The *Enterococcus faecalis* gene encoding the novel general stress protein Gsp62

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The *Enterococcus faecalis* general stress protein Gsp62 was purified using two-dimensional gel electrophoresis and its 25 N-terminal amino acid sequence determined. Analysis of the corresponding gene revealed that the *gsp62* product is a 172 aa protein. Transcriptional analysis of *gsp62* gave evidence for a monocistronic mRNA, the synthesis of which was induced at the onset of stationary phase and in response to heat shock, acid pH, detergents (i.e. SDS or bile salts), ethanol, tert-butyl hydroperoxide, sodium chloride and, to a lesser extent, hydrogen peroxide. 5' rapid amplification of cDNA ends by PCR experiments showed that *gsp62* transcription initiates 30 nt upstream of the ATG start codon. Although *gsp62* expression was induced in response to various stresses, its disruption had no significant effect on the cell survival after each individual stress. Two-dimensional protein gels from wild-type and mutant cells revealed no pleiotropic effect of the mutation on protein synthesis. Transcriptional fusions with the *lacL lacM* β -galactosidase genes showed that an inverted repeat located upstream of the promoter is required for transcriptional induction by environmental stresses but not by entrance into stationary phase. Two distinct mechanisms responding to different signals are thus involved in *gsp62* induction.

Keywords: *Enterococcus faecalis*, general stress protein, Gsp

INTRODUCTION

When grown under moderate stress conditions, bacteria may develop an adaptive response, allowing them to cope with subsequent more severe stresses. In general, this adaptation phenomenon appears to involve multiple genes encoding stress proteins, which can be specifically induced by a particular stress factor (specific stress proteins) or induced by several conditions (general stress proteins).

The Gram-positive bacterium *Enterococcus faecalis* is an ubiquitous micro-organism. Resident of the human and animal gut, it is introduced to the environment by means of faeces and subsequently disseminated to diverse niches. *Ent. faecalis* also has importance as a pathogen, ranking the second most important agent in

total nosocomial infections. Its survival in the external environment is linked to its exceptional aptitude for coping with harsh conditions (Jett *et al.*, 1994; Mundt, 1986). Physiological studies showed that *Ent. faecalis* is able to develop adaptive responses towards diverse stresses (Boutibonnes *et al.*, 1993; Flahaut *et al.*, 1996a, b, c; 1997a, b, c; 1998; Laplace *et al.*, 1996). Moreover, analysis of protein synthesis during incubation of exponentially growing cells of *Ent. faecalis* with sublethal stresses led to the detection of the overexpression of 167 proteins. Six of these are induced by at least six different stress conditions and probably play an important physiological role in the non-specific stress response. These general stress proteins were named Gsp62 to Gsp67 (Rincé *et al.*, 2000). Studies of proteins overexpressed during glucose exhaustion led to the identification of several glucose starvation proteins (Gls) and to the characterization of a seventh general stress protein, Gls24, which is involved in bile salt resistance (Giard *et al.*, 2000). Western blot analyses identified Gsp66 and Gsp67 as DnaK and GroEL chaperonins, respectively (Flahaut *et al.*, 1997b), and

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Abbreviations: tBOOH, tert-butyl hydroperoxide; 2-D, two-dimensional; IR, inverted repeat; RACE, rapid amplification of cDNA ends.

their induction characteristics implied that their genes belong to the same regulon (Rincé *et al.*, 2000). Recently, Gsp65 was identified as an organic hydroperoxide resistance protein (Ohr) involved in resistance to oxidative stress (Rincé *et al.*, 2001). mRNA studies revealed a transcriptional induction of *gsp65* in response to tert-butyl hydroperoxide (tBOOH), heat shock, acid pH, detergents (bile salts, SDS), ethanol, sodium chloride and H₂O₂.

In this paper, we describe Gsp62, a novel *Ent. faecalis* general stress protein. This was purified from two-dimensional (2-D) protein gels and its N-terminal sequence determined. The identification of the corresponding gene allowed sequence analyses, gene inactivation and expression studies.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Ent. faecalis* strains were grown at 37 °C without shaking in brain heart infusion medium (BHI, Difco) or in M17 medium (Terzaghi & Sandine, 1975) supplemented with 0.5% glucose (GM17). When necessary, erythromycin was added at a concentration of 150 µg ml⁻¹. Viability was determined by spreading 0.5 ml serial dilutions on BHI agar (1.5% w/v). *Escherichia coli* strain XL1Blue (Stratagene) and plasmid pUCB300 (Frère *et al.*, 1993) were used for cloning a *gsp62* internal fragment for integrational mutagenesis. *Esc. coli* strain DH5α (Life Technologies) and plasmids pBluescript SK⁺ (Stratagene) and pAK80 (Israelsen *et al.*, 1995) were used for cloning the *gsp62* promoter upstream of the *lacL* and *lacM* β-galactosidase genes. *Esc. coli* strains were cultivated under vigorous agitation at 37 °C in LB medium (Sambrook

et al., 1989) with ampicillin (100 µg ml⁻¹) or erythromycin (150 µg ml⁻¹) added when required.

Construction of a *gsp62* mutant by homologous recombination. To construct an insertional mutant with a disruption in *gsp62*, an internal fragment of 368 bp was PCR amplified with phosphorylated oligonucleotides P5 and P6 (Fig. 1a; Table 2), treated 30 min at 72 °C with 2.5 U *Pfu* polymerase (Stratagene) and cloned into the insertional vector pUCB300 previously digested with *Sma*I and dephosphorylated. The resulting plasmid obtained after transformation of *Esc. coli* XL1Blue was introduced into *Ent. faecalis* JH2-2. Integrations within *gsp62* were verified in erythromycin-resistant colonies by PCR using primers P1 and P4 (Fig. 1a; Table 2) (data not shown) and by Southern blot hybridization (Fig. 2).

Adaptation and challenge treatments. *Ent. faecalis* JH2-2 cultures (10 ml) were grown to an OD₆₀₀ of 0.5 (mid-exponential growth phase). Bacteria were harvested by centrifugation and resuspended in BHI medium (control culture). Adaptation was conducted in the same medium incubated at 50 °C (heat shock), or at 37 °C in medium supplied with (i) 4% (v/v) ethanol, (ii) 0.08% (w/v) bile salts (sodium cholate:sodium deoxycholate, 1:1 v/v), (iii) 0.01% (w/v) SDS, (iv) 6.5% (w/v) NaCl, (v) 2.4 mM H₂O₂, (vi) lactic acid to adjust pH to 4.8, (vii) NaOH to adjust pH to 10.5, (viii) 2 mM tBOOH. Adaptation treatments were performed for 30 min, except for NaCl (2 h).

Adapted and non-adapted cells (treated as described above) were harvested by centrifugation. Cells were resuspended in 10 ml BHI and incubated at 37 °C (control) or at 62 °C to determine resistance to a thermal challenge. Other challenges were carried out at 37 °C in medium supplied with (i) 22% (v/v) ethanol, (ii) 0.3% (w/v) bile salts, (iii) 0.017% (w/v) SDS, (iv) 28.5% (w/v) NaCl, (v) 45 mM H₂O₂, (vi) lactic acid to adjust pH to 3.2, (vii) NaOH to adjust pH to 11.9, (viii) 20 mM tBOOH. Challenges were performed for 30 min except

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>Ent. faecalis</i> strains		
ATCC 19433		Jones & Shattock (1960)
JH2-2	Rif ^r Fus ^r	Yagi & Clewell (1980)
<i>Esc. coli</i> strains		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacI^qZΔM15 Tn10(Tet^r)]</i>	Stratagene
DH5α	F ⁻ Φ80 <i>lacZΔM15 endA1 recA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 thi-1 ΔgyrA96 (ΔlacZYA-argF)U169</i>	Life Technologies
Plasmids		
pUCB300	<i>Esc. coli</i> plasmid vector; Amp ^r , Erm ^r	Frère <i>et al.</i> (1993)
pBluescript SK ⁺	<i>Esc. coli</i> plasmid vector; Amp ^r	Stratagene
pAK80	<i>Esc. coli-Ent. faecalis</i> shuttle; promoter probe vector for transcriptional fusions with <i>lacL-lacM</i> β-galactosidase genes; Erm ^r	Israelsen <i>et al.</i> (1995)
p17635	<i>Esc. coli</i> plasmid vector; Amp ^r , Kan ^r	Bardonnet & Blanco (1992)
pFWT62	pAK80 containing the wild-type <i>gsp62</i> promoter (region -246 to +107) fused to the <i>lacL-lacM</i> β-galactosidase genes	This study
pFDE62	pFWT62 with a 15 bp <i>Eco</i> RI fragment deleted in the <i>gsp62</i> promoter region	This study

Fig. 1. (a) Genetic organization of the *gsp62* chromosomal region. Large arrows represent the ORFs and their orientation shows the transcriptional direction. The nucleotide sequences of the *gsp62* promoter region, of the putative rho-independent terminator (T1) located immediately downstream of the *gsp62* stop codon, and of the inverted repeat lying upstream of the *gsp62* promoter (IR) are shown. The transcription initiation nucleotide (+1) and the putative -35 and -10 motifs are boxed. The *EcoRI* sites used to delete a part of the IR within the *gsp62* promoter are indicated. The sequence resulting from the deletion is presented and the IRs in the wild-type and in the deleted fragment are shown by convergent arrows. Primer positions are indicated by arrowheads. (b) Electrophoretogram obtained from 5' RACE PCR experiment. The sequence in the electrophoretogram was obtained using primer Pext and 5' A-tailed cDNA obtained with the 3'/5' RACE kit. The last base (C) upstream of the 16 nt A-tail corresponds to the first nucleotide transcribed. The corresponding G on the reverse complement strand is indicated above (+1).

Table 2. Primers used for PCR, mutagenesis and 5' RACE PCR experiments

Primer	Sequence (5'–3')*	Orientation†
P1	AGAAGTTGTGGATTTTGC	+
P4	CAATGTATCCGCGTAATTC	–
P5	GTAAACACAGAAACAGGGC	+
P6	GGAAACAAGCGTTGCATCT	–
Ppe	TTGACAGGGGCATTACTCTC	–
Pext	CTCTACCAATGTGAAGCCCTGTT	–
P62S	atgtaggATCCAAGCAATCGGCATTAG	+
P62NS	ttaTactgCAGGGGCATTACTCTCACC	–

* Bases indicated in lower-case letters are not complementary to the target sequence. Underlined nucleotides correspond to *Bam*HI and *Pst*I sites.

† +, Primer directed towards the 3' end of *gsp62*; –, primer in the opposite direction.

for the bile salts and SDS detergents (30 s). Samples (0.5 ml) were removed, diluted in 0.9% NaCl and poured in GM17 agar for the determination of c.f.u. numbers. Plates were

incubated at 37 °C for 48 h. Each point of an experiment is the mean of duplicate platings and all experiments were repeated at least twice.

Fig. 2. Southern blots of total DNA extracted from wild-type *Ent. faecalis* JH2-2 (lanes 1) and a *gsp62* mutant (lanes 2) and digested by *EcoRI*. Hybridizations were performed with ^{32}P -labelled PCR fragment P5-P6 (a) or ^{32}P -labelled pUCB300 vector (b).

Analysis of mRNA by Northern and dot blot experiments.

Total RNA of *Ent. faecalis* JH2-2 was isolated from exponentially growing cells, from cells entering stationary phase or from stressed cells by using the RNeasy Midi Kit (Qiagen). For Northern blots, 10 µg RNA was electrophoretically resolved per lane and transferred onto Hybond-N⁺ membranes (Amersham International) using standard procedures (Sambrook *et al.*, 1989). Sizes of transcripts were estimated by comparison with an RNA ladder (0.56–9.4 kb) (Amersham International). For dot blots, 1 µg total RNA from cells incubated 10 min under the different individual stress conditions was spotted onto Hybond-N⁺ membranes. Membrane-bound nucleic acids were hybridized at a temperature of 55 °C in 1 M sodium phosphate buffer (pH 7.0) containing 5 % SDS with a single-strand labelled probe complementary to *gsp62* mRNA. After hybridization, membranes were washed twice in 2× SSC, 0.1 % SDS (10 min), twice in 0.5× SSC, 0.1 % SDS (10 min) at 55 °C, and exposed to a storage phosphor screen (Packard Instrument Company) for 5 h.

Preparation of the single-strand labelled probe was as follows: first, a DNA fragment was amplified by PCR from chromosomal DNA of *Ent. faecalis* JH2-2 with the primers P4 and P5 (Fig. 1a; Table 2). The probe was then synthesized by elongating the oligonucleotide P4 with *Taq* DNA polymerase, 2 µM dCTP, dGTP and dTTP, 2 µCi (74 kBq) [α - ^{32}P]dATP and 10 ng of the previously obtained PCR DNA fragment as template. Thirty cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C were performed.

Mapping of the transcriptional start site. The 5' end of *gsp62* mRNA was mapped from a 5' RACE (rapid amplification of cDNA ends) PCR product obtained with the 3'/5' RACE kit (Roche Molecular Biochemicals) using primer P6 (Fig. 1a; Table 2) for the reverse transcriptase reaction and polyA tailing and primer Ppe for PCR amplification (Fig. 1a; Table 2). The cDNA was then purified, analysed on a 6 % polyacrylamide gel and sequenced by the dideoxy chain termination method with the ABI prism sequencing system (PE Biosystems) and primer Pext (Fig. 1a; Table 2).

Construction of promoter fusions. A 353 bp fragment containing the wild-type *gsp62* promoter (nucleotide region –246 to +107, relative to the transcriptional start site) was amplified by PCR using the primers P62S and P62NS (Fig. 1a; Table 2), digested with *Bam*HI and *Pst*I, and cloned into the *Bgl*II and *Pst*I sites of the promoter probe vector pAK80, creating plasmid pFWT62. The insert was sequenced to ensure that the PCR reaction did not induce any mutations. A variant of pFWT62, bearing an *Eco*RI deletion which removes a part of the inverted repeat located upstream of the promoter (Fig. 1a) was constructed as follows. First, the 353 bp fragment containing the wild-type *gsp62* promoter was cloned into the *Bam*HI and *Pst*I sites of the pBluescript vector and its sequence was determined to make sure that the PCR did not create any mutations. Then, the *Eco*RI site of the vector was eliminated by inserting the kanamycin resistance cassette from p17635 (Bardonnnet & Blanco, 1992) between the *Pst*I and *Apa*I sites. The resulting plasmid was linearized by *Eco*RI and self-ligated to obtain the 15 bp deletion within the *gsp62* promoter region. The resulting 338 bp fragment was excised by digestion with *Bam*HI and *Pst*I, purified and subcloned into the *Bgl*II and *Pst*I sites of pAK80, yielding plasmid pFDE62. For both constructions, conservation of the three stop codons immediately upstream of the ribosome-binding site of *lacL* in pAK80 ensured that the coding region of *gsp62* was not translationally fused to the reporter gene. Plasmids pFWT62 and pFDE62 were introduced by electroporation as described below.

β -Galactosidase assays. Cells grown in BHI and exposed to 4 % ethanol or 0.3 M sodium chloride were harvested by centrifugation and concentrated fivefold in Z buffer (Miller, 1972). Two hundred microlitres of bacterial suspension were treated as described by Israelsen *et al.* (1995) and β -galactosidase activity was expressed in Miller units calculated according to the equation: activity = $(\text{OD}_{420} \times 1000) / (\text{OD}_{600} \times 0.2 \times t_{(\text{min})})$ (0.2 corresponding to the volume of cell suspension in ml). Each point is the mean of at least three experiments.

Two-dimensional protein gel electrophoresis. Sample preparation and 2-D protein gel electrophoresis were carried out as described by Giard *et al.* (1997).

General molecular methods. Restriction endonucleases, T4 polynucleotide kinase, alkaline phosphatase and T4 DNA ligase were obtained from Roche Molecular Biochemicals, Amersham International and Eurogentec, and used according to the manufacturers' instructions. PCRs were carried out with 5 µg chromosomal DNA from *Ent. faecalis* JH2-2 and 20 pmol primers, using *Taq* DNA polymerase (Sigma) or Ready To Go PCR Beads (Pharmacia Biotech). When necessary, PCR products were purified using the QIAquick Kit (Qiagen). *Esc. coli* and *Ent. faecalis* were transformed by electroporation with the Gene Pulser Apparatus (Bio-Rad), as described by Dower *et al.* (1988) and Holo & Nes (1995), respectively. Plasmids were purified by using QIAprep Miniprep Kit (Qiagen). DNA and amino acid sequences were analysed using the Mac Vector program (Kodak, Scientific Imaging Systems) and database searches were performed with the BLAST program (Altschul *et al.*, 1990). Other standard techniques were carried out as described by Sambrook *et al.* (1989).

RESULTS

Identification of *gsp62*

Proteins extracted from a 50 ml culture of *Ent. faecalis* ATCC 19433 incubated for 2 h in the presence of 6.5 % NaCl were separated by preparative 2-D electrophoresis

Fig. 3. Autoradiogram of 2-D protein gels of *Ent. faecalis* JH2-2 (a) and a *gsp62* mutant (b). The pictures represent a part of the autoradiograms obtained after electrophoresis of proteins extracted from 5 ml culture aliquots of exponentially growing cells, pulse-labelled with 250 μ Ci (9.25 MBq) [35 S]methionine/cysteine 'protein-labelling mix' (New England Nuclear Co., 1000 Ci mmol $^{-1}$) during 30 min treatment with 0.08 % bile salts. The arrow shows the position of Gsp62.

and electroblotted onto a PVDF membrane. The general stress protein Gsp62 was then purified and N-terminal sequencing allowed the determination of the 25 N-terminal amino acid sequence MDISVIDATKVNAETGLHIGESNAP. This sequence has been identified in the stress proteome of *Ent. faecalis* (Giard *et al.*, 2001). Homology searches carried out with the BLAST program gave no significant similarities with sequences from databases. However, the corresponding ORF was obtained from the genomic sequence of *Ent. faecalis* (V583) available at <http://www.tigr.org>. The product of this ORF shares 24 aa with the 25 N-terminal amino acid sequence of Gsp62 (the alanine in position 13 of Gsp62 was substituted by a threonine in the sequence deduced from the *Ent. faecalis* V583 genome). Translation of the entire ORF revealed that it encodes a 172 aa protein with a calculated molecular mass of 19.5 kDa and a pI of 4.68. From this entire sequence, no significant similarities were found with proteins from databases. The hydrophobicity profile of Gsp62 showed a hydrophobic domain located between amino acids in positions 130 and 155 (data not shown). The nucleotide sequence immediately upstream of this ORF contains a putative ribosome-binding site (GAGG) located 6 bp upstream of the initiation codon (ATG). An inverted repeat (Δ G

= -33.8 kcal mol $^{-1}$), which could act as a rho-independent terminator, was observed immediately downstream of the *gsp62* termination codon TAA (Fig. 1a).

Phenotypic study of the *gsp62* mutant

Because of the induction of Gsp62 synthesis under stress conditions, we examined whether a knockout of the gene affected stress resistance. A *gsp62* mutant was constructed by homologous recombination as described in Methods. Growth studies of the *gsp62* mutant did not reveal any significant difference with respect to the wild-type JH2-2 strain when cultured at 37 °C in BHI broth. Gsp62 is thus dispensable for optimal growth. When assessing the survival of bacteria after different individual lethal treatments, we observed no significant differences between the two strains after heat, NaCl, pH, detergents, tBOOH and H₂O₂ challenges applied either with or without previous adaptation.

We examined whether the knockout confers modifications to the protein pattern observable on 2-D PAGE. 2-D PAGE of proteins extracted from exponentially growing JH2-2 and *gsp62* mutant cells exposed to 0.08 % bile salts (Fig. 3) or 2 mM H₂O₂ (data not shown) for

Fig. 4. (a) Northern blot hybridization of *Ent. faecalis* JH2-2 RNA extracted from exponentially growing cells (lane 1) and from cells incubated 10, 20 and 30 min with 0.08% bile salts (lanes 2, 3 and 4, respectively). Hybridization was performed with a single-strand DNA probe corresponding to the DNA region located between primers P4 and P5 (Fig. 1a; Table 2). The size of the transcript is indicated on the right. (b) Northern blot hybridization of *Ent. faecalis* JH2-2 RNA extracted from exponentially growing cells (lane 1) and from cells harvested at the entrance into stationary phase (lane 2). Hybridization was performed as in (a). (c) Dot blot hybridization of total RNA from *Ent. faecalis* JH2-2 cells harvested in exponential growth phase (dot 10), and from exponentially growing cells incubated 10 min at 50 °C, in the presence of 1.02 M NaCl, 4% ethanol, 0.08% bile salts, 0.01% SDS, 2.4 mM H₂O₂ or 2 mM tBOOH (dots 1–7, respectively), or in GM17 medium adjusted to pH 4.8 or 10.5 (dots 8 and 9). Hybridization was performed as in (a).

30 min confirmed the absence of Gsp62 in the mutant, but revealed no other significant difference in protein synthesis between the *gsp62* mutant and the wild-type strains.

Transcriptional analysis of *gsp62*

gsp62 is preceded by ORFs encoding proteins of unknown functions, while an ORF encoding a protein 80% identical to the ClpP protease of *Listeria monocytogenes* (Gaillot *et al.*, 2000) is located downstream of *gsp62* on the other DNA strand (Fig. 1a: ORFB). Northern blot analysis of *Ent. faecalis* RNA revealed a unique *gsp62* transcript of 0.6 kb (Fig. 4a, b), demonstrating that the *gsp62* mRNA is monocistronic. The Northern blot experiments also showed an unambigu-

Fig. 5. *gsp62* promoter activities during growth of *Ent. faecalis* JH2-2. The strain contains the β-galactosidase genes *lacL* and *lacM* under the control of the wild-type *gsp62* promoter in pFWT62 (a) or the promoter deleted of the *EcoRI* fragment (Fig. 1a) in pFDE62 (b). OD₆₀₀ (white symbols) and β-galactosidase activities (black symbols) were determined during the growth at 37 °C in BHI medium.

ous induction of *gsp62* transcription by the presence of bile salts, with a maximum mRNA accumulation observed 10 min after stress exposure (Fig. 4a) and by the entrance into stationary phase (Fig. 4b).

To analyse the effect of different stress conditions on *gsp62* expression, dot blot experiments were carried out on total RNA extracted from exponentially growing cells or from bacteria incubated for 10 min with a sublethal dose of various agents (Fig. 4c). Results revealed that the transcription of *gsp62* is also stimulated by the presence of 2 mM tBOOH, by an elevation of the temperature to 50 °C, by incubation at pH 4.8, in the presence of 0.01% SDS, 4% ethanol, and, to a lesser extent, with 1.02 M NaCl or 2.4 mM H₂O₂.

Mapping of the transcriptional start site of *gsp62*

To identify the promoter of *gsp62*, 5' RACE PCR experiments were carried out with RNA extracted from cells treated for 10 min with 2 mM tBOOH. A unique

Fig. 6. (a) *gsp62* promoter activities in *Ent. faecalis* JH2-2 cells containing plasmid pFWT62. OD₆₀₀ (white squares) was determined during the growth at 37 °C in BHI medium. After 120 min incubation, the culture was divided in two and either treated with 0.3 M NaCl (arrow) or untreated. β -Galactosidase activities were measured and expressed as the ratio of activity of the treated culture to activity of the untreated culture (induction factor: black symbols). (b) β -Galactosidase activities of *Ent. faecalis* JH2-2 cells containing plasmid pFWT62 or pFDE62. Measurements were carried out from exponentially growing cells untreated (1), or treated 10 min with 0.3 M NaCl (2) or 4% ethanol (3). Induction factors are the ratio of β -galactosidase activities of the tested condition to that obtained with the pFWT62 control in unstressed cells. Standard deviations for all conditions are not higher than 0.03.

band was observed after electrophoresis of the 5' RACE PCR product and the sequence of the corresponding cDNA showed that the transcriptional start site of *gsp62* lies 21 nt upstream of the ribosome-binding site (Fig. 1b). Seven base pairs upstream of this transcriptional start site, we found the putative -10 box TATACT separated by 17 bp from the putative -35 box ATGATT. This promoter matches the consensus sequence for promoters depending on the primary sigma factor

(TATAAT-16–19 bp-TTGACA, respectively). Immediately upstream of the -35 box of the *gsp62* promoter, an inverted repeat (IR) (AGgCACGAATTCA-7 bp-TGAATTCGTGtCT) was identified (Fig. 1a).

Construction of promoter fusions and β -galactosidase expression analyses

Two promoter fusions with the *lacL* and *lacM* β -galactosidase genes were constructed as described in Methods. The first fusion corresponded to the wild-type *gsp62* promoter (region -246 to $+107$) cloned upstream of the β -galactosidase genes of the pAK80 vector (plasmid pFWT62), while the second differed by a 15 bp deletion which removed a part of the IR located immediately upstream of the -35 box (Fig. 1a) (plasmid pFDE62).

β -Galactosidase activity was monitored during the growth of *Ent. faecalis* JH2-2 harbouring pFWT62 or pFDE62. A strong increase (6.8-fold) of the β -galactosidase activity was observed at the end of the exponential growth phase with the wild-type promoter (Fig. 5a). This confirms the *gsp62* transcriptional induction at the entry into stationary phase. The transcriptional induction at the entry into stationary phase (7.2-fold) was also observable with the deleted version of the promoter fragment (Fig. 5b), showing that this phenomenon does not rely on the IR located immediately upstream of the -35 box.

When a sublethal dose of salt (0.3 M sodium chloride) was added to the culture during the exponential growth phase of *Ent. faecalis* JH2-2 harbouring pFWT62, the β -galactosidase activity abruptly increased to reach an induction factor of 2.0 (which corresponded to 5.68 Miller units) 30 min after the addition of NaCl (Fig. 6a). The addition of ethanol (4%) had a similar effect on the β -galactosidase activity with the wild-type promoter fusion (induction factor of 1.8) (Fig. 6b). With the disruption of the IR, the β -galactosidase activity was half that of the wild-type (Fig. 6b); challenge with sodium chloride or ethanol resulted in hardly any induction of *gsp62*. This showed the involvement of the IR in the *gsp62* basal transcription and in its regulation by environmental stresses. Two distinct mechanisms are thus involved in induction of transcription in response to environmental and metabolic stresses.

DISCUSSION

To understand the mechanisms involved in *Ent. faecalis* stress resistance and particularly the multistress-resistance phenomenon, we chose to characterize general stress proteins. Our original biochemical studies of *Ent. faecalis* protein synthesis allowed the detection of seven general stress proteins (Gsp62–67 and Gls24), the synthesis of which was shown to be increased by different individual stresses (Giard *et al.*, 2000; Rincé *et al.*, 2000). In the present study, our aim was to characterize Gsp62. Gsp62 was purified after 2-D PAGE and its N-terminal sequence was determined. The

knowledge of the 25 N-terminal amino acids allowed us to identify the corresponding gene within the *Ent. faecalis* V538 genome and to conclude that Gsp62 does not share significant similarities with proteins from databases and can be considered as a novel general stress protein. *gsp62* mRNA studies revealed that this gene is transcribed as a 0.6 kb monocistronic mRNA and gave evidence for a transcriptional induction when cells were exposed to elevated temperature, a pH decrease or to the presence of tBOOH, ethanol, bile salts, SDS, NaCl and H₂O₂. These observations provide an unambiguous demonstration that Gsp62 is a general stress protein. *gsp62* promoter activity is also clearly stimulated when bacteria enter the stationary phase, probably as a consequence of nutrient starvation. The expression of *gsp62* is thus induced by both environmental and metabolic stresses. In this respect, *gsp62* resembles *Bacillus subtilis* *gsp* genes belonging to class II, the induction of which is under the sole control of the general stress secondary sigma factor σ^B (Hecker *et al.*, 1996; Hecker & Völker, 1998).

We recently identified and characterized *gsp65*, another general stress protein encoding gene in *Ent. faecalis* (Rincé *et al.*, 2001). The transcription of *gsp62* and of *gsp65* is induced to comparable levels by the same treatments, suggesting that those two genes belong to the same regulon.

5' RACE PCR product sequencing allowed us to locate the *gsp62* promoter, which matches the consensus sequence for the primary sigma factor. Immediately upstream of this promoter, an IR was identified. Upstream of the previously described *gsp65* promoter, an IR of distinct sequence was also identified (Rincé *et al.*, 2001). As *gsp65* is preceded by a gene located on the other DNA strand, we suggested that the *gsp65* IR was unlikely to be a rho-independent terminator, but rather a structure potentially involved in transcriptional regulation (Rincé *et al.*, 2001). Here, results from *gsp62* promoter fusions with β -galactosidase genes revealed that the disruption of the IR led to a reduction of the basal level of transcription and prevented environmental stress induction of *gsp62*. This IR is thus very likely a target of a regulator (activator) of general stress response in *Ent. faecalis*. The induction of *gsp62* expression in response to environmental stresses is thus not due to a secondary sigma factor. Whereas a gene encoding a σ^B homologue has been identified in some non-sporulating Gram-positive bacteria (Becker *et al.*, 1998; Wiedmann *et al.*, 1998; Wu *et al.*, 1996), no alternative sigma factor has been identified in the *Ent. faecalis* V538 chromosome sequence. Moreover, in *Lactococcus lactis*, which is closely related to *Ent. faecalis* and the genome of which has been entirely sequenced (Bolotin *et al.*, 1999), no σ^S or σ^B -like transcription factor has been found. Surprisingly, the deletion of a part of the IR had no effect on the activation of *gsp62* promoter in response to entry into stationary phase. This implies that in contrast to *B. subtilis* class II *gsp* genes, *gsp62* relies on two completely distinct pathways of induction responding to different signals.

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