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Expression of the Collagen Adhesin ace by *Enterococcus faecalis* Strain OG1RF is not Repressed by Ers but Requires the Ers box

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Abstract

Expression of adhesin to collagen of *Enterococcis faecalis* (*ace*), a known virulence factor, is increased by environmental signals such as the presence of serum, high temperature, and bile salts. Currently, the enterococcal regulator of survival (Ers) of *E. faecalis* strain JH2-2 is the only reported repressor of *ace*. Here, we show that for strain OG1RF, Ers is not involved in the regulation of *ace.* Our data showed similar levels of *ace* expression by OG1RF and its *Δers* derivative in the presence of bile salts, serum, and high temperature. Using *ace* promoter-*lacZ* fusions and site-directed mutagenesis, we confirmed these results and further showed that, while the previously designated Ers box is important for increased expression from the *ace* promoter of OG1RF, the region responsible for the increase is bigger than the Ers box. In summary, these results indicate that, in strain OG1RF, Ers is not a repressor of *ace* expression. Although JH2-2 and OG1RF differ by 6 nucleotides in the region upstream of *ace* as well as in production of Fsr and gelatinase, the reason(s) for the difference in *ace* expression between JH2-2 and OG1RF and for increased *ace* expression in bile, serum and at 46°C remain(s) to be determined.

Keywords

Enterococcus faecalis; *ace*; regulation; bile salts; virulence

INTRODUCTION

Enterococcus faecalis is a gram-positive commensal bacterium recognized as an important cause of nosocomial infections (Arias & Murray, 2012). At the first step of infection, bacteria adhere to host tissue and the family of bacterial surface proteins known as

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MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) appear to play an important role in this process (Patti *et al.*, 1994). The most studied MSCRAMM of *E. faecalis* is Ace (Adhesin to collagen of *E. faecalis*), which plays a major role in experimental *E. faecalis* infections and is presumed to mediate its attachment to host tissues via its interaction with collagen I and IV (Rich *et al.*, 1999). In different models such as the murine macrophage, urinary tract infection and experimental endocarditis, *ace* null mutants were clearly attenuated suggesting that Ace is an important virulence factor (Lebreton *et al.*, 2009; Singh *et al.*, 2010; Nallapareddy *et al.*, 2011). In addition, anti-recombinant Ace antibodies showed a protective effect against *E. faecalis* experimental endocarditis (Singh *et al.*, 2010).

It was recently shown that the presence of Ace on the cell surface of *E. faecalis* varies during the growth cycle in a strain-dependent way (Pinkston *et al.*, 2011). In *E. faecalis* strain OG1RF, Ace increases on the cell surface in early exponential phase and diminishes in stationary phase (Hall *et al.*, 2007; Pinkston *et al.*, 2011); the stationary phase decrease was shown to be dependent on a post-translational process mediated by gelatinase (GelE), which cleaves Ace from the cell surface (Nallapareddy *et al.*, 2000; Pinkston *et al.*, 2011). Strains that do not produce GelE, such as JH2-2, maintain Ace on their surface even in stationary phase (Pinkston *et al.*, 2011).

Expression of *ace* by *E. faecalis* is also well known to be increased by environmental stimuli such as the presence of serum, bile salts, urine, and at 46°C (Shepard & Gilmore, 2002; Nallapareddy & Murray, 2006; Lebreton *et al.*, 2009), but how *ace* is regulated is largely unknown. Lebreton and coworkers (Lebreton *et al.*, 2009) showed that, with *E. faecalis* strain JH2-2, the enterococcal regulator of survival (Ers) negatively regulates *ace* expression while it positively regulates other genes (Riboulet-Bisson *et al.*, 2008, 2009; Lebreton *et al.*, 2009). When bile salts were added, expression of *ers* by JH2-2 decreased and *ace* expression increased. However, *ers* expression was reported as not being changed by high temperature (46°C), nor the addition of collagen or horse serum, suggesting that other factors regulate *ace* expression under these conditions (Lebreton *et al.*, 2009).

To better understand the regulation of this important virulence factor, we studied *ace* expression in the well studied *E. faecalis* strain OG1RF (Bourgogne *et al.*, 2008) and in its *ers* deletion mutant (Δ*ers*) by northern and western blot analysis. In addition, we studied the importance of the putative Ers binding motif using *lacZ* fusion plasmids with deletions/substitutions in and adjacent to this motif.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions

The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) broth, Brain Heart Infusion (BHI) broth, M17 medium supplemented with 0.5% glucose (M17-G) or MM9YEG with 10 mM *p*-chloro-phenylalanine (p-Cl-Phe, Sigma-Aldrich Co.) (Kristich *et al.*, 2007). For some experiments, BHI was supplemented with 40% (v/v) horse serum (BHIS). To investigate *ace* expression with bile salts, strain OG1RF and its *Δers* mutant were grown in M17-G media. At the early exponential phase of growth (2.5 hr), bile salts (1:1 mixture of sodium cholate and sodium deoxycholate, Sigma-Aldrich Co.) were added to the final concentrations of 0.02, 0.04, 0.06, and 0.08% as indicated in Fig. 1A. The antibiotic concentrations used for bacterial selection were as follows: 200 μg/ml erythromycin, 50 μg/ml ampicillin, and 25 μg/ml gentamicin (all from Sigma-Aldrich Co.) for *Escherichia coli* and 10 μg/ml erythromycin and 125 μg/ml of gentamicin for *E. faecalis*.

Construction of *E. faecalis* mutants

A non-polar, unmarked, in-frame deletion of *ers* was created using pHOU1 as described previously (Panesso *et al.*, 2011). Briefly, pHOU1 containing the *ers* deletion construct (Supplementary Table for construction primers) was introduced into strain OG1RF by electroporation, and selected on BHI agar with 125 μg/ml gentamicin and 200 μg/ml X-Gal (Gold Biotechnology Inc.). Colonies containing the presumed first crossover recombination event were then streaked onto MM9YEG supplemented with 10 mM p-Cl-Phe and incubated at 37°C overnight to allow excision of pHOU1 from the *E. faecalis* chromosome. Resulting colonies were screened for loss of the plasmid (loss of both gentamicin resistance and β-galactosidase activity) and deletion of the target gene was confirmed by PCR using gene flanking primers (Supplementary Table). Mutants were confirmed by DNA sequencing of the deleted region and by pulsed field gel electrophoresis (PFGE) after SmaI digestion as reported previously (Murray *et al.*, 1993).

RNA analysis and identification of the transcription initiation site

Total RNA was isolated as follows: an overnight culture was re-inoculated into 20 ml BHI (or M17-G) to a starting OD_{600} of 0.05. At mid-exponential phase (OD_{600} = 0.5), 5 ml of culture was mixed with 10 ml RNAprotect reagent (Qiagen) and collected (3,900 rpm for 10 min) for RNA isolation. The pellet was resuspended in 1ml RNAwiz (Ambion) and disrupted by bead beating for 1 min using a Mini BeadBeater (BioSpec Products). RNA was extracted according to the protocol of RNAwiz and cleaned with the RNeasy Mini kit (Qiagen). Total RNA (10 μg/lane) was separated in a 1% agarose gel. 16S and 23S rRNA bands in the gel were stained by ethidium bromide and used as loading controls. Northern blot analysis was performed with internal probes for *ace*, *ers* and *sylA* genes. The transcriptional regulator gene, *sylA*, was used as a control for RNA quality and bile salts effect because its expression was previously reported to be increased in the presence of bile salts (Michaux *et al.*, 2011). Primers to amplify an internal probe of each gene are shown in Supplementary Table. RadPrime DNA labeling kit (Invitrogen) was used to labeling the probes with [α-^{32}P]-dCTP (PerkinElmer Inc.).

The 5'/3' RACE kit 2nd Generation (Roche) was used to map the transcriptional start site of the *ace* gene. RNA was extracted from strain OG1RF grown in BHIS at 37 °C to mid-exponential phase (OD_{600} = 0.5). cDNA of the *ace* gene was generated using primer AceSP1, followed by addition of a poly(A) tail by a terminal transferase. Nested primer AceSP2 and either ACEF1099R or AceSP3 primer were used for further PCR. cDNA generated was subcloned in pBluescript and sequenced by Genewiz Inc. (South Plainfield, NJ).

Construction of the *ace::lacZ* fusion plasmids and β-galactosidase assay

Six *lacZ* fusion plasmids were created containing the following variations of the *ace* promoter: P_{435}, P_{176}, $P_{\Delta CAAA}$, $P_{\Delta TTGTA}$, P_{GTC}, and P_{GGGC}; the fusions were constructed in pKAF7, a promoter-less *lacZ* plasmid (Fox *et al.*, 2009). The upstream promoter region of *ace* was amplified with primer pairs, AcePromF2b/AcePromR and AcePromF3b/AcePromR, and ligated into pKAF7 to make P_{435} and P_{176}, respectively. P_{435} includes 101 bp of the upstream gene EF1098, the 334 bp intergenic region, and 27 bp of the *ace* gene. P_{176} consists of 176 bp from the *ace* start codon up to the predicted Ers box previously described (Lebreton *et al.*, 2009) and 27 bp of the *ace* gene. Plasmids designated $P_{\Delta CAAA}$, $P_{\Delta TTGTA}$, P_{GTC}, and P_{GGGC} were created by site-directed mutagenesis using P_{435} as a template. Plasmids $P_{\Delta CAAA}$ and $P_{\Delta TTGTA}$ are deletion derivatives of the Ers box region whereas plasmids P_{GTC} and P_{GGGC} are replacement constructs (GTC replaces ACA and GGGC replaces AATG). Deletion/substitution constructs were created using the primer pairs DelErsF1/DelErsR, DelupErsF/DelupErsR, ErsGGCF/ErsGGCR and CovGGGCF/

CovGGGCR (Supplementary Table). Fusion constructs were electroporated into the parent strain OG1RF and its Δ*ers* mutant. Independent duplicate/triplicate cultures were assessed for β-galactosidase activity as described previously (Hammerstrom *et al.*, 2011).

Mutanolysin extraction and western blotting

Surface proteins of *E. faecalis* were prepared by mutanolysin treatment described previously (Nallapareddy *et al.*, 2000) and separated in 10% SDS-PAGE (10 μg/lane). Proteins transferred to a nitrocellulose membrane were incubated with anti-Ace monoclonal antibody 70 (Pinkston *et al.*, 2011) and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS AND DISCUSSION

Currently, Ers of *E. faecalis* JH2-2 is the only transcriptional regulator reported for regulation of *ace* expression. Lebreton*, et al.*, showed an interaction between Ers and the *ace* promoter using electrophoretic mobility shift assay and that purified recombinant Ers protein bound a 400 bp DNA fragment containing the *ace* promoter, but also required the presence of crude extract of the *Δers* strain (Lebreton*, et al.*, 2009). In addition, RT-qPCR data showed that out of the 8 genes of JH2-2 containing a putative Ers box in its promoter region, only *ace* expression was significantly (5–7 fold) increased in the *Δers* strain while the other genes were expressed similarly in both JH2-2 and its *Δers* mutant (Lebreton *et al.*, 2009). Therefore, Ers was inferred to be a repressor of *ace* expression (Lebreton *et al.*, 2009).

To further investigate how Ers is involved in *ace* expression, we generated an *ers* null (*Δers*) strain in *E. faecalis* OG1RF. Comparison of the *ers* ORF sequence of OG1RF and JH2-2 showed 100% identity between strains. The mutant strain, *Δers,* contains a non-polar in-frame deletion and lacks 534 bp (out of 654 bp) of the *ers* ORF (Supplementary Table for primer sequence). Comparison of growth of OG1RF and *Δers* in BHI, in BHI with serum, bile salts, and high temperature using BHI broth showed no significant differences between two strains (data not shown).

Effect of bile salts on *ace* and *ers* expression

The effect of bile salts on the expression of *ace* and *ers* was studied by northern blot analysis of OG1RF and its Δ*ers* mutant grown in M17-G supplemented with increased amounts of bile salts (0 - 0.08%). Addition of bile salts inhibited the growth of OG1RF (Fig. 1A) and Δ*ers* (data not shown) in the same concentration dependent manner. High concentrations of bile salts (0.06% and 0.08%) severely inhibited growth of both strains that had not recovered by 24 hrs (data not shown). Lower concentrations of bile salts (0.02 and 0.04%) initially inhibited growth but then growth resumed (Fig. 1A). The growth decrease of OG1RF in 0.02 and 0.04% is in agreement with the growth curve of JH2-2 in 0.08% bile salt condition previously reported (Michaux *et al.*, 2011).

Figure 1B shows the northern blot analysis of OG1RF and its *Δers* derivative grown in 0%, 0.02%, and 0.04% bile salts concentrations. Expression of *ers* in strain OG1RF was decreased by increasing the bile salts concentration; *Δers* did not show an *ers* signal, as expected. Expression of *ace* in both OG1RF and its *Δers* mutant was very low and similar to each other without bile salts (0%) or in 0.02% suggesting Ers is not a repressor in these conditions. In 0.04% bile salts concentration, *ace* expression increased approximately equally in both OG1RF and its *Δers* mutant, further indicating that Ers is not a repressor of *ace* in strain OG1RF. The increase in *ace* expression of OG1RF in 0.04% bile salts appears similar to the increase in *ace* expression by JH2-2 at the single concentration previously reported (0.08%) (Lebreton *et al.*, 2009); our results for OG1RF differ from those previously

published for JH2-2 showing 5–7 fold increase of *ace* expression of *Δers* strain compared to its parental strain (Lebreton *et al.*, 2009).

Effect of serum and high temperature on *ace* and *ers* expression

In addition to bile salts, the effect of Ers on *ace* expression was investigated under two other stress conditions previously reported to induce *ace* expression: growth in the presence of serum and growth at 46°C. Figure 2A shows northern blots of *ace* and *ers* transcripts from OG1RF and Δ*ers* grown in BHIS at 37 °C and in BHI broth at 46 °C. Strain *Δers* grown in BHIS showed similar *ace* levels to the parent strain OG1RF (Fig. 2A). Also, after growth at 46°C, *ace* expression of the Δ*ers* mutant was similar to that of OG1RF (Fig. 2A). Expression of *ace* was about 3-fold higher in both strains grown in BHI at 46°C compared to BHIS. However, expression of *ers* was similar for OG1RF grown in both stress conditions indicating again that Ers is not a repressor of *ace* expression. As previously reported (Nallapareddy & Murray, 2006), *ace* expression was increased in OG1RF grown in BHIS compared to BHI alone (Fig. 1B and Fig. 3). We also performed western blotting using OG1RF and its Δ*ers* mutant grown in BHIS with anti-Ace monoclonal antibody 70 (Pinkston *et al.*, 2011) (Fig. 2B). Addition of serum increased the presence of Ace on the cell surface but Ace amounts in OG1RF and *Δers* were similar when grown in BHIS condition. These observations were comparable to the changes in transcriptional activity shown in Figures 1 and 3.

Promoter region and requirement for an intact Ers box for increased expression of *ace*

To characterize the promoter structure of *ace,* we first determined the transcriptional start site of *ace* using *E. faecalis* OG1RF grown in BHIS. Using 5'/3' RACE kit, the transcriptional start site (+1) of *ace* was identified at 57 bp upstream of the start codon (data not shown). This site was previously identified as the transcriptional start site of *ace* in strain JH2-2 grown in the presence of bile salts (Lebreton *et al.*, 2009). Therefore, the transcriptional start site of *ace* appears to be the same regardless of different conditions (serum or bile salts) or clonal lineages (ST1 for OG1RF and ST8 for JH2-2, Ruiz-Garbajosa *et al.*, 2006).

Expression of *ace* after growth in BHIS was also investigated by β-galactosidase reporter gene assay (Fig. 3). We generated 6 different *ace* promoter fusions using the promoter-less *lacZ* plasmid pKAF7: P_{435}, P_{176}, $P_{\Delta CAAA}$, $P_{\Delta TTGTA}$, P_{GTC}, and P_{GGGC} (Fig. 3 and Supplementary Figure) and transformed them into *E. faecalis* OG1RF and into Δ*ers*. β-galactosidase activitity was first determined for OG1RF(P_{435}) grown in BHI and in BHIS; results showed that β-galactosidase activities increased about 4–5 fold when OG1RF(P_{435}) was grown in BHIS compared to BHI alone (Fig. 3). Production of β-galactosidase activity by Δ*ers*(P_{435}) in BHIS was similar to that in OG1RF(P_{435}) and consistent with the northern results in Fig. 2.

OG1RF(P_{176}) (contains 176 bp upstream of *ace* start codon but lacks the predicted Ers box) showed about half the β-galactosidase activity compared to OG1RF(P_{435}) suggesting that the upstream region containing the previously designated Ers box is important for *ace* induction. These results were corroborated using deletions derived from P_{435} ($P_{\Delta CAAA}$, and $P_{\Delta TTGTA}$) and substitutions (P_{GTC} and P_{GGGC}) within the predicted Ers box and its surrounding region (Fig. 3). Like OG1RF(P_{176}), the two deletion constructs, $P_{\Delta CAAA}$ and $P_{\Delta TTGTA}$, showed reduced β-galactosidase activities as did the substitution constructs, P_{GTC} and P_{GGGC} (Fig. 3). Among the constructs, OG1RF(P_{GGGC}) showed the lowest β-galactosidase activity in BHIS indicating that the region immediately downstream of the Ers box is also very important for the *ace* induction. Therefore, unknown transcriptional

regulator(s) other than Ers could bind to the putative Ers binding site and regulate *ace* expression by responding to environmental signals.

Although we showed that Ers in OG1RF is not involved in the regulation of *ace,* we also recognize that there is a difference in the *ace* upstream regions of JH2-2 and OG1RF, as shown in the Supplementary Figure, with 6 mismatches out of 334 nucleotides, including one mismatch located in the putative -35 box (Supplementary Figure). The 6 different nucleotides might play a role in the regulation of this important virulence factor. Another notable difference between JH2-2 and OG1RF is that the former is phenotypically a gelatinase (GelE) non-producer. Maintenance of Ace on the cell surface depends on the expression of GelE, which in turns depends on the expression of a complete Fsr system, a system that is present in OG1RF but largely absent in JH2-2. The influence of the loss of this global regulator (Bourgogne *et al.,* 2006) on the interaction of Ers and *ace* expression is unknown.

In summary, we showed that regulation of *ace* of *E. faecalis* OG1RF under stress conditions requires the putative Ers box, but not the transcriptional regulator Ers. Contrary to JH2-2, in OG1RF, the increase in *ace* expression in the presence of bile salts occurs independently of Ers. Future studies are needed to address these differences as well as the mechanism for the effect of stress conditions on *ace* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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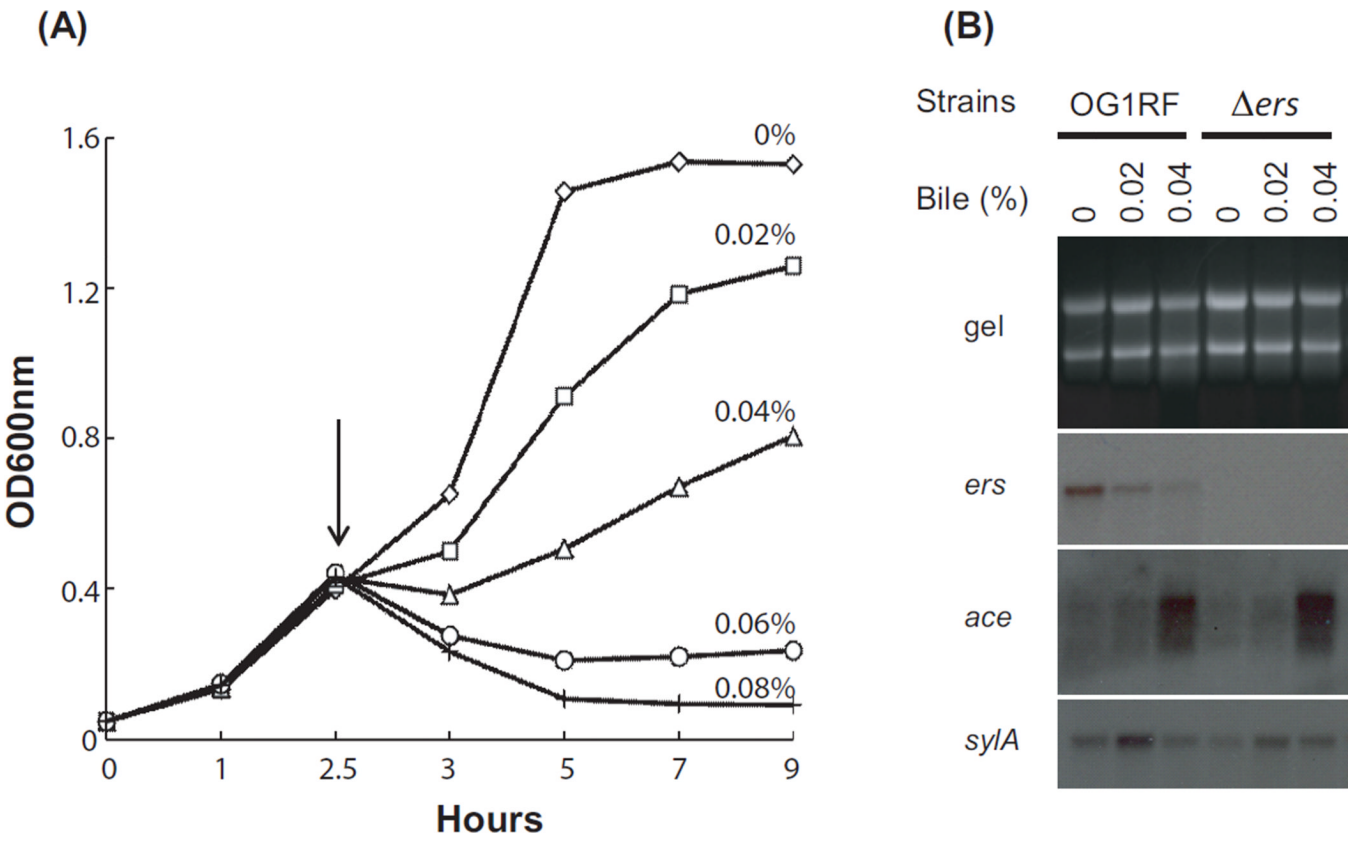

Figure 1.

Effect of bile salts on growth and gene expression of *E. faecalis*. (A) Growth curve of *E. faecalis* OG1RF grown in M17-G medium with and without bile salts and (B) northern blot analysis. Bile salts were added at 2.5 hr (indicated by arrow) and sampled at 3 hr (after 30 minutes incubation). Final concentrations of bile salts are indicated with different symbols: diamonds (0% bile salts), squares (0.02%), triangles (0.04%), circles (0.06%), and crosses (0.08%). Total RNA was separated in 1% agarose gel (gel), transferred, and hybridized with internal probes of *ers, ace*, and *sylA* genes.

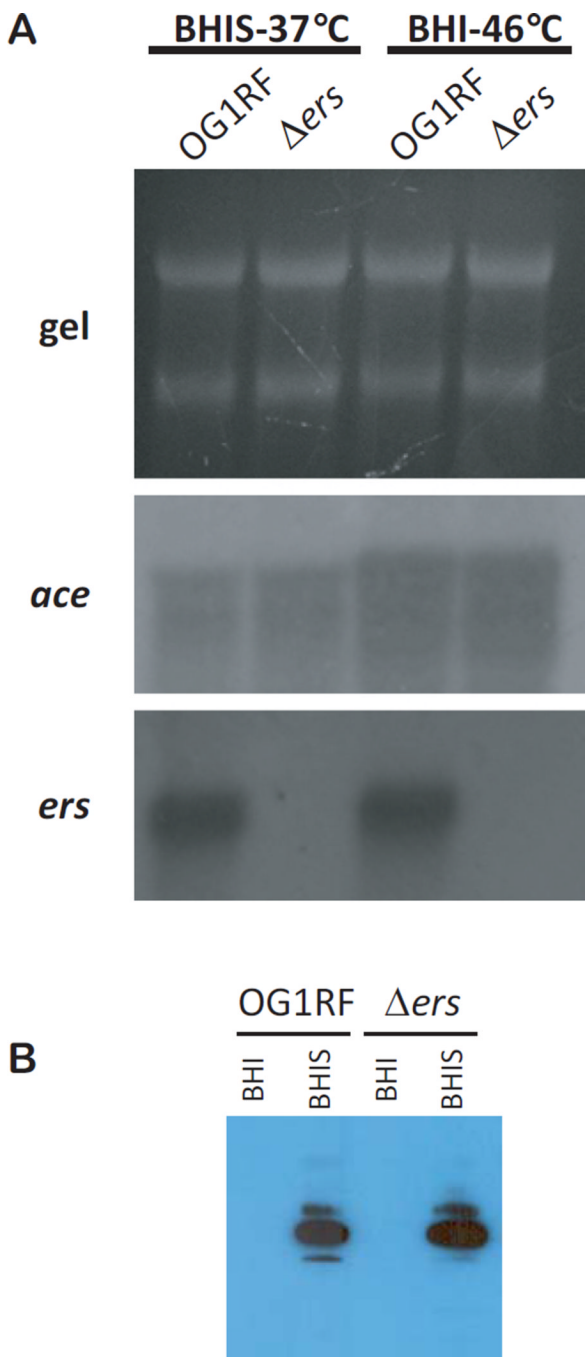

Figure 2.

Expression of *ace*/Ace by *E. faecalis* OG1RF and its *Δers* derivative. (A) Transcriptional changes by strains grown in BHI with serum at 37°C and in BHI at 46°C. Northern blot analysis was performed with total RNA (gel, 10 μg/lane) and hybridized with internal probes of *ace* and *ers*. (B) Surface expression of Ace after growth with serum. Western blot analysis was performed as described in the Materials and Methods.

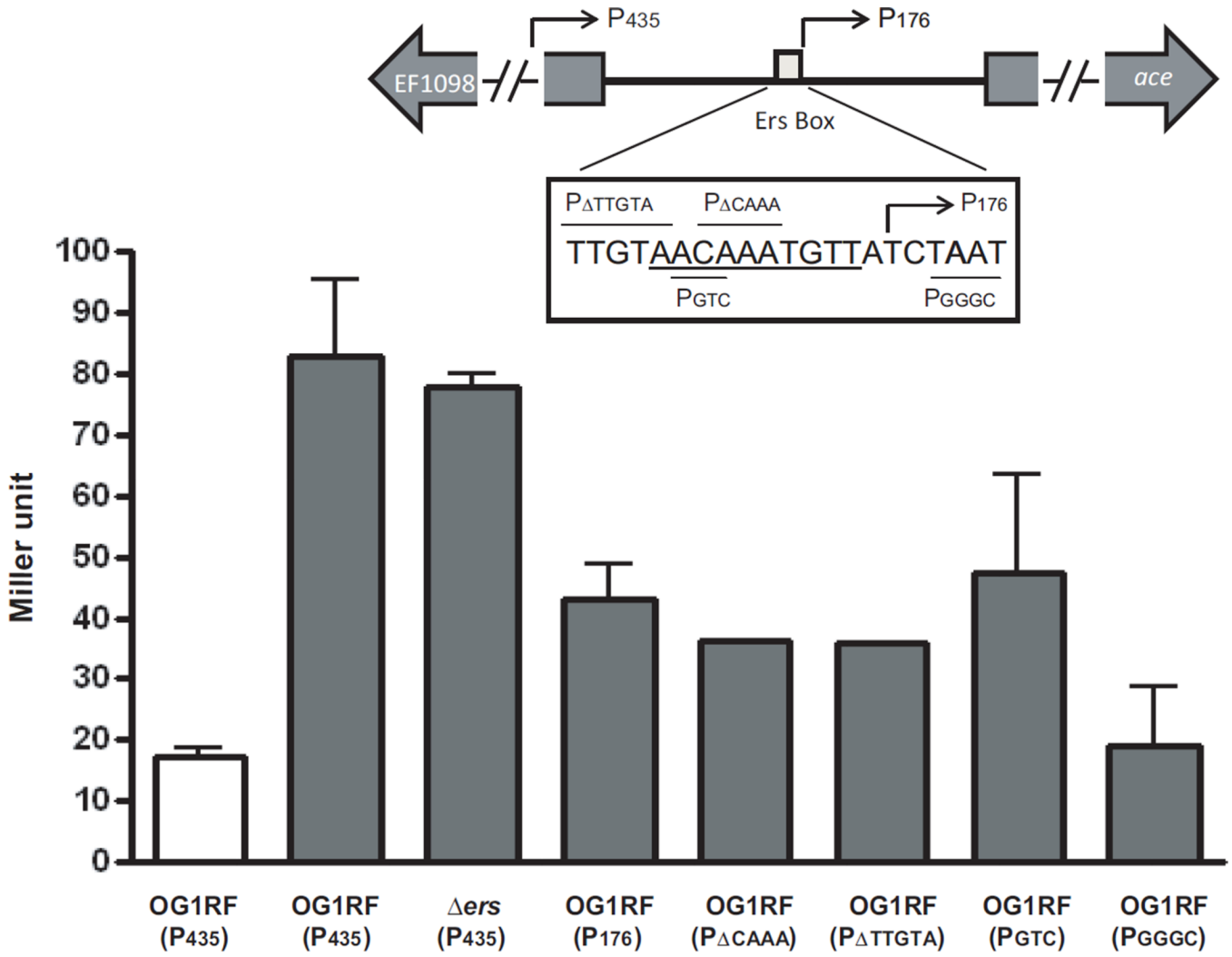

Figure 3.

Effect of changes around the putative Ers box on expression from the *ace* promoter. When grown in the presence of serum, β-galactosidase activities of P_{435} and its derivatives were determined. Sequence information of fusion plasmids is indicated in the box. The putative Ers binding site (AACAAATGTTA) is underlined. Deletion constructs are indicated above the sequence whereas substitution constructs are indicated below the sequence. P_{176}, indicated with the arrow, extends from the Ers box to 27 bp inside the *ace* gene. Bars with standard error indicate the means of the duplicate/triplicate assay using strains collected at early exponential phase. Strains were grown in BHI (white bar) or BHIS (gray bar).

Table 1

Bacteria strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source or reference
E. faecalis		
OG1RF	Fus^r, Rif^r	(Bourgogne *et al.*, 2008)
TX5696	OG1RF Δ*ers* (EF0074); non-polar, in-frame deletion of *ers*; Fus^r, Rif^r	This study
E. coli		
DH5α	*E. coli* host strain used for routine cloning	Stratagene
EC1000	*E. coli* host strain, provides RepA	(Leenhouts *et al.*, 1996)
Plasmids		
pBluescript	*E. coli* cloning vector; Ap^r	Agilent Technologies
pHOU1	Plasmid for mutagenesis; Gm^r	(Panesso *et al.*, 2011)
pKAF7	Reporter plasmid with *lacZ* from pCJK47; Em^r	(Fox *et al.*, 2009)
pTEX6075b	P_{435}; pKAF7 with 435 bp upstream of the *ace* start codon and 27 bp of the *ace* gene; Em^r	This study
pTEX6075c	P_{176}; pKAF7 with 176 bp upstream promoter region of the *ace* start codon and 27 bp of the *ace* gene; Em^r	This study
pTX6080f	$P_{\Delta CAAA}$; P_{435} with 4 bp CAAA deleted near the Ers box; Em^r	This study
pTX6080g	$P_{\Delta TTGTA}$; P_{435} with 5 bp TTGTA deleted from the Ers box; Em^r	This study
pTX6080h	P_{GTC}; P_{435} substitution of ACA in the Ers box with GTC; Em^r	This study
pTX6080i	P_{GGGC}; substitution of TAAT near the Ers box with GGGC, Em^r	This study

Ap, ampicillin; Em, erythromycin; Fus, fusidic acid; Gm, gentamicin; Rif, rifampicin.