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FEMS Microbiology Letters 138 (1996) 49–54

FEMS MICROBIOLOGY LETTERS

Relationship between stress response towards bile salts, acid and heat treatment in *Enterococcus faecalis*

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Received 19 January 1996; revised 15 February 1996; accepted 22 February 1996

Abstract

Stress tolerance and cross-protection in *Enterococcus faecalis* ATCC19433 were examined after exposure to bile salts, acid or heat shock. Bile salts and heat adapted cells demonstrated induced homologous tolerance and cross-resistance. No cross-protection of heat adapted cells against acid stress is observed and pretreatment with bile salts even sensitized the cells to this challenge. Whole-cell protein extract analysis revealed that each treatment induced a battery of stress proteins. Some of these polypeptides are induced by more than one treatment. The greatest overlap is observed between bile salts and heat treatments. Eighteen stress proteins, including DnaK and GroEL, are common between these stresses.

Keywords: *Enterococcus faecalis*; Stress protein; Cross-protection; Environmental stress

1. Introduction

How microbial cells sense and cope with shifts in environmental parameters is nowadays one of the best ways to elucidate the physiology of living cells. Over the last 20 years, a number of experiments have shown that bacteria develop an adaptive response when grown under moderate stress conditions. In general, the adaptation appears to involve multiple genes as indicated by changes in extracted proteins separated by two-dimensional gel electrophoresis [1–5].

Enterococci, Gram-positive bacteria, are commensal microorganisms adapted to the nutrient-enriched, oxygen-depleted, ecologically complex environments of the oral cavity, vaginal and gastrointestinal tracts. Used as an indicator of fecal contamination in water and food products, *Enterococcus faecalis* can also be responsible for serious human and animal diseases [6]. Because *E. faecalis* survives heating to 60°C for 30 min in neutral medium, in a wide range of pH (from 3.5 to 11), and has relatively high salt tolerance (6.5% NaCl), it copes with moderate stress conditions and withstands imperfect hygiene in food procedures. Characterization of behaviour in response to severe stresses in *E. faecalis* is important in particular when processes such as heating or acidity are used to control foodborne pathogens.

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PII S0378-1097(96)00080-8

General properties of the heat shock response of *E. faecalis* have already been characterized [7]. While the understanding of mechanisms of enterococcal virulence and antibiotic resistance has received widespread attention [6], only limited data are available concerning other responses to environmental fluctuations. Besides, other common growth-limiting conditions like heat, bile salts and acid stresses are of special interest because they may occur frequently in the natural environment of enteric bacteria such as *E. faecalis*.

In this report, we describe the response of *E. faecalis* to bile salts, acid and heat shifts. We determine the degree of tolerance of stress adapted bacteria to more extreme treatments (homologous tolerance) or to other types of lethal conditions (cross-protection) and we attempt to correlate these physiological phenomena to changes observed on the protein level.

2. Materials and methods

2.1. Micro-organism and growth medium

The bacterial strain employed in this study was *Enterococcus faecalis* subsp. *faecalis*, type strain ATCC19433. Growth was carried out in Brain Heart Infusion Broth (BHIB, Difco) at 37°C without shaking.

2.2. Adaptation and challenge conditions

Cultures were grown to the mid-exponential growth phase (OD_{600} of 0.6). Bacterial cells were harvested by centrifugation and resuspended in fresh BHIB (non-adapted control culture). Adaptation (30 min or 3 h) was conducted in the same medium at 37°C (i) with 0.08% bile salts (sodium cholate, sodium deoxycholate 1:1), (ii) adjusted to pH 4.8 with lactic acid or (iii) at 50°C (heat shock). After centrifugation, adapted and non-adapted cell pellets were adjusted to an OD_{600} of 1.0 in fresh BHIB. To measure tolerance to stresses, cells were treated at 37°C with (i) 0.3% bile salts, (ii) pH 3.2 (adjusted with lactic acid) or (iii) heat (62°C). Samples (0.5 ml) were removed at the desired intervals of time and diluted in ice-cold 0.9% NaCl. 0.1 ml of each sample was plated onto glucose M17 medium [8] to measure colony forming unit capacity (CFU). Plates were incubated at 37°C for 48 h before colonies were counted. Survival was determined as the ratio of CFU after challenge treatment to CFU at time zero. In all experiments each determination is the average of duplicate platings; all experiments were repeated a minimum of three times.

2.3. Pulse labelling and two-dimensional protein electrophoresis

A 1 ml portion of bacterial suspension was labelled with 200 μCi [^{35}S]methionine/cysteine (New England Nuclear) for 30 min. Cells were pelleted, washed twice in 0.9% NaCl and suspended in 500 μl protoplast buffer (25 mM Tris pH 7.0, 0.1 mg ml^{-1} lysozyme, 1.5 mM phenyl methyl sulfonyl fluoride, 50 $\mu g\ ml^{-1}$ chloramphenicol, 0.5 M sucrose) for 10 min at 37°C. Cell pellets were resuspended in 200 μl lysis buffer (0.3% SDS, 170 mM DTT, 28 mM Tris · HCl, 22 mM Tris). After 5 min at 100°C, the samples were centrifuged (4°C, 12 000 $\times g$, 10 min). The supernatant was treated with 24 μl of solution (24 mM Tris, 475 mM Tris · HCl, 50 mM $MgCl_2$, 1 mg ml^{-1} DNase I, 0.25 mg ml^{-1} RNase A) for 15 min at 4°C. Proteins were precipitated with 4 volumes of cooled acetone, in ice for 20 min. After centrifugation (4°C, 12 000 $\times g$, 10 min) the protein pellet was suspended in 50 μl of sample buffer (Pharmacia). Protein extracts were examined by the O'Farrel procedure [9]. Equivalent amounts of radioactivity from each sample were loaded onto 1-D gels. Isoclectric focusing was performed using Immobiline Dry Strips (pH 4.0 to 7.0, Pharmacia) as recommended by the supplier. The strips were equilibrated twice in IEF gel equilibration buffer (Millipore) for 10 min and were placed on top of a 14% SDS-polyacrylamide gel for second dimension electrophoresis (Millipore Investigator™ 2-D electrophoresis system, Bedford, MA). Dried gels were exposed to Hyperfilm-MP (Amersham) at −80°C for 7 weeks. The spots were quantified using the 2D Analyzer computer program (BioImage Systems Corp., USA).

3. Results

3.1. Stress tolerance and cross-protection induced by bile salts, acid and heat treatments

We determined the adaptation conditions of stress which conferred maximum resistance on cells when exposed to the homologous lethal stress. Under our culture conditions the strongest induced tolerance to bile salts (0.3%), pH 3.2 and heat (62°C) was obtained under the following conditions: 0.08% bile salts, pH 4.8 and 50°C for 30 min (data not shown).

Against bile salt treatment, bacterial survival dropped dramatically within seconds: after 15 s treatment the survival was 0.05% (Table 1), and did not change further during a 30 min exposure (data not shown). Acid (pH 3.2) and heat (62°C) challenge for 30 min both decreased the surviving fraction to 0.006% (Table 1).

The degree of tolerance was also determined after 3 h of exposure to the adaptation conditions. For bile salts and acid stress, adaptation for either 30 min or 3 h leads to a comparable protection against a homologous challenge. On the other hand, 3 h heat adapted cells are less thermotolerant than 30 min adapted cells (3.1% versus 39.1% survival).

We investigated whether adapted cells acquired tolerance against other environmental stresses. Heat adapted cells showed significant cross-protection against bile salts (18.6% survival of adapted versus 0.05% survival of control cells). Inversely, pretreatment with bile salts induced significant thermotolerance (0.08% versus 0.006% survival for adapted and control cells, respectively). However, heat shock failed to induce acid tolerance (Table 1). Acid adapted cells exhibited a very slight augmentation of heat resistance (0.02% versus 0.006% survival) but acid pretreatment resulted in no cross-protection towards bile salts challenge (Table 1). Moreover, bile salt adaptation leads to an increased sensibility to the acid challenge (0.0003% versus 0.006% survival). Furthermore, the results show that induction of tolerance is maximal with stresses of homologous nature.

3.2. Changes in protein pattern and analysis of overlapping proteins

Fig. 1 shows the analysis of polypeptide changes between control (A) and pretreated cells (B, C and

Table 1
Development of tolerance and cross-protection in adapted growing cells of *E. faecalis* ATCC19433

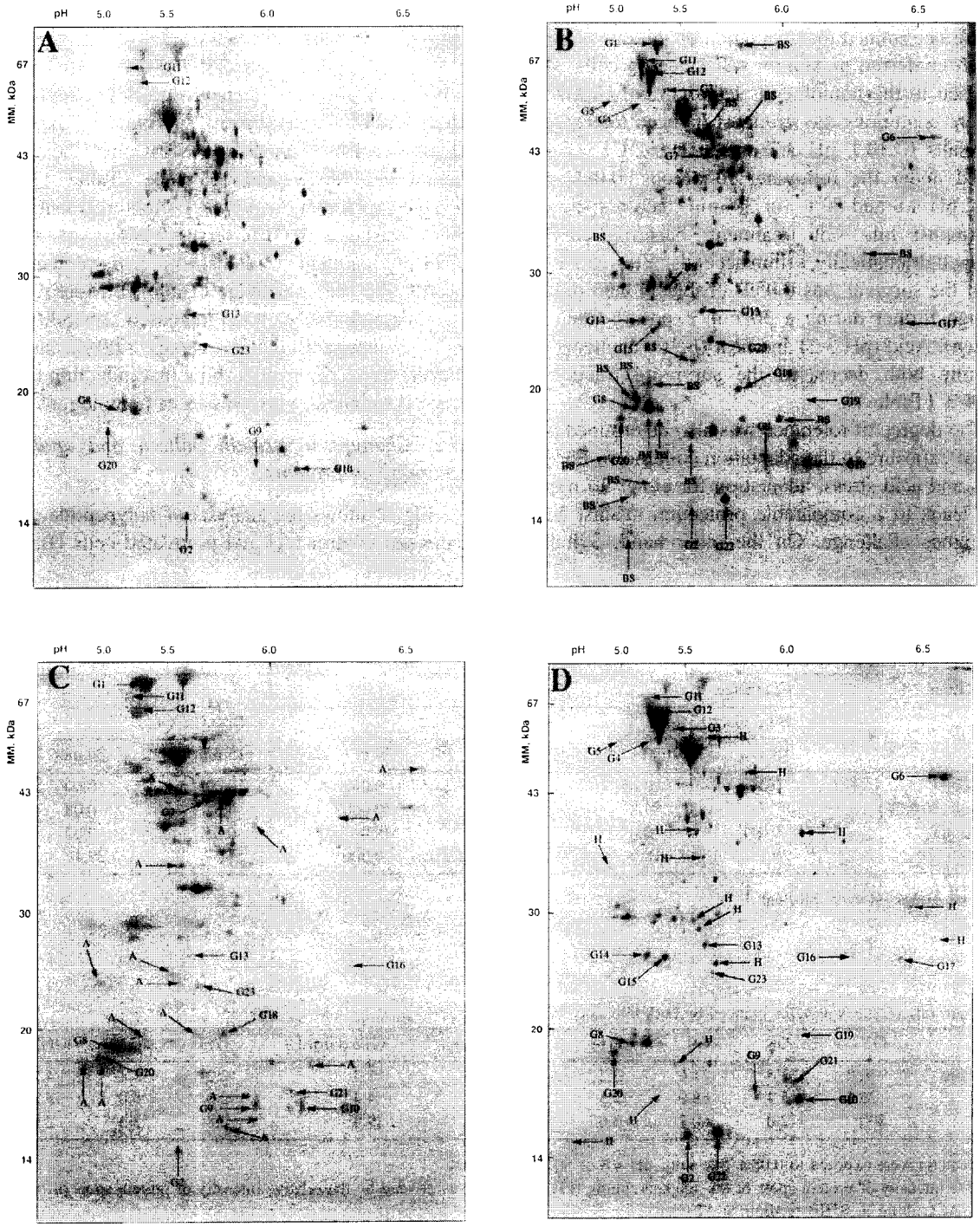
Adaptation	Challenge					
	Bile salts (0.3%)		Acid (3.2)		Heat (62°C)	
	15 s	30 s	15 min	30 min	15 min	30 min
Control	0.05	0.05	0.4	0.006	0.02	0.006
Bile salts (0.08%)	87.4	86.8	0.01	0.0003	4.9	0.08
Acid (4.8)	0.07	0.07	83.5	78.8	0.05	0.02
Heat (50°C)	20.1	18.6	0.6	0.006	55.3	39.1

Data are expressed as % survival.

Table 2
Induction rate of stress proteins induced by both bile salts, acid and heat in *E. faecalis* ATCC19433

	G2	G8	G9	G10	G11 (DnaK)	G12 (GroEL)	G13	G20	G23
Bile salts	30.2	2.8	21.6	3.2	5.4	13.3	2.2	3.1	4.5
Acid	26.6	5.4	88.0	2.5	2.1	4.4	2.0	5.0	4.3
Heat	200	2.6	60.7	3.6	9.2	29.6	2.8	3.3	2.0

The bacteria were exposed to 0.08% bile salts, pH 4.8 or 50°C for 30 min.
Relative intensity of protein spots of the adapted culture (Fig. 1B,C,D) was divided by the relative intensity of protein spots of the control (Fig. 1A) (culture in BHIB medium at 37°C).



D) of *E. faecalis*. Exposure of *E. faecalis* to several individual environmental stresses resulted in changes of a large number of proteins. Synthesis of some proteins decreased to a minor level. On the other hand, 44, 32 and 34 polypeptides were induced by bile salts, acid and heat treatments, respectively. Nine proteins, including the major heat shock proteins DnaK and GroEL, were induced by all three treatments (Table 2). The most spectacular induction ratios are observed for G2 after the heat shock treatment and G9 after treatment by acid and heat. According to the terminology defined by Hecker and Völker [10], the nine proteins induced by all treatments examined here may be regarded as general stress proteins in *E. faecalis*.

4. Discussion

A remarkable observation is the nearly instantaneous toxicity by lethal concentrations of bile salts. This detergent could act by solubilization of membrane proteins, a property widely used in protein biochemistry methods. Despite the action of this agent, fairly different from heat and acid action, adaptation to bile salts leads to a significant cross-protection towards the heat challenge. The pretreatment with bile salts results in the induction of a subset of the heat shock proteins (18 out of 21 bile salt stress polypeptides are also induced by heat), which may be responsible for the observed cross-protection effect. This shows that bile salt and heat shock responses are closely related in *E. faecalis*, further confirmed by the nearly maximal cross-protection effect induced by heat-adapted cells towards the bile salt challenge. However, in comparison to heat-induced thermotolerance, that induced by bile salts is less pronounced (Table 1). Therefore, accessory or 'partner' proteins may be necessary for protection against heat stress, and these proteins seem to be in the fraction of heat specific polypeptides which are not induced by bile salts or other stimuli. Nevertheless, the stress response to bile salts provides a multilevel defence concomitant with increase of the synthesis of numerous stress proteins.

Despite some overlap of stress proteins between the acid regulon and the other stresses, no significant acid-induced cross-protection is observed. Furthermore, adaptation by heat confers no protection against acid, and bile salt adaptation even provokes sensitization of cells towards the acid challenge. These combined data show that the acid stress triggers a rather specific response in *E. faecalis*.

Strikingly, the tolerance towards bile salts and acid challenges in *E. faecalis* is further improved or maintained after prolonged exposure to adaptation conditions. Because of its lifestyle, *E. faecalis* tolerates long periods of bile salt stress and acidic conditions and subsequently seems to have developed non-transient responses. Heat tolerance, on the other hand, decreases after prolonged exposure; this indicates that the heat shock response in *E. faecalis* has a largely transient character, which is analogous to findings in other organisms [1].

In conclusion, the results show that *E. faecalis*, living in vastly differing environments, is able to both distinguish and appropriately adjust to these alternative surroundings. The present work stresses that induction of tolerance is rapid, extended and maximal with homologous stresses. The cross-protection effects observed here show that the adaptability of *E. faecalis* is indeed remarkable. The results are of potential importance for food contamination involving *E. faecalis* in that shocked organisms would become tolerant to detergent or thermic treatment. Moreover, *E. faecalis* exposed to mild acidity becomes acid-habituated and if subsequently ingested by man could resist stomach acidity or if expelled into the external environment could survive in acidulated foods. Killing of *E. faecalis* is highly

Fig. 1. Autoradiograms of ^{35}S-labelled proteins in *E. faecalis* ATCC19433 during exponential growth and after the imposition of stress. Growth, labelling and gel running were performed in two independent experiments and the subsequent data analysis was accordingly based on comparisons of the sample duplicates. A, control; B, 0.08% bile salts; C, pH 4.8; D, 50°C. The different groups of stress proteins are marked with capital letters. BS, bile salt specific stress proteins; A, acid specific stress proteins; H, heat specific stress proteins; G, stress proteins common to two or three stresses (Table 2). Of the G proteins, two were immunologically identified as homologues of DnaK (G11) and GroEL (G12) in *E. faecalis*, by using polyclonal antibodies directed against these Hsp of *E. coli* (data not shown).

dependent on the physiological state of the cells prior to exposure to lethal conditions, indicating that bacteria should be compared to a black box which retains momentarily the marks of cellular traumatisms.

Acknowledgements

This work was partly financed by the Agence de l'Eau Seine-Normandie. S. Flahaut is the recipient of an award from the Ministère de la Recherche et de l'Enseignement Supérieur of France.

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