ELSEVIER

FEMS Microbiology Letters 252 (2005) 137–141

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Implication of *hypR* in the virulence and oxidative stress response of *Enterococcus faecalis*

Nicolas Verneuil [a], Alain Rincé [a], Maurizio Sanguinetti [b], Yanick Auffray [a], Axel Hartke [a], Jean-Christophe Giard [a,*]

[a] *Laboratoire de Microbiologie de l'Environnement, EA 956 soutenue par l'INRA, IRBA, Université de Caen, 14032 Caen Cedex, France*
[b] *Institute of Microbiology, Catholic University of Sacred Heart, L. go F. Vito 1, 00168 Rome, Italy*

Received 6 July 2005; received in revised form 22 August 2005; accepted 26 August 2005

First published online 9 September 2005

Edited by M.R. Soria

Abstract

HypR has recently been described as the first transcriptional regulator involved in the oxidative stress response and in the intracellular survival of *Enterococcus faecalis* within macrophages. In order to characterize the HypR regulon, real-time quantitative RT-PCR experiments were performed. The expression of four genes involved in the oxidative stress response encoding catalase, glutathione reductase, and the two subunits of alkyl hydroperoxide reductase were down regulated in the *hypR* background under H_2O_2 condition. These findings show that HypR acts as a transcriptional activator, especially during oxidative stress. In addition, DNAse I footprinting assays allowed us to identify the HypR-protected DNA regions corresponding to the "HypR box" in the *hypR* promoter. Moreover, the effect of the *hypR* mutation on the virulence of *E. faecalis* was evaluated in comparison with the wild-type JH2-2 strain using a mouse peritonitis model. Our results revealed that HypR appears to be an important virulence factor in *E. faecalis*.

Keywords: *Enterococcus faecalis*; HypR; Oxidative stress; Virulence

1. Introduction

While *Enterococcus faecalis* is a ubiquitous bacterium commonly found in the intestinal tract of humans, it can be the causative agent of severe infections. The intrinsic as well as acquired resistances of this bacterium towards antibiotics or environmental challenges contribute to its emergence as a prominent nosocomial pathogen. Over the last 15 years, studies have identified some *E. faecalis* virulence factors such as the surface adhesins Esp, AS, Ace, and EfaA, the secreted toxin cytolysin (Cyl), the secreted proteases GelE and SrpE, the two cell wall polysaccharides CpsA-K and Epa, and the general stress protein Gls24 [1–3]. Moreover, some genes that may contribute to *E. faecalis* virulence were identified from the sequence of the pathogenicity island of the MMH594 strain [4]. In addition, transcriptional regulators involved in the stress response and/or the potential of virulence in *E. faecalis* have recently been studied. This is the case for the sigma factor SigV, the two-component system Err05 or EtaRS (important for virulence of *E. faecalis* in a mouse peritonitis model), and the Fsr system controlling expression of GelE and SprE, or HypR [5–9]. The latter is involved in the oxidative stress response and in the survival within murine macrophages. The *hypR* mutant appeared highly sensitive to

* Corresponding author. Tel.: +330 2 31 56 54 10; fax: +330 2 31 56 53 11.
E-mail address: jean-christophe.giard@unicaen.fr (J.-C. Giard).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.08.043

oxidative challenge caused by hydrogen peroxide compared to the wild type strain. Moreover, it has been proven that HypR controlled directly the transcription of *hypR* itself and of the *ahpCF* operon (for alkyl hydroperoxide reductase) [9]. In the present study, we have examined the importance of HypR in regulation of other genes known to be involved in the oxidative stress response. We also evaluated the role of HypR in lethality in an animal model.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. faecalis strains used in this study were the wild type JH2-2 and the *hypR* mutant previously constructed [10]. Cells were cultivated at 37 °C without shaking in 10 ml of semi-synthetic medium (Bacto Folic AOAC Medium, Difco, Detroit, Mich.) supplemented with 0.2% glucose (w/v).

2.2. RNA isolation, and real-time quantitative PCR

Total RNAs of *E. faecalis* were isolated from exponentially growing or stressed cells (30 min with 2.4 mM H_2O_2, which has been shown to be the best adaptation condition for *E. faecalis* [11]) by using the RNeasy Midi Kit (Qiagen). Primers used to amplify around 100 bp were designed using the Primer3 software available at the web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Two micrograms of total RNA were reverse transcribed with random hexamer primers. Five microliters of the resulting cDNA synthesis reaction mixture (dilution 10^{-2}) were used as matrix for the real-time quantitative PCR using the BioRad iCycler iQ detection system (Bio-Rad Laboratories, Richmond, Calif., USA). Experiments were performed three times and in duplicate with three different RNA samples. C_T (threshold cycle) was converted to "*n*-fold difference" by comparing mRNA abundance in the JH2-2 wild type strain (harvested in the middle of the growing phase or after 30 min in presence of 2.4 mM H_2O_2) to that obtained with the *hypR* mutant strain. The *n*-fold difference was calculated by the formula ($n = 2^{-x}$) when C_T mutant $< C_T$ JH2-2 and ($n = -2^{x}$) when C_T mutant $> C_T$ JH2-2 with $x = (C_T \text{ mutant} - C_T \text{ JH2-2})$. A value greater than 1 corresponds to a relative increase and a negative value reflects a relative decrease compared to the expression in the wild type strain. Statistical comparison of means was performed using Student's *t* test.

2.3. Mouse peritonitis model

This model was previously described by Teng et al. [7]. Bacterial cells were incubated in BHI broth overnight at 37 °C under constant agitation. The cells density was adjusted in order to obtain around 6×10^7 CFU ml^{-1} (in 0.85% saline solution). Ten mice per group [outbred ICR female mice, 4–6 weeks old (Harlan Italy S.r.l., San Pietro al Natisone, Udine, Italy)] were injected intraperitoneally with 1 ml of each bacterial inoculum made in 25% sterile rat faecal extract (SRFE). The number of surviving mice was recorded each 3 h and curves were obtained by the Kaplan–Meier method and compared by log-rank test using the GraphPad Prism software (GraphPad Software Inc., San Diego, Calif.). Comparisons with P values <0.05 were considered to be significant. All strains were tested three times.

2.4. DNase I footprint analysis

DNase I footprint experiments were performed as follows. A 465-bp DNA fragment in the *hypR* promoter region was generated by PCR with primers *hyp*R1 (5′-CGTGGCAAGAAGATTCCTTAC-3′) and *hyp*R2 (5′-GCTAAATATTCTCCTTCAGGTG-3′), which was previously end-labelled with [γ-^{32}P]-dATP using T4 polynucleotides kinase (Amersham Biosciences, Little Chalfont, UK). This DNA amplicon contained 193 bp of the *hypR* coding sequence, as well as 272 bp upstream of the *hypR* translation start. For DNase I footprint experiments, the ^{32}P-end-labeled DNA fragment was purified and 5 µl were incubated with 10 µl HypR protein extract (1 µg) in a 20 µl solution containing 5 µl of binding buffer 4X (80 mM Tris–HCl pH 7.5, 4 mM $CaCl_2$, 4 mM DTT, 40 µg ml^{-1} of poly(dI − dC)/poly(dI − dC) and 0.04% BSA). After 15 min incubation, DNase I (Amersham Biosciences) was added at a final concentration of 1×10^{-4} U. The digestion was carried out at room temperature and stopped after 1 min by adding 5 µl of a solution containing 10 mM EDTA pH 7.5, 97.5% deionized formamide, 0.3% each Bromophenol Blue and Xylene cyanol FF. Sequencing reactions were performed with the same labelled primer using the T7 sequencing™ kit (Amersham Biosciences) according to the user manual. Digests and their corresponding sequences were then incubated at 94 °C during 5 min and analyzed on 6% denaturing polyacrylamide gels.

3. Results

3.1. Identification of HypR-regulated genes in E. faecalis

In a recent study, we showed that the transcriptional regulator HypR was involved in the oxidative stress response in *E. faecalis* [9]. Real-time quantitative PCR was carried out in order to identify potential new members of the HypR regulon. Eleven genes known or suspected

Table 1
Change in abundance of *E. faecalis* mRNA of genes with a potential role in oxidative stress response in *hypR* mutant strain, relative to expression level in the JH2-2 wild type cells

ORF	Function	Gene	Expo[a]	H_2O_2[b]
ef0463	Superoxide dismutase	*sodA*	**+1.8**[c]	**−4.2**
ef1597	Catalase	*kat*	−1.2	**−12.1**
ef3270	Glutathione reductase		−1.5	**−6**
ef1211	NADH peroxidase	*npr*	+2.1	−2.6
ef1586	NADH oxidase		+1.3	−1.8
ef1338	Thioredoxine reductase		−1.4	−8
ef2738/2739	Alkyl hydroperoxide reductase	*ahpCF*	−1.3	**−6**
ef3233	DNA binding protein	*dps*	−1.4	+1.1
ef1585	PerR regulator	*perR*	−1.2	−5.2
ef0453	Ohr regulator	*ohr*	+1.5	−1.7
23Sr RNA	Ribosome RNA		1	1

[a] RNA extracted from cells harvested in exponential growing phase.
[b] RNA extracted from cells harvested after H_2O_2 treatment (30 min with 2.4 mM H_2O_2).
[c] Fold change relative to wild type cultured in the same condition. Values in boldface type represent changes with statistical significance ($P < 0.05$).

to play a role in the oxidative stress response in *E. faecalis* (nine enzymes and two regulators) were selected from the annotation of the genome available at the TIGR web site and from genes listed by Paulsen et al. [12]. Amplifications of 23S rRNA were used as internal controls. Quantifications were performed by comparison between values obtained from amplification of cDNA derived from *hypR* mutant strains and those obtained from the JH2-2 wild type strain. Expression of selected genes was determined in cells harvested in the middle of exponential growth phase and after 30 min in presence of 2.4 mM H_2O_2. Results are shown in Table 1. Four of the 11 genes tested (encoding catalase, glutathione reductase, and the operon including peroxiredoxine reductase, and alkyl peroxide reductase) exhibited significant decrease of level of expression (12.1, 6, and 6-fold reduction, respectively) when the *hypR* mutant was incubated in the presence of H_2O_2 (Table 1). Thus, HypR appears to be a transcriptional activator specifically active in stressed cells.

While a statistically significant difference was observed for the *sodA* transcript between the wild type and *hypR* mutant, the absolute magnitude of the difference was modest (Table 1). Moreover, supported by our Northern blot experiments [9], we suppose that this result does not reflect a major role in regulation of *sodA* by HypR.

3.2. Identification of the HypR binding sequence

To identify the DNA binding site recognized by HypR within the *hypR* sequence promoter, a DNase I footprint analysis was conducted. Two HypR protected-regions separated by 5 bp were located at positions −29 to −7 and −1 to +27 (Fig. 1). This large protected region (50 nucleotides) covered the transcriptional start site and the putative −10 box, suggesting that *hypR* may be negatively autoregulated. An inverted-repeat (ATACTTAT-N_{10}-ATAAGTAT), containing the transcription start point (base underlined), was located within the DNA region covered by HypR (Fig. 1). Moreover, gel retardation assays realized with different DNA fragments obtained by fragmentation of the same *hypR* promoter region confirm that this HypR-protected sequence was the single site of HypR fixation (data not shown). Attempts to identify other DNA targets by blasting the *hypR* binding motif against the entire genome of *E. faecalis* did not find additional HypR-binding sites.

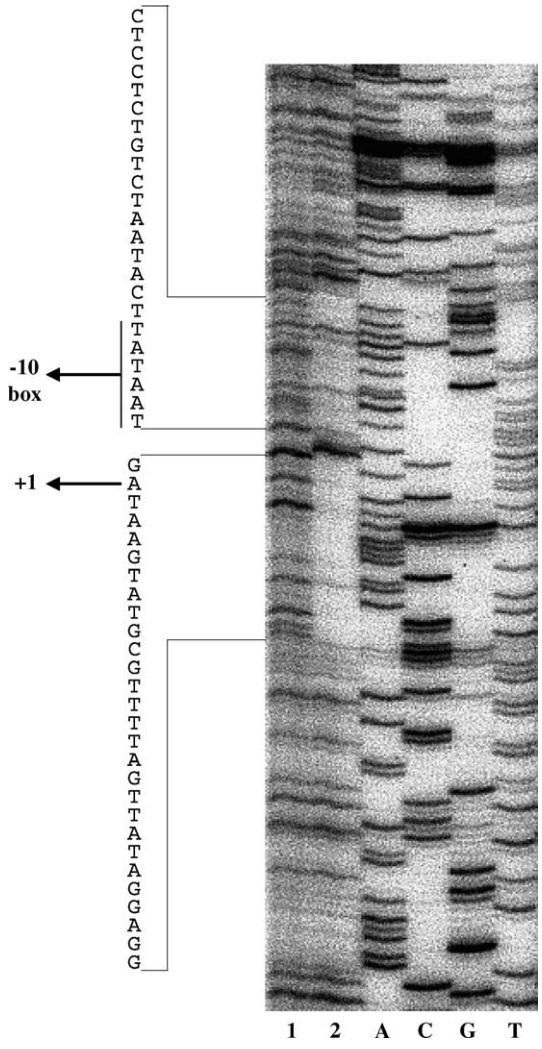

Fig. 1. DNAse I footprinting analysis of the HypR binding site into the *hypR* promoter region. PCR-generated DNA incubated without HypR (lane 1) or with 1 μg HypR (lane 2) was digested with DNAse I. The two HypR-protected DNA sequences are indicated on the left. Arrows indicate the +1 of transcription and the putative −10 box. The sequencing reactions (ACGT) are shown on the right.

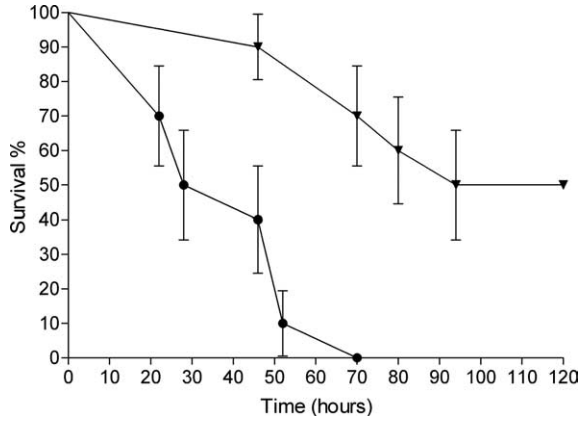

Fig. 2. Kaplan–Meier survival curves (±standard deviation) of peritoneally infected mice after injection with around 6×10^7 CFU of *E. faecalis* JH2-2 (black ring) and *hypR* mutant (black triangle).

3.3. Effect of the hypR mutation on the virulence of E. faecalis

Since the *hypR* mutant was severely affected in survival within macrophages [9], it appeared interesting to determine the contribution of this regulator to virulence in a mouse model. Intraperitoneal infection of the JH2-2 strain (around 6×10^7 CFU) resulted in the death of all the animals after 70 h (Fig. 2). On the other hand, killing by the *hypR* mutant was significantly attenuated. As shown in Fig. 2, 50% of mice infected with the *hypR* mutant cells were still alive after 120 h. These findings demonstrate that the *hypR* gene contributes to the virulence of *E. faecalis* in the mouse peritonitis model.

4. Discussion

We used a functional genomic approach to investigate the mechanism of defense against toxic oxygen species in the gram positive bacterium *E. faecalis* responsible for hospital-based infections. Because this regulator was shown to be involved in the oxidative stress response in *E. faecalis* [9], it appeared interesting to characterize the implication of HypR in the expression of genes with known or suspected roles in the H_2O_2 stress response. Transcription of genes encoding catalase (*katA*), glutathione reductase (*ef3270*), and the two subunits of the alkyl hydroperoxide reductase (*ahpC* and *ahpF*) was decreased in the *hypR* mutant. Results for the two genes of the *ahpCF* operon were in agreement with previous data obtained by Northern blot and gel shift analysis [9]. Likewise, the absence of changes in the expression of *sodA* and *npr* genes in the *hypR* mutant has already been observed [9]. Except for *katA*, the HypR regulated genes were not H_2O_2 inducible. Therefore, the role of HypR appeared to maintain transcription of these loci at a level comparable to naive cells under oxidative stress conditions. In *Bacillus subtilis*, PerR has been identified as the major regulator of the oxidative stress response [13]. A gene coding a PerR homologous protein is also present in *E. faecalis*. However, real-time quantitative PCR assays did not allow us to detect significant variations of expression of the same set of genes tested in this study in an *E. faecalis perR* background (unpublished results). Our data brought to light the fact that the regulation of the oxidative stress response diverges from that of the gram positive bacteria model *B. subtilis* and that the transcriptional regulator HypR appears to be the major regulator of the peroxide stress response in *E. faecalis*.

In order to precisely characterize the HypR regulon, we tried to identify a putative "HypR-box". DNAse I footprinting revealed a 50-bp sequence protected by the regulator including an inverted-repeat structure. Unfortunately, no consensus motif could be proposed by comparison with the promoter regions of HypR-regulated genes previously characterized. These HypR-protected sequences contained six T-N_{11}-A corresponding to the putative consensus LysR-type binding site [14,15]. Note that the size of HypR footprint is consistent with the 50-nt OxyR protected region in *E. coli* [16]. Sequence alignments revealed that HypR was the most closely related protein to OxyR (also member of the LysR family) which controls a regulon that mediates the oxidative-stress response in *E. coli* [10].

HypR was previously shown to be involved in *E. faecalis* survival within murin macrophages, reinforcing the hypothesis according to which the oxidative stress response leads to the production of antioxidant enzymes that inactivate ROS generated by the oxidative burst of phagocytic cells [9]. In this study, we used an in vivo model to demonstrate that the absence of HypR increased the survival of the infected mice. These results strengthen the hypothesis that this regulator may be an important virulence factor of *E. faecalis*. Further work is being undertaken to characterize the HypR regulon and the precise role of this regulator in the pathogenesis of *E. faecalis*.

Acknowledgments

The expert technical assistance of Annick Blandin, Béatrice Gillot, Marie-Jeanne Pigny, and Eliette Riboulet was greatly appreciated. We thank A. Benachour, J.-M. Laplace, and V. Pichereau for helpful discussions.

References

[1] Gilmore, M.S., Coburn, P.S., Nallapareddy, S.R. and Murray, B.E. (2002) Enterococcal virulence In: The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance (Gilmore,

B.N., Ed.), pp. 301–354. American Society for Microbiology, Washington, DC.
[2] Giard, J.-C., Rince, A., Capiaux, H., Auffray, Y. and Hartke, A. (2000) Inactivation of the stress- and starvation-inducible *gls24* operon has a pleiotrophic effect on cell morphology, stress sensitivity, and gene expression in *Enterococcus faecalis*. J. Bacteriol. 182, 4512–4520.
[3] Teng, F., Nannini, E.C. and Murray, B.E. (2005) Importance of *gls24* in virulence and stress response of *Enterococcus faecalis* and use of the Gls24 protein as a possible immunotherapy target. J. Infect. Dis. 191, 472–480.
[4] Shankar, N., Baghdayan, A.S. and Gilmore, M.S. (2002) Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. Nature 417, 746–750.
[5] Benachour, A., Muller, C., Dabrowski-Coton, M., Le Breton, Y., Giard, J.-C., Rince, A., Auffray, Y. and Hartke, A. (2005) The *Enterococcus faecalis* sigV protein is an extracytoplasmic function sigma factor contributing to survival following heat, acid, and ethanol treatments. J. Bacteriol. 187, 1022–1035.
[6] Le Breton, Y., Boel, G., Benachour, A., Prevost, H., Auffray, Y. and Rince, A. (2003) Molecular characterization of *Enterococcus faecalis* two-component signal transduction pathways related to environmental stresses. Environ. Microbiol. 5, 329–337.
[7] Teng, F., Wang, L., Singh, K.V., Murray, B.E. and Weinstck, G.M. (2002) Involvement of PhoP–PhoS homologs in *Enterococcus faecalis* virulence. Infect. Immun. 70, 1991–1996.
[8] Qin, X., Singh, K.V., Weinstock, G.M. and Murray, B.E. (2000) Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infect. Immun. 68, 2579–2586.
[9] Verneuil, N., Sanguinetti, M., Le Breton, Y., Posteraro, B., Fadda, G., Auffray, Y., Hartke, A. and Giard, J-C. (2004) Effects of *Enterococcus faecalis hypR* gene encoding a new transcriptional regulator on oxidative stress response and intracellular survival within macrophages. Infect. Immun. 72, 4424–4431.
[10] Verneuil, N., Le Breton, Y., Hartke, A., Giard, J.-C. and Auffray, Y. (2004) Identification of a new oxidative stress transcriptional regulator in *Enterococcus faecalis*. Lait 84, 69–76.
[11] Flahaut, S., Laplace, J.-M., Frère, J. and Auffray, Y. (1998) The oxidative stress response in *Enterococcus faecalis*: relationship between H_2O_2 tolerance and H_2O_2 stress proteins. Lett. Appl. Microbiol. 26, 259–264.
[12] Paulsen, I.T., Banerjei, L., Myers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., Tettelin, H., Dodson, R.J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R.T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K.A., Dougherty, B.A. and Fraser, C.M. (2003) Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science 299, 2071–2074.
[13] Chen, L., Keramati, L. and Helmann, J.D. (1995) Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. Proc. Natl. Acad. Sci. USA 92, 8190–8198.
[14] Goethals, K., van Montagu, M. and Holsters, M. (1992) Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins. Proc. Natl. Acad. Sci. USA 89, 1646–1650.
[15] Schell, M.A. (1993) Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. 47, 597–626.
[16] Tartaglia, L.A., Gimeno, C.J., Storz, G. and Ames, B.N. (1992) Multidegenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. 267, 2038–2045.