 mM Tris-HCl, 22 mM Tris) was added. The samples were heated at 100°C for 5 min and then were centrifuged (4°C, 12,000 × *g*, 10 min). The resulting supernatant was treated with 24 μl of a buffer containing 24 mM Tris, 475 mM Tris-HCl, 50 mM $MgCl_2$, 1 mg of DNase I (Sigma Chemical Co.) per ml, and 0.25 mg of RNase A (Sigma Chemical Co.) per ml. The reaction was disrupted after 15 min at 4°C with 4 volumes of cooled acetone, and proteins were precipitated for 20 min in ice. The precipitate was collected by centrifugation at 12,000 × *g* for 10 min and suspended in 50 μl of isoelectric focusing buffer (9 M urea, 55 mM dithiothreitol, 2% [vol/vol] pH 4 to 8 ampholytes [Millipore, Bedford, Mass.], 0.5% Triton X-100). A 2-μl sample was counted by liquid scintillation spectrometry to determine the level of incorporation of radioactive label.

Two-dimensional electrophoresis. Protein extracts were subjected to high-resolution two-dimensional electrophoresis according to the method described by O'Farrel (11) and modified by Görg et al. (5). Equal amounts of radioactivity (approximately 550,000 cpm) were loaded onto a first-dimension gel. Isoelectric focusing was performed with Immobiline Dry Strip (pH 4.0 to 7.0) (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the recommendations of the manufacturer. Isoelectric focusing was achieved when the total running time yielded 48,500 V · h. The strips were equilibrated in an isoelectric focusing gel equilibration buffer (0.3 M Tris base, 0.075 M Tris-HCl, 3% [wt/vol] SDS, 50 mM dithiothreitol, 0.01% [wt/vol] bromophenol blue) twice for 10 min each. Each strip was placed on top of a uniform SDS–14% (wt/vol) polyacrylamide gel for second-dimension electrophoresis with the Millipore Investigator two-dimensional electrophoresis system. The gels were dried and exposed to Hyperfilm-MP (Amersham Int., Buckinghamshire, United Kingdom) at −80°C for 7 weeks before being developed. The spots were quantified with the two-dimensional gel computer analysis program 2-D Analyzer (BioImage, B. I. Systems Corp.). The quantitative analysis of spots was normalized against the control gel used as a standard.

RESULTS

Tolerance to bile salts and SDS. To examine the role of detergent pretreatments in protection against challenges with bile salts and SDS, we first determined the effect of these two chemicals on survival of cells from the exponential growth phase. After a 30-min challenge, survival of nonadapted cells was less than 0.1% (data not shown). It is noteworthy that killing by detergents is extremely rapid; 15 s of stress exposure provoked 1,000-fold decreased survival (Fig. 1). Cells were then exposed to concentrations of 0.017% SDS or 0.3% bile salts for 30 s with or without conditioning pretreatments.

Furthermore, we determined the stress adaptation conditions which conferred maximum resistance to cells when exposed to the homologous lethal stress. For a 30-min adaptation period, the strongest induced tolerance to bile salts (0.3%) and SDS (0.017%) was obtained with 0.08% bile salts and 0.01% SDS, respectively (data not shown).

The kinetics of induction of tolerance was determined at 5 s up to 30 min of exposure to the adaptation conditions previously defined (Fig. 1). A 5-s exposure to 0.08% bile salts or 0.01% SDS was sufficient to induce significant homologous tolerances (tolerance factors of bile salt- and SDS-adapted cells of 14 and 5 after 30 s of exposure, respectively) (Fig. 1). Adaptation for 30 min led to nearly 100% protection of cells (tolerance factors of 1,600 and 200 for bile salt- and SDS-pretreated cells, respectively) (Fig. 2).

We investigated whether stresses caused by bile salts and SDS could induce cross-protection. Cells adapted to bile salts for 1 or 30 min showed significant cross-protection against SDS challenge (Fig. 2). Inversely, SDS-adapted cells with short or long exposures became tolerant to treatment with bile salts (Fig. 2). In this context, we observed that the degree of cross-

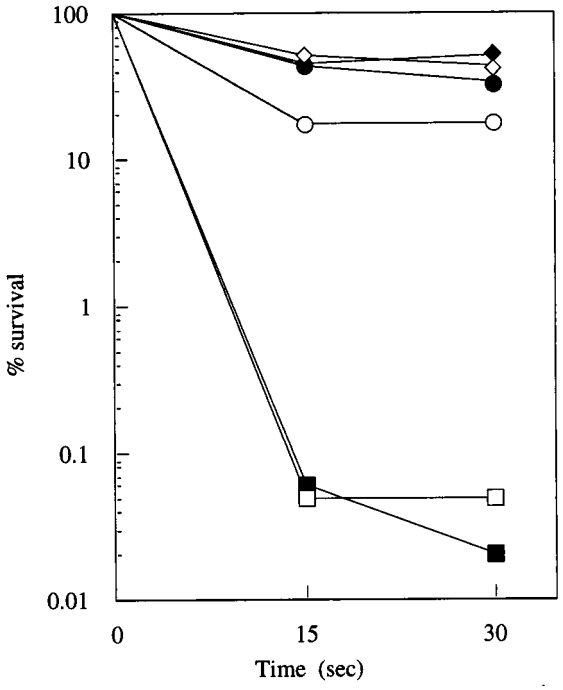


FIG. 2. Bile salt- and SDS-induced cross-protection in *A. faecalis* ATCC 19433. For the determination of cross-protection, bacteria were cultivated for 1 min (circles) or 30 min (diamonds) with 0.08% bile salts (solid symbols) or 0.01% SDS (open symbols) and then challenged with 0.017% SDS or 0.3% bile salts, respectively. The efficiency of lethal treatment with bile salts (□) or SDS (■) was determined by challenge of nonadapted cells.

protection is comparable with that of homologous tolerances (Fig. 1 and 2). However, bile salts were a better inducer of SDS tolerance than SDS was of bile salt tolerance.

Analysis of the bile salt and SDS shock proteins. Two-dimensional gel electrophoresis was used to resolve the total ^{35}S-labelled cell proteins of *E. faecalis* cells treated with 0.08% bile salts (Fig. 3B) or 0.01% SDS (Fig. 3C) for 30 min. Comparison of these two two-dimensional gels and the control gel (Fig. 3A) showed the induction of 45 bile salts and 34 SDS stress proteins. Seventeen bile salt stress proteins increased their relative rate of synthesis to at least fivefold. Out of them, eight proteins exhibited more than 10-fold increases. On the other hand, no SDS stress proteins displayed impressive changes (more than a fivefold increase) in their relative rate of synthesis. Two proteins were previously immunologically identified as the homologous DnaK and GroEL proteins by antibodies directed against heat shock proteins of *E. coli* (data not shown). These polypeptides were strongly induced by a bile salt shock. Their relative rates of synthesis increased 10- and 72-fold, respectively (Fig. 3B). SDS stress did not provoke any induction of these two homologous heat shock proteins (Fig. 3C). Furthermore, an overlap of 12 stress proteins exists between bile salt- and SDS-induced polypeptides (Fig. 2B and C). These proteins are arranged by decreasing molecular mass in Table 1. A complementary study with uniform SDS–10% polyacrylamide gels in the second dimension to analyze high-molecular-weight proteins revealed no significant changes of spot intensities compared with those of control cells (data not shown).

Effect of protein synthesis inhibition on stress tolerance. In order to investigate whether induction of stress proteins would be involved in acquired resistances, chloramphenicol was added during the 30-min adaptation period. The development of bile salt tolerance is not at all compromised by the blockage of protein synthesis prior to challenge in adapted *E. faecalis* cells. (Survival of bile salt-adapted cells was 89.9% with chloramphenicol versus 86.8% without, and survival of SDS-adapted cells was 74.7% in the presence of the antibiotic versus 78.7% without it.)

DISCUSSION

Microbial inhabitants in the gastrointestinal tract such as *E. faecalis* are presumably challenged by toxic detergent-like compounds such as bile salts in the duodenal loop. Expelled into the external environment, enteric bacteria must also adapt to survive in the presence of domestic and industrial detergent pollutants.

In *E. faecalis*, adaptive pretreatments with bile salts and SDS induced nearly maximal levels of survival in response to homologous challenge. An adaptation exposure time as short as 5 s was sufficient to induce significant resistance to detergents. To our knowledge, this is the first report about extremely short exposure, or "flash adaptation," which induced tolerance in bacteria. Moreover, extremely strong detergent cross-resistances have been observed with cells adapted to bile salts and SDS. This shows that physiological responses to bile salts and SDS are very closely related in *E. faecalis.*

As part of the adaptive response to these two stimuli, *E. faecalis* induced numerous detergent stress proteins (45 proteins in response to bile salts, 34 proteins in response to SDS). No stress protein has a molecular mass higher than 81.0 kDa. Out of them, an overlap of 12 polypeptides between the treatment with bile salts and that with SDS was observed. However, bile salt stress proteins showed more impressive changes in their relative rate of synthesis than SDS-shocked proteins. Moreover, the protein pattern induced by bile salts displayed a large number of stress proteins which were different from those induced by SDS treatment. Therefore, on the protein

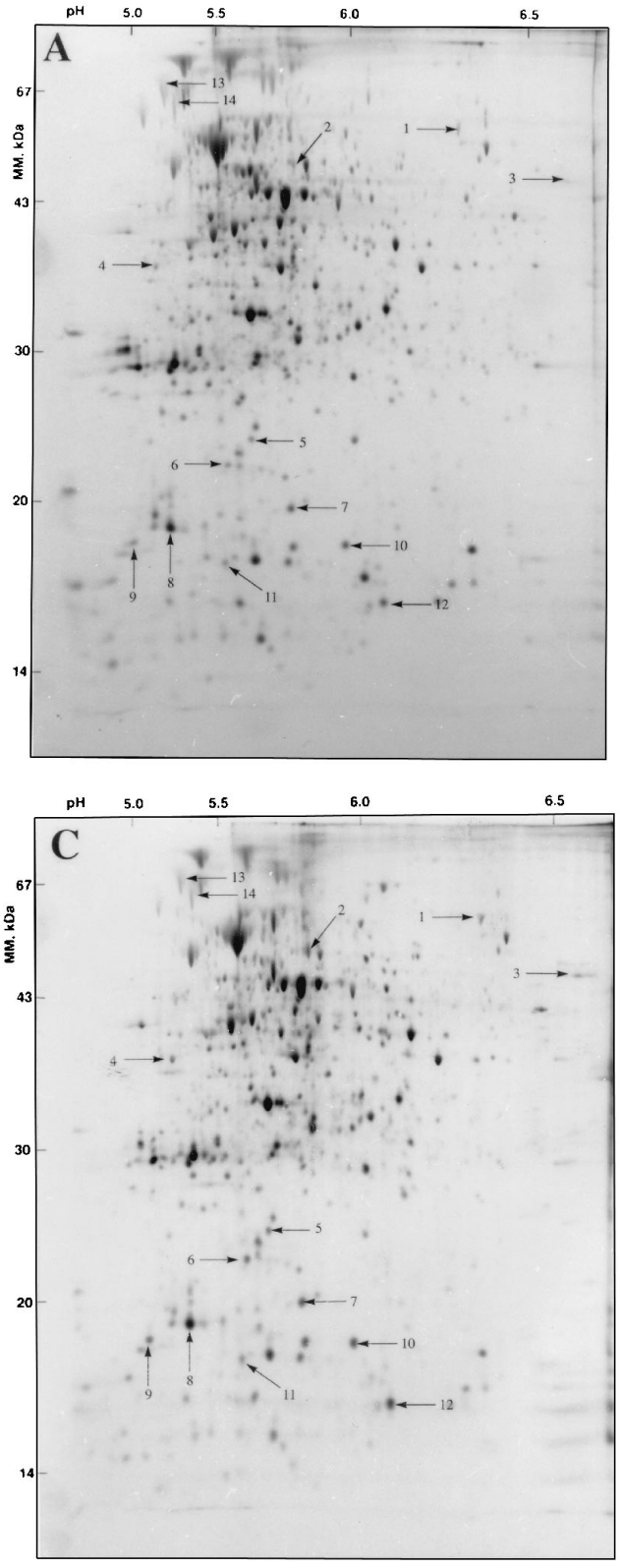
TABLE 1. Analysis of stress proteins induced by 0.08% bile salts and by 0.01% SDS in *E. faecalis* ATCC 19433

Spot	Estimated		Fold increase in stress proteins induced by both[c]:	
	Mol mass (kDa)[a]	pI[b]	Bile salts	SDS
1	54.5	6.35	2.0	2.0
2	47.0	5.80	2.9	4.0
3	45.0	6.55	9.4	3.0
4	36.0	5.20	2.1	2.1
5	24.0	5.70	4.5	2.1
6	22.5	5.60	3.8	4.0
7	20.0	5.80	2.1	2.1
8	19.5	5.30	2.5	2.1
9	19.0	5.05	3.1	2.3
10	19.0	6.00	2.0	2.0
11	18.0	5.60	3.1	2.0
12	16.5	6.10	3.2	2.0

[a] Extrapolated mass from five marker proteins used with molecular weights of 67,000, 43,000, 30,000, 20,100, and 14,400 (LMW electrophoresis calibration kit; Pharmacia).

[b] Extrapolated from pI standards, with carbamylated carbonic anhydrase with a molecular mass of 29 kDa and a pI of 4.8 to 6.7 (carbamylate calibration kit; Pharmacia).

[c] The relative intensity of protein spot of the adapted culture was divided by the relative intensity of protein spot of the control culture in BHI at 37°C. The spots were quantified by the two-dimensional gel computer analysis program 2-D Analyzer (BioImage).



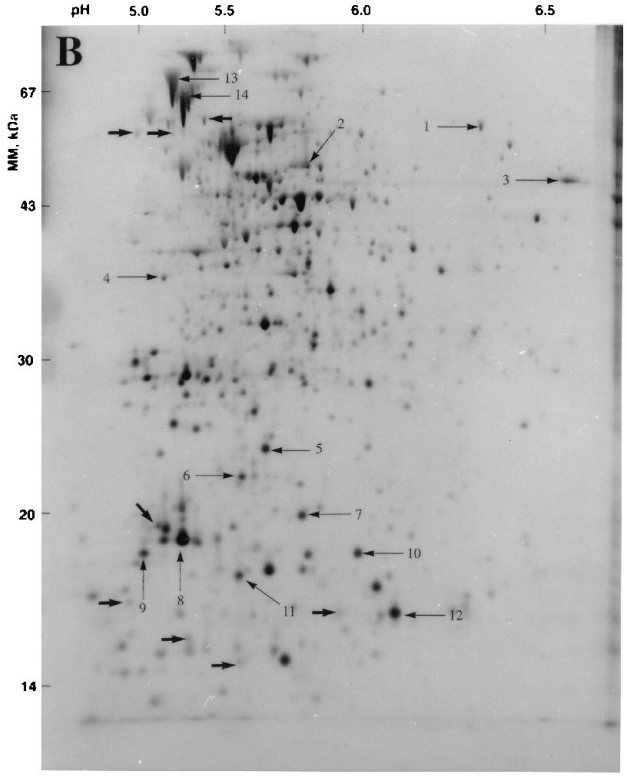


FIG. 3. ^{35}S-labelled protein gels after bidimensional electrophoresis (SDS–14% polyacrylamide gel electrophoresis). (A) Control. (B) Cells treated with bile salts (0.08% for 30 min). (C) Cells treated with SDS (0.01% for 30 min). Growth, labelling, and gel running were performed in two independent experiments, and the subsequent analysis was based on comparisons of the duplicate samples. The proteins 1 to 12 correspond to the overlap between bile salt and SDS stress proteins. The DnaK and GroEL homologous proteins are indicated by 13 and 14, respectively. Bile salt stress proteins induced more than 10-fold are indicated by short, thick arrows in panel B. MM, molecular mass.

level, the responses to treatments with bile salts and SDS are less related than the similarities observed on the physiological level. This was further confirmed by lack of induction of the two homologous heat shock proteins DnaK and GroEL by SDS, although stress caused by bile salts (Fig. 3) and heat shock (4) strongly induced this subset of heat shock proteins in *E. faecalis*. Thus, the data presented may indicate the involvement of a common regulatory mechanism during the period of adaptation to heat and bile salts. However, the 12 proteins overlapping between the profiles of bile salts and SDS have an unknown function. Furthermore, we observed no significant decrease in resistance when protein synthesis was inhibited during adaptation. Therefore, the mechanism(s) leading to tolerance toward detergent treatments seems to be protein synthesis independent.

The results showed that the detergent responses appear to be unique among a variety of stress responses in that tolerances could be induced by a flash contact with the stress and that the physiological responses to the biological and synthetic detergents in *E. faecalis* were comparable. However, the protein battery synthesized under these stress conditions appears to involve different genetic changes. Therefore, bile salts cannot be a substitute for SDS. In this case, SDS stress is not the appropriate model to use in the biochemical study of enteric bacteria.

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