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Enterococcal Rgg-Like Regulator ElrR Activates Expression of the *elrA* Operon

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The *Enterococcus faecalis* leucine-rich protein ElrA promotes virulence by stimulating bacterial persistence in macrophages and production of the interleukin-6 (IL-6) cytokine. The ElrA protein is encoded within an operon that is poorly expressed under laboratory conditions but induced *in vivo*. In this study, we identify *ef2687* (renamed *elrR*), which encodes a member of the Rgg (regulator gene for glucosyltransferase) family of putative regulatory proteins. Using quantitative reverse transcription-PCR, translational *lacZ* fusions, and electrophoretic mobility shift assays, we demonstrate that ElrR positively regulates expression of *elrA*. These results correlate with the attenuated virulence of the Δ*elrR* strain in a mouse peritonitis model. Virulence of simple and double *elrR* and *elrA* deletion mutants also suggests a remaining ElrR-independent expression of *elrA in vivo* and additional virulence-related genes controlled by ElrR.

Enterococcus faecalis is a commensal bacterium of the intestinal tract and an opportunistic pathogen responsible for a variety of community-acquired and health care-associated infections (1, 2). The dual lifestyle of *E. faecalis* relies on multiple factors, including adhesins, secreted proteases, surface polysaccharides, and transcriptional regulators (3–5). One of the *E. faecalis* genes reported to be involved in virulence, *elrA*, encodes a protein with an N-terminal domain containing leucine-rich repeats (LRRs) and a C-terminal WxL domain, which promotes noncovalent association with the bacterial cell surface (6, 7). LRR-containing proteins are ubiquitous and are frequently involved in protein-protein interactions in a variety of functions such as ligand-receptor interactions, signal transduction, and adhesion processes (8). ElrA shares structural features with the members of the internalin multigenic family of *Listeria monocytogenes* (9). We previously demonstrated that inactivation of the *elrA* gene significantly reduces *E. faecalis* virulence in a mouse peritonitis model and that ElrA contributes to bacterial persistence in macrophages and increases the host inflammatory response by stimulating the production of the cytokine interleukin-6 (IL-6) *in vivo* (6). We also reported that *elrA* is the first gene of an operon that is poorly transcribed under laboratory growth conditions and is induced *in vivo* in mice (6), suggesting tight environmental control of *elrA* expression.

Quorum sensing in low-G+C Gram-positive bacteria seems to rely on short peptides, which either interact with two-component systems or are internalized and interact with peptide-responsive transcription factors (10). The peptide-associated transcriptional regulators described to date belong to the RNPP superfamily, which includes the Rap (aspartyl phosphate phosphatase), NprR (neutral protease regulator), and PlcR (phospholipase C regulator) regulators of *Bacillus* species and PrgX of *E. faecalis*. All of these proteins contain tandem tetratricopeptide repeats (TPRs), which interact with cytoplasmic peptide signals (11, 12). Recently, based on predicted structural similarities, Rgg family transcription factors have been proposed as candidates for members of the RNPP superfamily (13, 14). Rgg stands for regulator gene for glucosyltransferase of *Streptococcus gordonii* (15), the first member of a large family of regulators exclusively found in the order *Lactobacillales* and the family *Listeriaceae* (14). Rgg regulators are characterized by an N-terminal XRE-type helix-turn-helix (HTH) DNA-binding domain and a C-terminal region rich in predicted alpha-helices, and they share structural similarity with PlcR and PrgX of the RNPP family (13, 16). In addition to participating in various physiological functions, such as the biosynthesis of extracellular polysaccharides, the stress response, and natural competence (13, 17–23), Rgg-type regulators control the expression of genes involved in virulence in *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus suis* (21, 24–28). Most Rgg-type regulators activate the transcription of adjacent genes (29–31), and the direct interaction between the activator and the corresponding promoter region has been demonstrated in several cases (14, 20, 24, 25, 30). However, some Rgg-type proteins in *S. pyogenes*, *S. agalactiae*, and *S. suis* are global regulators acting both as activators and as repressors (25, 26, 28, 32).

The present study reports the characterization of the first *E. faecalis* chromosomally encoded Rgg-like protein, named ElrR for *e*nterococcal *l*eucine-*r*ich protein *r*egulator, which is identified as a positive regulator of *elrA* expression and most likely controls additional virulence-related genes in *E. faecalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *E. faecalis* strain used in this study was OG1RF (33). *Escherichia coli* strains TG1 (34) and TG1 *repA* (35) were used for cloning and plasmid propagation. Plasmids used in this study are listed in Table 1. Enterococci were grown in M17 broth or agar supplemented with 0.5% glucose (M17G) at 37°C without aeration. *E. coli*

Received 31 January 2013 Accepted 29 April 2013
Published ahead of print 3 May 2013
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doi:10.1128/JB.00121-13

TABLE 1 Plasmids used in this study

Plasmid	Relevant characteristics[a,b]	Reference or source
p3TET	Tet^r (*tetM*), ori ColE1	36
pET2818	Amp^r, ori ColE1	37
pGhost9	Erm^r, ori pWV01, *repA*(Ts)	38
pJIM2242	Erm^r, ori pWV01	35
pIL253	Erm^r, ori pAMβ1	39
pMutin2	*rrnBt$_1$t$_2$*-λ*t$_0$* (Term)	40
pMutin4	*lacZ*	40
pUC18	Amp^r, ori ColE1	41
pVE3916	Cm^r, ori pWV01, *repA*	42
pVE14040	Cm^r, ori pWV01, *repA* with P$_{aphA3}$::*elrR*	This study
pVE14041	Amp^r, pUC18 with P$_{aphA3}$	This study
pVE14042	Amp^r, pUC18 with P$_{aphA3}$::*elrR*	This study
pVE14043	Amp^r Erm^r, pUC18/pIL253 with P$_{aphA3}$::*elrR*	This study
pVE14044	Erm^r, ori pWV01, *repA*(Ts) with Δ*elrR*	This study
pVE14045	Amp^r, ori ColE1with *elrR*::6×His	This study
pVE14147	Erm^r, ori pWV01 with *elrR*::6×His	This study
pVE14161	Tet^r, ori ColE1, ori pAMβ1	This study
pVE14162	Amp^r Erm^r, pUC18/pIL253 with P$_{aphA3}$::*elrR*::6×His	This study
pVE14165	Tet^r, ori pWV01, Term with *lacZ*	This study
pVE14167	Tet^r, ori pWV01, Term, P$_{elrA}$::*lacZ*	This study
pVE14173	Tet^r, ori pWV01, Term 0, Up$_{elrR}$-*elrR*-P$_{elrA}$::*lacZ*	This study
pVE14174	Tet^r, ori ColE1, ori pAMβ1, Term, *lacZ*	This study
pVE14181	Tet^r, ori ColE1, ori pWV01, Term, P$_{aphA3}$-*elrR*-P$_{elrA}$::*lacZ*	This study
pVE14182	Tet^r, ori ColE1, ori pAMβ1, Term, P$_{elrA}$::*lacZ*	This study
pVE14183	Tet^r, ori ColE1, ori pAMβ1, Term, P$_{aphA3}$-*elrR*-P$_{elrA}$::*lacZ*	This study
pVE14186	Tet^r, ori ColE1, ori pAMβ1, Term, Up$_{elrR}$-*elrR*-P$_{elrA}$::*lacZ*	This study
pVE14188	Tet^r, ori ColE1, ori pAMβ1, Term, P$_{aphA3}$-*elrR$_{stop}$*-P$_{elrA}$::*lacZ*	This study
pVE14189	Tet^r, ori ColE1, ori pAMβ1, Term, *lacZ*	This study
pVE14190	Tet^r, ori ColE1, ori pAMβ1, Term, P$_{elrA[-82\ to\ +3]}$::*lacZ*	This study
pVE14191	Tet^r, ori ColE1, ori pAMβ1, Term, P$_{elrA[-190\ to\ +3]}$::*lacZ*	This study
pVE14194	Tet^r, ori ColE1, ori pAMβ1, Term, P$_{elrA[-583\ to\ +3]}$::*lacZ*	This study
pVE14195	Tet^r, ori ColE1, ori pAMβ1, *lacZ*	This study
pVE14196	Tet^r, ori ColE1, ori pAMβ1, P$_{elrA[-82\ to\ +3]}$::*lacZ*	This study
pVE14197	Tet^r, ori ColE1, ori pAMβ1, P$_{elrA[-190\ to\ +3]}$::*lacZ*	This study
pVE14198	Tet^r, ori ColE1, ori pAMβ1, P$_{elrA[-583\ to\ +3]}$::*lacZ*	This study

[a] Term corresponds to *t1*, *t2*, and *t0* transcriptional terminators from pMutin2.
[b] Brackets indicate positions relative to the *elrA* start codon.

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)[a]
OEF212	CTCTTCTGCCGATGAAGTTTCTGG
OEF262	TTTCCAGAGGATCATTCGCCGTTCAATTTCTTCACC
OEF264	TCTCGAGGTATTGAACTCCTTCCAC
OEF273	AAGGATCCTGACGGAAGAAGATATTCGC
OEF274	TGGCATGCGACAAATTCTCAATTATGCC
OEF279	TCCCATGGAACAAAAAGAATATATGGAATATCGG
OEF280	TTAGGATCCGTTTTCTTTTATTTCTTTTAAAC
OEF281	CCTGCAGACAAGAAAACGGTGCTC
OEF324	CGAATGATCCTCTGGAAA
OEF333	TTCACCTAGCGGCCGATATTC
OEF339	GCAACGAAATGGTGGAACAG
OEF340	AAGGCATCGGCAATCTCTAAG
OEF353	FAM-AGGCATAATTGAGAATTTGTC
OEF354	FAM-GGAGGATGCGATTGTTTCG
OEF355	FAM-ATTAATTTGGACAGTTTGTCG
OEF501	TTGTCGACCATAAACTTCACCTCCTG
OEF502	AAGGATCCGGGAGGTTCTGGCAAAG
OEF504	AAGCATGCCGTTTCCAACTTCGTTAC
OEF594	FAM-CGAATGATCCTCTGGAAA
OEF651	FAM-GCCGCTAGGTGAAGAAATTG
OEF652	FAM-CGATAATCTGCAACGATTCG
OEF658	GATGCCACAAAAGGACTGTTTGA
OEF659	CAGGTTGGACGACTGTTCCA
OEF689	AAGCATGCGGCATAATTGAGAATTTGTC
OEF690	AAGCATGCTTAATTTGGACAGTTTGTCG
OEF694	AAGCATGCGAGTGTGTTGATAGTGC
OEF695	TTTTCTTAAGCTCCTTTCGCTTTCTTC
FR.lac5	ATGGGATAGGTCACGTTGGTGTAGAT

[a] Underlined nucleotides specify the restriction enzyme site used for cloning. FAM indicates coupling with 6-carboxyfluorescein.

strains were grown aerobically in Luria-Bertani broth or agar at 37°C. Antibiotics used were ampicillin (80 μg/ml), tetracycline (12.5 μg/ml for *E. coli* and 5 μg/ml for *E. faecalis*), and erythromycin (150 μg/ml for *E. coli* and 30 μg/ml for *E. faecalis*).

General DNA techniques. General molecular biology techniques were performed by standard methods (43). Restriction enzymes, T4 DNA ligase, and the Klenow DNA polymerase fragment were used according to the manufacturer's instructions. PCR amplification was performed using a Mastercycler apparatus (Eppendorf). Oligonucleotides used in this work are listed in Table 2. When necessary, PCR products and DNA restriction fragments were purified with QIAquick kits (Qiagen). Plasmids were purified using QIAprep Miniprep or Midiprep kits (Qiagen). Electrotransformation of *E. coli* and *E. faecalis* was carried out as described previously (7), using a Gene Pulser apparatus (Bio-Rad Laboratories). Plasmid inserts were sequenced by GATC Biotech.

Labeling *elrR* with a 6×His coding sequence tag and Western blotting. The chromosomal *elrR* gene was tagged by integrating the conditionally replicating plasmid pVE14147. A 1.28-kb EaeI-EcoRI fragment from pVE14045 (see below) was cloned in pJIM2242, generating pVE14147, which was introduced into strain OG1RFΔ*gelE* (44) by electroporation. An integrant of pVE14147 was obtained as previously described (45), generating a strain expressing chromosomal ElrR-6×His under the control of its own promoter. Another OG1RF strain expressing ElrR-6×His under the control of the constitutively expressed *E. faecalis* P$_{aphA3}$ promoter (46) on a replicating plasmid was used as a positive control. The ElrR-6×His-encoding sequence was fused to the P$_{aphA3}$ promoter. For this purpose, plasmids pVE14045 and pVE14043 (see below) were ligated at their BglI and EagI sites to generate pVE14162. Protein extracts and Western blotting were performed as previously described (7). ElrR-6×His was detected with a mouse monoclonal antipolyhistidine antibody (Zymed Laboratories) diluted 1:5,000. Bound primary antibodies were detected with horseradish peroxidase-coupled anti-mouse antibodies diluted 1:5,000 (GE Healthcare).

Deletion of *elrR* in OG1RF and in Δ*elrA* strains. Strain Δ*elrR* was constructed by deleting the *elrR* coding sequence in strain OG1RF by double-crossover recombination, using pVE14044, a pG$^+$host9-derivative plasmid (38). Two DNA fragments encompassing the 5′ and 3′ ends of the *elrR* gene were amplified by PCR from OG1RF chromosomal DNA with primer pairs OEF281-OEF262 and OEF324-OEF264, respectively. The two PCR products were fused by PCR using the external primers OEF281 and OEF264. The resulting product was digested with PstI and

XhoI and cloned in pG$^+$host9, yielding pVE14044, which was then introduced into OG1RF. A markerless in-frame *elrR* deletion mutant was selected as previously described (7), and the deletion of *elrR* was confirmed by sequencing of the chromosomal locus.

The Δ*elrR* Δ*elrA* strain, with a markerless in-frame Δ*elrR* Δ*elrA* deletion, was constructed by deleting the *elrR* coding sequence in the Δ*elrA* strain (6). For this purpose, an 853-bp AhdI-KpnI fragment from the pG$^+$host9 derivative plasmid that carried the Δ*elrA* construct (7) was ligated with a 4.88-kb AhdI-KpnI fragment of pVE14044, generating pVE14143, which carries the double deletion of *elrR* and *elrA*. Plasmid pVE14143 was introduced into the Δ*elrA* strain, and the Δ*elrR* Δ*elrA* deletion mutant was obtained as described above.

Construction of strain OG1RF overproducing ElrR. To overproduce ElrR, the corresponding gene was cloned on a plasmid under the control of the *E. faecalis* P_{aphA3} promoter. The entire *elrR* gene was amplified by PCR using primers OEF273 and OEF274, and the resulting product was digested with BamHI and SphI and cloned in pVE14041 (Table 1). The resulting plasmid, pVE14042, was fused to plasmid pIL253 at the EcoRI site, to obtain pVE14043, which was introduced into the OG1RF strain, yielding the *elrR*$^+$ strain.

Construction of *lacZ* reporter plasmids. Translational *lacZ* fusions were constructed using pVE14165, which carries a promoterless *lacZ* gene devoid of the first 15 codons and preceded by rho-independent transcription terminators (Term) (63) and a pWV01 replication origin and a *tetM* tetracycline resistance marker (36). The region encompassing the *elrA* promoter region from positions −583 to +3 with respect to the *elrA* start codon was recovered by PCR using oligonucleotides OEF501 and OEF504 as primers. The region from positions −1688 to +3 with respect to the *elrA* start codon, which includes the entire *elrR* gene plus the *elrA* promoter region and start codon, was amplified by PCR using primers OEF501 and OEF502. The resulting fragments were digested with SphI and SalI and ligated to the SphI and SalI sites of pVE14165 to obtain plasmids pVE14167 and pVE14173, which carried the P_{elrA}::*lacZ* and Up_{elrR}-*elrR*-P_{elrA}::*lacZ* translational fusions, respectively. To ensure the expression of the *elrR* gene in the latter plasmid construct, the EagI-EcoRI upstream region of the *elrR* gene in pVE14173 was replaced by the 0.5-kb EagI-EcoRI fragment from pVE14042, which contained the constitutive P_{aphA3} promoter (46). The resulting plasmid pVE14181 carried the translational fusion P_{aphA3}-*elrR*-P_{elrA}::*lacZ*.

To convert these plasmids into *E. faecalis* replicative plasmids, the pWV01 origin was replaced by a pAMβ1 replicon from pVE14161 (Table 1). The 4.67-kb SnaBI-BstEII fragment from pVE14161 was ligated to BstEII-SwaI fragments from pVE14167 (6.26 kb), pVE14173 (7.267 kb), and pVE14181 (7.56 kb) to yield pVE14182, pVE14186, and pVE14183, respectively. The plasmid pVE14186, which carries the fusion P_{aphA3}-$elrR_{stop}$-P_{elrA}::*lacZ*, in which the *elrR* open reading frame (ORF) was disrupted, was obtained by digesting pVE14183 with EagI and self-ligating after filling in the ends with the Klenow fragment.

The plasmids pVE14174, pVE14189, and pVE14195 were used as negative controls for the *lacZ* fusions. pVE14174 was constructed by ligating a 4.67-kb BstEII and SnaBI fragment of pVE14161 to a 5.7-kb BstEII and SwaI fragment of pVE14165. pVE14189 resulted from the replacement of the 875-bp SapI-NheI fragment of pVE14174 with the 551-bp EcoRI-NheI fragment of pVE14161 after filling in the SapI and EcoRI ends with the Klenow fragment. The 7.25-kb SphI-AflII fragment from pVE14189 was ligated to the *tetM* gene amplified by PCR using primers OEF694 and OEF695. The resulting plasmid pVE14195 lacks the transcriptional terminators and has the *tetM* and *lacZ* genes in opposite orientation.

Plasmids pVE14190 and pVE14196, pVE14191 and pVE14197, and pVE14194 and pVE14198 contained fragments of the *elrA* promoter region from positions −80, −190, and −583 bp relative to the *elrA* start codon fused to *lacZ*, respectively. They were generated by cloning PCR-amplified fragments using primer OEF690, OEF689, or OEF504 paired with OEF501 in pVE14189 and pVE14195, respectively.

Ectopic expression of ElrR was performed by cloning *elrR* on the pWV01-derivative plasmid pVE3916 (42). For this purpose, the 1.5-kb PvuII-SphI fragment from pVE14042 was ligated to pVE3916 to generate pVE14040.

RNA isolation, Northern blotting, quantitative reverse transcription-PCR (qRT-PCR), and transcription start site (TSS) mapping. Total RNA was extracted as described previously (47). Northern blotting assays were performed on 40 μg of total RNA separated on a 0.9% denaturing agarose gel as previously described (48). Specific oligonucleotides OEF212 and OEF333 were used to detect *elrA* and *elrR* RNAs, respectively. Oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB Biolabs) according to the recommendations of the manufacturer (NEB Biolabs). An RNA molecular weight ladder was from Ambion (Millenium RNA; Ambion).

Single-stranded cDNA libraries to analyze RNA samples via quantitative reverse transcription-PCR (qRT-PCR) amplification were prepared as described previously (47). The RNA and cDNA quality and concentration were assessed using a ND-100 spectrophotometer (Nanodrop) and the 2100 Bioanalyzer (Agilent). Primers for qRT-PCR were designed using Primer Express software (Applied Biosystems) and used to amplify PCR fragments of about 150 bp. *elrA* primers were OEF658 and OEF659, and *recA* primers were OEF339 and OEF340 (Table 2). PCRs were performed on an ABI Prism 7900 HT (Applied Biosystems) in triplicate with two amounts (1 ng or 0.2 ng) of cDNA in a 20-μl volume containing forward and reverse primers at 300 nM and Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's recommendations. Results were normalized by using the *recA* gene. The relative mRNA expression level of the *elrA* gene in each sample was calculated using the comparative cycle threshold (C_T) as described previously (49). Analysis was performed from cDNA synthesized from RNA extracted from three independent cultures.

Expression of the *elrA* gene was assessed *in vitro* using RNA extracted from liquid cultures of *E. faecalis* strains with an RNeasy minikit (Qiagen) including an RNase-free DNase treatment step and *in vivo* using RNA extracted from peritoneal cavity fluids of *E. faecalis*-infected mice (see below) with the RNeasy minikit, after the cells were suspended in RNAlater solution (Qiagen) as described previously (6). Real-time qRT-PCR was performed in an iCycler iQ system (Bio-Rad Laboratories), using *rpoB* as the normalization gene. The *elrA* primers and probe (OEF246, OEF247, and OEF248) and the *rpoB* primers and probe (OEF249, OEF250, and OEF251) were used in RT-PCRs as previously reported (6). The relative mRNA expression level of the *elrA* gene in each sample was tested in triplicate and calculated using the abovementioned comparative cycle threshold method. For all qRT-PCR analyses, statistical significance between the mean ratios of genes was evaluated by a two-sided paired *t* test by using GraphPad Prism version 4.03 for Windows (GraphPad Software). A P value of <0.05 was considered significant.

The *elrA* transcription start site was mapped in strains that carried *lacZ* fusions using the 5′-tag RACE (rapid amplification of cDNA ends) method (47). Specific amplification of *lacZ* transcripts from the tagged cDNA library was performed using the FR.lac5 and FR.DNA5 oligonucleotides (47). The authenticity of amplicons was confirmed by sequencing after cloning the PCR products in the pCRII-Topo vector (Invitrogen), and the transcription start site was deduced as the first nucleotide after the FR.DNA5 sequence.

β-Galactosidase assays. *E. faecalis* strains were grown as described for RNA isolation. Frozen pellets of cells harvested in mid-exponential phase (optical density at 600 nm [OD_{600}], ~1) were thawed and permeabilized by vigorous mixing in Z buffer (50) containing 1% (vol/vol) toluene. β-Galactosidase activity was assayed at 37°C and expressed in Miller units (MU), which were calculated as follows: $OD_{420} \times 1{,}000/(t \times v \times OD_{600})$, where OD_{420} is optical density at 420 nm of *o*-nitrophenol released, t is time of assay in minutes, v is volume of the cell culture in milliliters, and OD_{600} is optical density at 600 nm of the cell culture used (50).

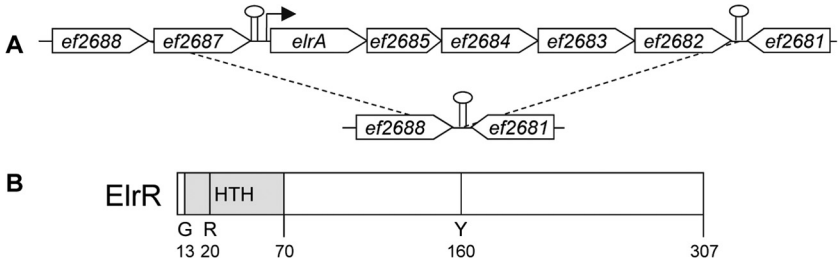

FIG 1 Gene organization and natural deletion of the *elrA* operon in *E. faecalis* isolates. (A) Schematic representation of the chromosomal region of the *elrA* operon. Putative rho-independent transcriptional terminators are indicated as lollipops. The promoter region of the *elrA* operon is represented by a black-headed arrow. (B) Schematic representation of the predicted ElrR product. HTH indicates the helix-turn-helix domain. G, R, and Y indicate glycine, arginine, and tyrosine residues, respectively. Numbers refer to the amino acid positions in ElrR.

Overexpression and purification of recombinant ElrR-6×His in *E. coli*. A DNA fragment containing the full-length *elrR* open reading frame was amplified by PCR from *E. faecalis* OG1RF genomic DNA using primers OEF279 and OEF280. The resulting PCR fragment was digested with NcoI and BamHI and inserted into the pET2818 vector to generate pVE14045, which encodes the ElrR-6×His tag. After verification by sequencing, pVE14045 was introduced into *E. coli* BL21(DE3). Protein expression was induced at an OD_{600} of 0.6 by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h at 37°C. Cells were harvested by centrifugation at 5,700 × *g* for 8 min at 4°C and resuspended in binding buffer (50 mM $NaHPO_4$, pH 8, 300 mM NaCl, 10 mM imidazole) containing 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], 10 μg ml^{-1} RNase A, and 5 μg ml^{-1} DNase I. The cells were broken using a cell disrupter system (Constant Systems Ltd.). Cell debris was removed by centrifugation at 3,000 × *g* for 15 min at 4°C, and the supernatant fluid was loaded on a Select affinity column (Sigma) equilibrated with binding buffer according to the supplier's instructions. The elution was carried out with binding buffer containing an imidazole gradient (20 to 250 mM). Eluted fractions were analyzed individually by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). ElrR-containing fractions were dialyzed overnight in a Slide-A-Lyzer (10-kDa cutoff; Pierce Perbio) against Tris-HCl, pH 7.5, and 1 mM dithiothreitol (DTT). Protein concentrations were determined by the Bradford assay (Bio-Rad), and purified ElrR was stored in 15% glycerol at −20°C.

Electrophoretic mobility shift assay (EMSA). 5′ fluorescent oligonucleotides labeled with 6-carboxyfluorescein (FAM) (Eurogentec) were used in PCRs to create labeled DNA fragments. The 188-bp, 296-bp, and 492-bp fragments contained the *elrA* promoter region from positions −83, −191, and −387 to +105 bp relative to the *elrA* start codon, respectively. They were generated from OG1RF DNA using the primer OEF355, OEF353, or OEF594 paired with primer OEF354, respectively, and purified using the PureLink PCR purification kit (Invitrogen). Nonspecific labeled DNA, which corresponds to a 288-bp internal fragment of the *elrR* coding sequence, was amplified with primers OEF651 and OEF652. Binding reactions were performed with purified protein and 5 to 3 ng of probe for 30 min at room temperature in binding buffer [5% glycerol, 40 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 0.3 mM bovine serum albumin, and 10 μg/ml poly(dI-dC)] in a final volume of 20 μl. Reaction mixtures were resolved at room temperature on a 12% polyacrylamide gel in Tris-buffered EDTA (TBE) buffer (Invitrogen) and visualized using a Typhoon fluorescence imager (GE Healthcare). Where specified, reactions were carried out in the presence of a more-than-100-fold excess of nonlabeled competitor DNA. Gel shift experiments were performed with two independent batches of purified ElrR.

Mouse peritonitis model of virulence. The mouse experiments were performed under a protocol approved by the Institutional Animal Use and Care Committee at the Università Cattolica del Sacro Cuore, Rome, Italy (permit number Z21, 1 November 2010), and authorized by the Italian Ministry of Health, according to the Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians of the Service for Animal Welfare.

Testing of the OG1RF, Δ*elrA*, Δ*elrR*, Δ*elrR* Δ*elrA*, and Δ*elrR*-complemented strains was performed as described previously (6). The inoculum size was confirmed by determining the number of CFU on brain heart infusion (BHI) agar. Each inoculum was 10-fold diluted in 25% sterile rat fecal extract prepared from a single batch as previously described (51). Groups of 4- to 6-week-old (22 to 25 g) outbred (ICR) female mice (Harlan Italy SRL) were challenged intraperitoneally with 1 ml of each bacterial inoculum, housed five per cage, and fed *ad libitum*. A control group of mice was injected with 25% sterile rat fecal extract only. Survival was monitored every 3 to 6 h. Survival estimates were constructed by the Kaplan-Meier method and compared by log rank analysis. In another set of experiments, groups of mice were killed 20 h postinfection, and fluids recovered from the animal peritoneal cavities were serially diluted in saline solution for RT-PCR analyses as described above. All statistical analyses were performed using the aforementioned GraphPad Prism software, and comparisons with *P* values of <0.05 were considered to be significant.

RESULTS AND DISCUSSION

***elrR*, an Rgg-like regulator-encoding gene upstream of the *elrA* operon.** PCR screening of our laboratory collection of enterococcal isolates using an array of primers nested and outside the *elrA* operon had revealed several strains that lacked the entire operon (from *ef2686* to *ef2682*) (6). In those strains, only primers located upstream of the *ef2687* gene and downstream of the *ef2682* gene yielded a PCR product corresponding to the junction between *ef2688* and *ef2681* (Fig. 1A). Despite the predicted presence of a rho-independent transcription terminator (according to the programs TransTerm [52] and ARNold finding terminators [53]) between them, the systematic association of the *ef2687* gene and the *elrA* operon (88% of the strains tested) suggested that these genes are functionally related. The EF2687 product, here referred to as ElrR for *e*nterococcal *l*eucine-*r*ich protein *r*egulator, is annotated as a conserved domain protein in the V583 genome at the J. Craig Venter Institute website (http://cmr.jcvi.org/tigr-scripts/CMR/shared/GenePage.cgi?locus=EF2687) and is predicted to be a 307-residue polypeptide, of which the first 230 amino acids share 36% identity with those of *S. gordonii* Rgg. *In silico* analysis using NCBI PSI-BLAST revealed a significant similarity of the first 174 amino acids with the N-terminal putative DNA-binding helix-turn-helix motif of the Rgg-like regulators. Moreover, ElrR contains the invariant glycine (G13), the arginine (R20), and the aromatic residue tyrosine (Y160) corresponding to G4, R11, and W149 of *S. pyogenes* RopB (16) (Fig. 1B). The putative presence of a DNA-binding domain in ElrR was supported by other predictions using Protein Fold Recognition servers PHYRE2 (54) and

COMPASS (55) on the GeneSilico structure prediction Metaserver, which identified PrgX of *E. faecalis* as the best match to ElrR over the first 220 amino acid residues. Moreover, during the course of this work ElrR was reported to be a member of the Rgg-like proteins (14). Interestingly, the *E. faecalis* V583 genome has 5 chromosomally encoded (EF0073, EF1224, EF1316, EF1599, and ElrR) and two plasmid-encoded (EFA0004 and EFB0005) Rgg-like proteins. Among them, ElrR is phylogenetically related to EF0073, EFA0004, and EFB0005 (14), supporting the hypothesis that ElrR is an Rgg family member. Structure prediction algorithms have led to the proposal that Rgg proteins are members of the RNPP family (11, 14, 18). The carboxy-terminal regions of RNPP family proteins have predicted folds consistent with TPRs which mediate protein-protein or protein-peptide interactions (12, 56). As observed for PrgX, PlcR, and Rgg proteins, no tetratricopeptide repeats (TPRs) could be predicted from the primary sequence of ElrR; however, the secondary structure of ElrR predicted by PSIPRED (57) reveals alpha-helices as reported for other Rgg-like proteins that align with the TPR helices of PlcR and PrgX (13). Both PrgX and PlcR regulate the transcription of their target genes in response to a signal peptide. Peptide binding to PlcR enhances DNA binding through drastic conformational and oligomerization changes promoting transcription activation of cognate target genes (58, 59). In contrast, PrgX responds to two competing signaling peptides and acts as a transcriptional repressor. The change of PrgX from the tetrameric to the dimeric state allows access to the promoter region of the PrgX-regulated gene (58, 60). With the exception of RopB, which binds an N-terminal secretion signal sequence of a distantly encoded lipoprotein (61), the Rgg signaling molecules characterized so far are short peptides encoded by small adjacent genes of the peptide-associated Rgg subfamilies (14, 18, 62). In the case of *elrR*, no short coding sequence was detected upstream or downstream, as for the majority of predicted Rggs, suggesting that if any signaling molecule is involved, it is encoded at another locus or imported from the extracellular medium. All these characteristics suggest that ElrR belongs to the RNPP family and could act as a transcriptional regulator.

ElrR expression under laboratory conditions. To get some insight into *elrR* expression in *E. faecalis*, we attempted to characterize the *elrR* mRNA by Northern blotting of RNA extracted from bacteria grown under standard laboratory conditions. *elrR* mRNA could not be detected when expressed from its chromosomal locus (data not shown). To rule out the possibility that the *elrR* transcript might be particularly unstable, we inserted a 6×His tag coding sequence at the 5′ end of the *elrR* gene and searched for ElrR-6×His by Western blotting using anti-His tag antibodies (Fig. 2). We also failed to detect the ElrR-6×His protein expressed from the chromosomal locus. In contrast, ElrR-6×His was detected under the same growth conditions in a strain that carries pVE14162, a plasmid which contains ElrR-6×His under the control of the constitutively expressed *E. faecalis* P_{aphA3} promoter (46). These observations indicated that ElrR expression is extremely low under *in vitro* conditions. They also validated the use of P_{aphA3} to overcome the lack of ElrR expression under these conditions.

ElrR activates *elrA* expression. To determine whether ElrR has any impact on *elrA* expression, an in-frame deletion mutant of *elrR* (Δ*elrR*) and a strain that expresses *elrR* in *trans* under the control of P_{aphA3} (*elrR*$^+$) were constructed in *E. faecalis* strain OG1RF. Levels of *elrA* transcripts were first analyzed by Northern blotting hybridization on total RNA prepared from the wild-type (wt), Δ*elrR*, and *elrR*$^+$ strains. No *elrA* transcript was detected in any of the strains analyzed (data not shown). Since *elrA* was previously shown to be induced *in vivo*, it is likely that the expression level of *elrA* transcript is too low to be detected by Northern blotting under laboratory growth conditions. We then compared the levels of *elrA* transcript in the wt, Δ*elrR*, and *elrR*$^+$ strains using quantitative RT-PCR. The *recA* transcript tested as an internal control gave C_T values from 21.2 to 24.2; high C_T values from 29.4 to 34.4 were systematically obtained with *elrA*-specific primers, confirming a very low level of *elrA* transcript. The specific amplification of *elrA* cDNA was ascertained by the lack of amplification using cDNA extracted from the Δ*elrA* strain. The transcript level of *elrA* was increased 2.31- ± 0.39-fold ($P < 0.000001$) in the *elrR*$^+$ strain, indicating that the constitutive expression of *elrR* increases the level of *elrA* transcripts. In contrast, no significant difference in the levels of *elrA* transcript was noticed between the Δ*elrR* and wt strains, further supporting the idea that *elrR* is not expressed at significant levels during growth under the tested conditions.

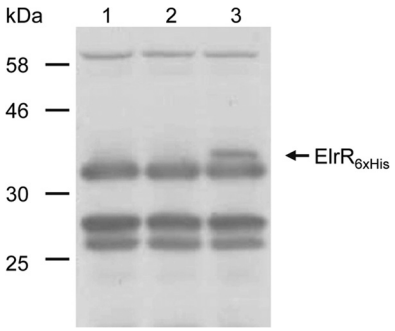

FIG 2 Western immunoblot analysis of ElrR-6×His under laboratory growth conditions from OG1RF derivative strains. Cytoplasmic and cell wall proteins from strains expressing chromosomal ElrR (lane 1), chromosomal ElrR-6×His (lane 2), and ElrR-6×His under the control of P_{aphA3} on a plasmid (lane 3) were extracted. Equivalent amounts of proteins were analyzed using an antipolyhistidine polyclonal rabbit antibody. The band corresponding to ElrR-6×His is indicated by an arrow. Other bands are nonspecific and correspond to cross-reacting signal.

To get further insights into the role of ElrR in *elrA* expression, we created an array of translational *elrA*::*lacZ* fusions (Fig. 3). We used a multicopy plasmid to compare the levels of expression of the P_{elrA}::*lacZ* and the promoterless *lacZ* fusions, carried by pVE14182 and pVE14174, respectively. Although the β-galactosidase activity of the P_{elrA}::*lacZ* fusion was low (1.29 ± 0.24 Miller units), it was still more than 8-fold higher than the basal level detected for the control strain carrying the promoterless *lacZ* fusion, i.e., the background level of the *lacZ* reporter system used, 0.15 ± 0.04 Miller units. This result shows the functionality of the P_{elrA}, enabling us to further investigate the role of *elrR* in P_{elrA}::*lacZ* activity. β-Galactosidase activities of the other constructs were compared and normalized with that of P_{elrA}::*lacZ* (Fig. 3). A comparable activity ratio was detected in the strain that harbored the *elrR* gene and 483 bp of upstream DNA, suggesting that *elrR* may not be expressed (Up_{elrR}-*elrR*-P_{elrA}::*lacZ*). In contrast, when *elrR* was expressed under the control of the constitutive promoter P_{aphA3}, the β-galactosidase ratio was increased more than 10-fold, indicating that the P_{aphA3}-driven expression of *elrR* stimulated P_{elrA}::*lacZ* expression. Due to the potential read-through of the

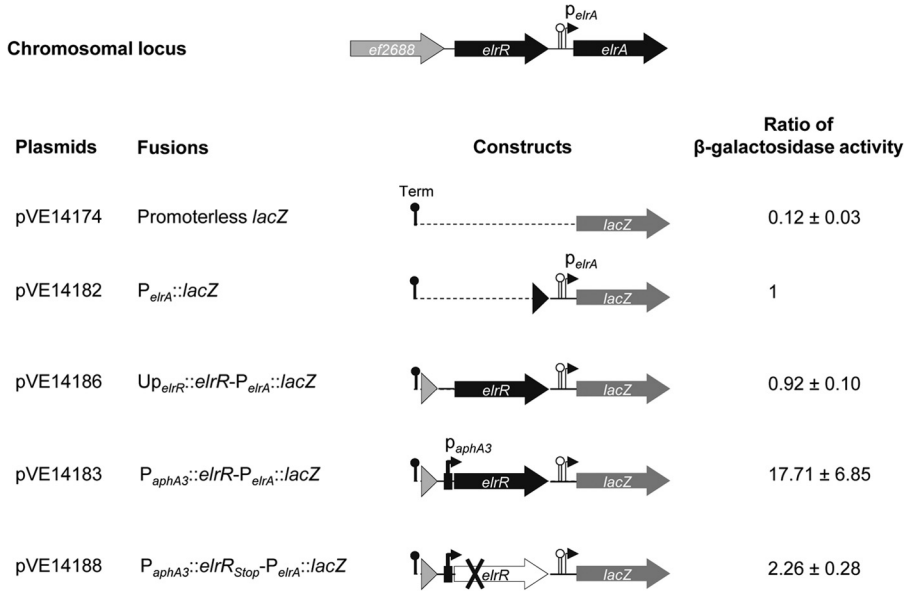

FIG 3 Schematic representation of *lacZ* translational fusions and their relative β-galactosidase activities. At top is shown the genomic organization of the *elrR-elrA* region with the P_{elrA} promoter, and the potential rho-independent transcription terminator is indicated by a lollipop. DNA fragments encompassing the *elrA* upstream region were fused to the *lacZ* coding sequence. Transcription from *tetM* of the vector pVE14174 was arrested by rho-independent transcription terminators, Term (black lollipop). Ratios of β-galactosidase activities were normalized to the activity measured for P_{elrA}::*lacZ* (1.29 ± 0.24 Miller units). Data provided are the averages of five independent experiments.

elrR transcription terminator from P_{aphA3}-driven expression and to demonstrate the activator effect of ElrR on the expression of P_{elrA}::*lacZ*, a translational stop codon was introduced into the ElrR-encoding sequence, leading to the plasmid construct pVE14188. The β-galactosidase ratio of this new construct (P_{aphA3}::$elrR_{stop}$-P_{elrA}::*lacZ*) was only 2-fold higher than that with P_{elrA}::*lacZ*, demonstrating that most of the activity of the P_{aphA3}::$elrR_{stop}$-P_{elrA}::*lacZ* fusion is due to the activator effect of ElrR on P_{elrA}::*lacZ* (~10-fold) and that the possible transcriptional read-through at the *elrR* terminator has a minor effect on the P_{elrA}::*lacZ* expression (~2-fold). To confirm *elrA* transactivation by ElrR, we expressed the *elrR* gene in *trans* under the control of the P_{aphA3} promoter on plasmid pVE14040, which is compatible with the P_{elrA}::*lacZ* plasmid. The β-galactosidase activity of the strain that expresses both *elrR* and P_{elrA}::*lacZ* on compatible plasmids (8.8 ± 1.2 MU) was ~14-fold higher than that of the control strain, which carries pVE3916 and P_{elrA}::*lacZ* (0.6 ± 0.1 MU). These data further demonstrate that ElrR activates *elrA* expression in *trans*.

Rgg family regulators positively regulate their own expression, and their activity is modulated by short hydrophobic peptides in *S. mutans*, *S. thermophilus*, and *S. pyogenes* (13, 14, 62–65). Recently, Shelburne et al. identified a lipoprotein-derived inhibitory peptide that interacts with RopB of *S. pyogenes* and therefore could modulate RopB-dependent expression of *speB* (61). Our data indicate that the 483-bp DNA region upstream of *elrR* is not sufficient either to express *elrR* or to reach a detectable effect on *elrA* expression under laboratory conditions. However, we show that the expression of *elrR* from a strong surrogate promoter activates *elrA* expression regardless of the need for a signaling molecule. Based on the structural predictions and despite the absence of reliable prediction of a short coding sequence adjacent to *elrR*, we cannot exclude the possibility that ElrR regulatory activity relies on a signaling molecule that may not be expressed or available under laboratory conditions.

ElrR specifically binds to the *elrA* promoter region. The suggestion that ElrR is an Rgg family member, i.e., the presence of the HTH DNA-binding motif and the positive effect of the ElrR protein on the $P_{elrA::lacZ}$ fusion (see above), prompted us to assay the direct binding of ElrR to the *elrA* promoter region by electrophoretic mobility shift assays (EMSAs). The assays were performed using purified ElrR and a 492-bp DNA fragment (probe S) encompassing the *elrA* promoter region from positions −387 to +105 bp relative to the start codon of *elrA* (6) or a 288-bp DNA fragment internal to the ElrR coding sequence as a negative control (Fig. 4). ElrR provoked a mobility shift of the DNA fragment containing the *elrA* promoter region in a protein concentration-dependent manner (Fig. 4, left panel, lanes 2 to 4). No band shift was observed with the control DNA fragment (NS in Fig. 4). The ElrR binding specificity was further confirmed by showing that addition of unlabeled competitor DNA reduced the amount of shifting of the fluorescently labeled S probe DNA (Fig. 4, left panel, lane 6), whereas an excess of the nonspecific DNA fragment had no effect on ElrR binding to the *elrA* promoter region (Fig. 4, left panel, lane 8). This result shows that ElrR binds specifically to the *elrA* promoter and suggests that the positive effect of ElrR on *elrA* expression is due to direct interaction with the *elrA* promoter region.

To narrow down the *elrA* promoter region recognized by ElrR, an EMSA was performed with four additional DNA probes, which covered the *elrA* promoter region from positions −387 to +105 bp relative to the start codon of *elrA* (Fig. 4, right panel). A shift was observed with probes that encompassed the region from positions −387 to −56 bp (probe 1) and from −191 to +105 bp (probe 3). It is noteworthy, however, that the retardation efficacy was decreased compared to that for probe S. Given the lack of mobility shift for probes corresponding to positions −387 to −144 bp (probe 2) and −83 to +105 bp (probe 4), the shift of probes 1 and 3 points toward the 136-bp region from positions −191 to −56 bp, which includes an inverted repeat DNA se-

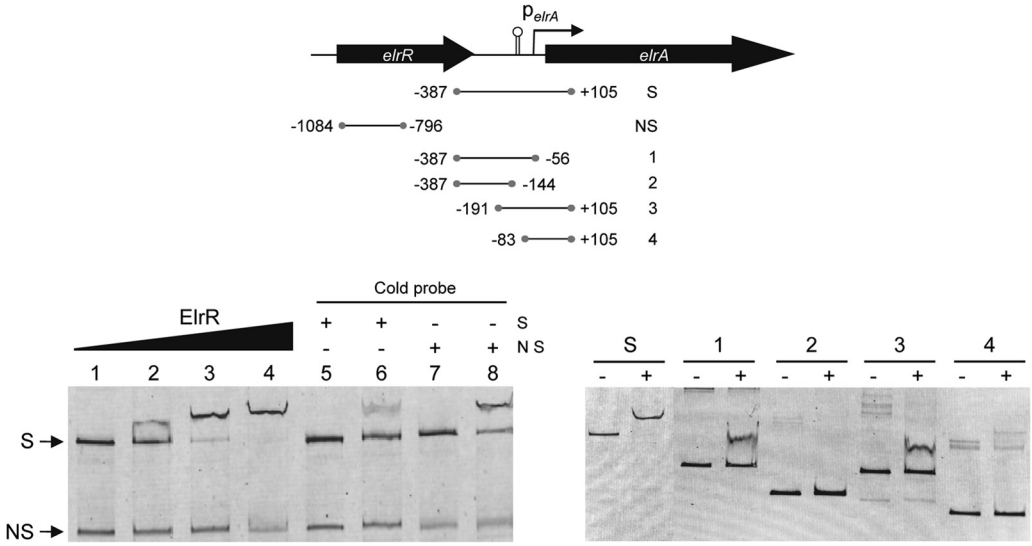

FIG 4 ElrR specifically binds the *elrA* promoter region. (Top) Map of the intergenic region upstream of the *E. faecalis elrA* operon, with the ORFs *elrR* and *elrA*. The transcription orientation of *elrA* and *elrR* is indicated by thick arrows, and the thin arrow indicates the promoter of the *elrA* operon (P_{elrA}). The lollipop indicates a 50-bp inverted repeat at the DNA level and the predicted transcription terminator. Lines below represent DNA probes used for EMSAs; numbers correspond to the location of the extremities relative to the start codon of the *elrA* gene. (Bottom) EMSA of *elrA* promoter region with ElrR. In the left panel, 3 ng of a 0.5-kb fluorescently labeled DNA fragment encompassing the upstream region of P_{elrA} (S for "specific") and 5 ng of a fluorescently labeled DNA control fragment (NS for "nonspecific"), corresponding to a 0.3-kb internal fragment of the *elrR* gene, were incubated with 0, 1, 1.5, and 2 μM purified ElrR (lanes 1 to 4, respectively). An excess of cold S (lanes 5 and 6) and NS (lanes 7 and 8) DNA probes was added to the binding reaction mixtures in the absence (lanes 5 and 7) or presence (lanes 6 and 8) of 1.5 μM ElrR. In the right panel, EMSA of DNA probes encompassing nested fragments of the P_{elrA} region that were incubated without (−) or with (+) 1.5 μM ElrR. S and 1 to 4 correspond to the DNA probes shown on the map above.

quence predicted to be the transcription terminator of *elrR*, as the region required for binding. However, attempts to observe a mobility shift for the postulated minimal ElrR binding region failed (data not shown), indicating that ElrR binding *in vitro* requires sequences flanking both sides of the *elrA* promoter region from positions −191 to −56 bp or a specific DNA conformation. This observation agrees with the apparent decreased binding efficiency of ElrR on probes 1 and 3 compared to that on the extended probe S (Fig. 4), suggesting that the tightness of ElrR binding is influenced by the position of the binding site within the probe. As is the case for other Rgg-like regulators (14, 20, 24–26, 30), our data demonstrate that ElrR does not require the presence of a signaling molecule to bind DNA, and yet further experiments are needed to determine if ElrR binding in the absence of any signal is sufficient to promote transcription.

To correlate ElrR binding and transcriptional activity, we constructed additional *lacZ* translational fusions, which contained 583, 190, or 82 bp upstream of the *elrA* start codon (Fig. 5). The resulting plasmids were introduced into a strain expressing *elrR* in *trans* from pVE14040 or harboring the empty plasmid pVE3916, used as a negative control. The strains carrying fusions from pVE14194, pVE14191, and pVE14190 that include different elements of the *elrA* promoter region displayed similar levels of β-galactosidase activity in response to ElrR expression compared to the control strains (Fig. 5, top). Since we hypothesized that a terminator-like structure (i.e., inverted repeat sequence on DNA) could be recognized by ElrR, we cannot rule out the possibility that the terminator ("Term" in Fig. 5) used to silence transcription from the *tetM* promoter upstream of the *lacZ* fusions may be targeted by ElrR. We therefore constructed a second vector carrying *lacZ* fusions where "Term" was removed and the *tetM* cassette was inverted (Fig. 5, bottom). In the two sets of vectors, the *elrA*::*lacZ* fusions responded specifically to the presence of ElrR and with the same magnitude, demonstrating that the 82 bp upstream of the *elrA* ORF are specifically targeted by ElrR *in vivo* and indicating that ElrR-dependent activation of *elrA* occurs at the previously mapped proximal promoter of *elrA* (6). Transcription start site (TSS) mapping was performed using the 5′-tag RACE method (47) on P_{elrA}::*lacZ* transcripts expressed from fusions containing the *elrA* promoter region and ElrR provided in *cis* (pVE14183) or in *trans* (pVE14194). Regardless of how ElrR was provided, all TSSs mapped for *elrA*::*lacZ* transcripts were located at 21 nucleotides upstream of the translation initiation codon of ElrA corresponding to t1, the *elrA* proximal promoter previously mapped (6). An identical TSS was mapped in strains expressing the P_{elrA}::*lacZ* fusion where ElrR was either inactivated by a stop codon (pVE14188, *cis* construct) or absent from the vector (pVE3916) when provided in *trans*. We previously mapped a second *elrA* transcription start site (t2) within the 50-bp inverted repeat at position −104 relative to the start codon of *elrA* (6). The absence of detectable t2 in these experiments further supports the idea that the long inverted repeat is not required for ElrR-dependent activation of *elrA* (Fig. 5). Thus, our data show that ElrR acts on the *elrA* proximal promoter (Fig. 6). The two predicted boxes for this promoter, −35 (TTGAAC) and −10 (TATAAT), are separated by 21 bp, a fairly long element (66). Interestingly, regulators of the MerR family bind between the −35 and −10 boxes separated by long spacers and induce DNA distortion, activating transcription (67, 68). It is conceivable that ElrR may act in a similar manner. Although we cannot rule out the possibility that the N-terminal histidine tag modifies ElrR binding capacities, the apparent discrepancy between the *in vivo* ElrR-dependent activation of *elrA*

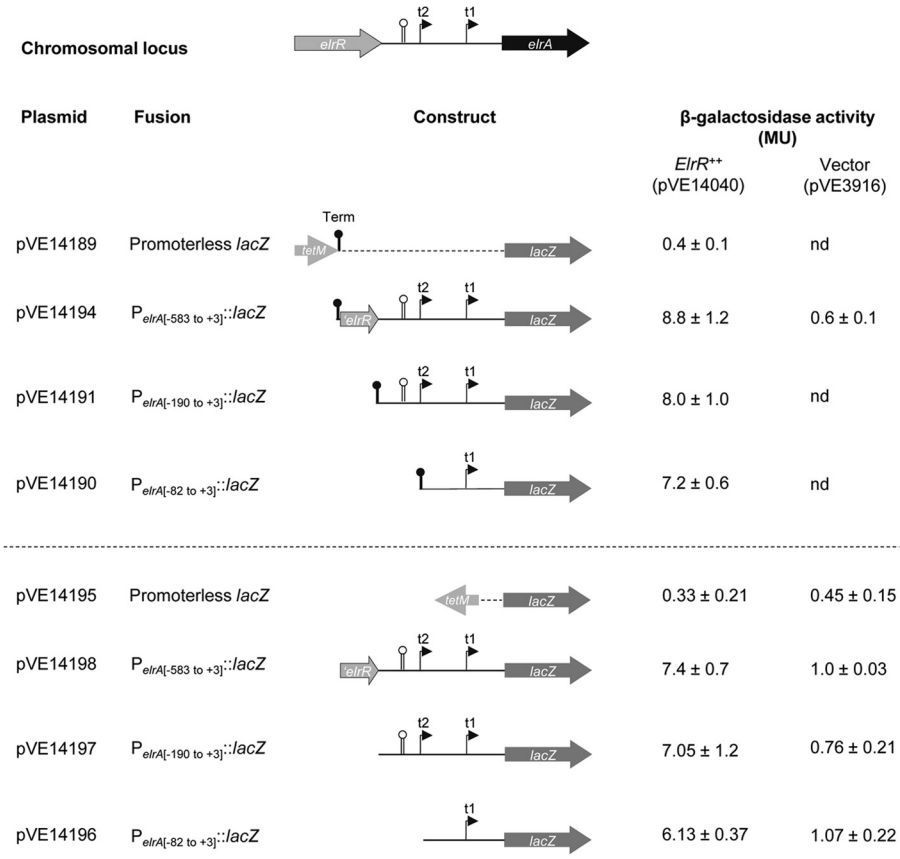

FIG 5 Schematic representation of the *lacZ* translational fusions and their corresponding β-galactosidase activities with *elrR* provided in *trans*. At the top, the *elrA* promoter region is schematized with the two transcription start sites t1 and t2 and the 50-bp inverted repeat indicated by the lollipop as defined previously (6). DNA fragments encompassing *elrA* promoter regions were fused to the *lacZ* coding sequence in two vectors. Transcription from *tetM* of vector pVE14189 was stopped by rho-independent transcription terminators, Term (black lollipop). *tetM* of vector pVE14195 was reversed and Term terminators were removed compared to pVE14189. β-Galactosidase activities of strains expressing *elrR* (*ElrR*$^{++}$) and plasmid control (Vector) are the averages of at least three independent experiments. nd, not done.

expression and the *in vitro* ElrR binding region may reflect changes in conformation or oligomerization of ElrR, which would act differently on the *elrA* promoter region. Noticeably, *in vitro* binding of Rgg1358 of *S. thermophilus* to the promoter region of its target genes does not require the small hydrophobic activating peptide that is necessary for transcription activation *in vivo* (14), suggesting that binding does not necessarily imply transcription activation. In the case of PrgX, which acts as a repressor by binding as a tetramer to two operator sites upstream of the *prgQ* operon, its binding capacity is modified by the activating pheromone cCF10,

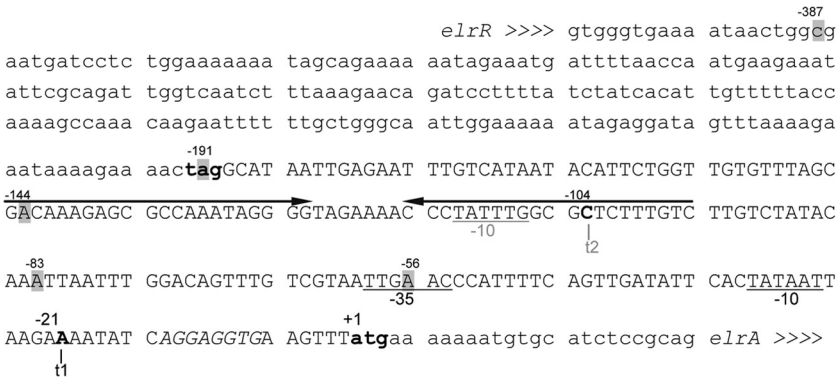

FIG 6 Nucleotide sequence of *elrA* promoter region. Lowercase letters indicate *elrR* and *elrA* coding sequences; the stop and start codons are in bold. The 50-bp inverted repeat is indicated by convergent arrows. Numbering refers to the translation start site of *elrA* (+1). The two transcriptional start sites t1 and t2 of *elrA* previously mapped are shown (6); t2 was not found under the conditions used in this work. The putative −35 and −10 boxes are underlined. The ribosome binding site for *elrA* is shown by italics. Shaded letters correspond to extremities of DNA probes used for EMSA (Fig. 4).

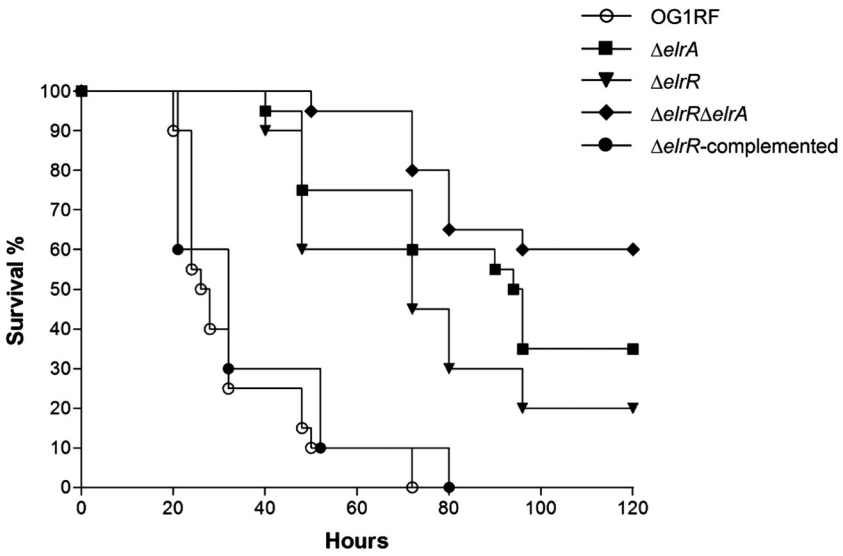

FIG 7 Effect of *elrR* inactivation on *E. faecalis* virulence. Kaplan-Meier survival analysis in a mouse peritonitis model with the OG1RF wild-type strain (open circles), the Δ*elrA* mutant strain (squares), the Δ*elrR* mutant strain (inverted triangles), the Δ*elrR* Δ*elrA* double mutant strain (diamonds), and the *elrR*-complemented strain (closed circles). Data were pooled from two independent experiments, except for the Δ*elrR*-complemented strain. A total of 20 mice were infected intraperitoneally with 1.4×10^9 or 0.85×10^9, 1.3×10^9 or 0.9×10^9, 1.5×10^9 or 1.1×10^9, and 1.3×10^9 or 0.98×10^9 CFU of OG1RF, Δ*elrA*, Δ*elrR*, and Δ*elrR* Δ*elrA* strains, respectively. A total of 10 mice were infected with 1.6×10^9 CFU of the Δ*elrR*-complemented strain. For each pairwise comparison, OG1RF/Δ*elrR*, Δ*elrR*/Δ*elrR* Δ*elrA*, and Δ*elrA*/Δ*elrR* Δ*elrA*, *P* values were <0.0001, <0.005, and <0.15, respectively.

which switches the oligomeric status of the regulator from a tetramer to a dimer and relieves repression (60, 69, 70). More recently, it was also proposed that Rgg3-mediated repression in *S. pyogenes* was relieved upon interaction with the signaling peptide (71). At this stage of our knowledge, further investigation is required to analyze the *elrR*-dependent activation of *elrA* at the molecular level.

ElrR inactivation attenuates *E. faecalis* virulence in a mouse peritonitis model. In several bacterial species, Rgg family members participate in virulence by regulating expression of virulence factors (72–75). We previously showed that *elrA* expression is enhanced *in vivo*, stimulates the host inflammatory response, and contributes to virulence (6). Added to the positive effect of ElrR on *elrA* expression (see above), these data led us to investigate the effects of ElrR on *E. faecalis* virulence. Using a mouse peritonitis model (76), the virulence of the isogenic single-mutant Δ*elrR* and Δ*elrA* strains and the double-deletion Δ*elrR* Δ*elrA* mutant was compared to that of the parental strain OG1RF and the Δ*elrR*-complemented strain. For each infection, mice were injected intraperitoneally with $\sim 10^9$ CFU and mortality rates were compared. Mortality was delayed and significantly reduced for mice infected with the Δ*elrR* strain; 20% of the mice were still alive after 120 h, whereas 100% of mice died after 75 h when infected with the OG1RF strain (Fig. 7). Survival analysis after infection with the Δ*elrR* Δ*elrA* and Δ*elrA* strains showed that only 40% of the mice infected with the Δ*elrR* Δ*elrA* mutant died within 5 days, while 65% of the mice died after infection with the Δ*elrA* strain. Attenuation of a strain deleted for *elrR* could be expected from its positive regulatory effect on ElrA, previously implicated in *E. faecalis* virulence in the same virulence model. Although not significant, the slight delay in virulence of the Δ*elrA* strain compared with the Δ*elrR* strain suggests that inactivation of *elrR* does not totally abrogate *elrA* expression *in vivo*. Unexpectedly, concomitant inactivation of the *elrA* and *elrR* genes had a cumulative effect on virulence attenuation, strongly suggesting that *elrR* regulates genes other than *elrA*. As *elrA* belongs to an operon, the other genes of the operon (*ef2685* to *ef2682*) are obvious candidates that may participate in increased virulence attenuation of the Δ*elrR* Δ*elrA* strain. Since we had previously reported that *elrA* was upregulated *in vivo* in mice (6), we compared the *elrA* transcript levels of OG1RF and Δ*elrR* strains in mice at 20 h postinfection. Under these experimental conditions, the level of *elrA* transcript found in the enterococci recovered from peritoneal cavity fluids of mice infected with $\sim 1 \times 10^9$ CFU was about 4-fold higher in the wild-type strain than in the Δ*elrR* strain, indicating that ElrR activates *elrA* expression also *in vivo*.

WxL proteins are present in a subset of low-GC Gram-positive species, including *E. faecalis*, *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Listeria monocytogenes*, and *Bacillus cereus* (7, 77, 78). We previously showed that the C-terminal WxL domain, which contains two conserved motifs, Trp-x-Leu, of secreted proteins including ElrA, promotes noncovalent association to the bacterial cell surface (7). The *elrA* operon is representative of the organization of other WxL protein-encoding clusters, which encode at least two WxL domain proteins, a small LPXTG protein, and a predicted membrane-anchored protein with a conserved DUF916 domain. These encoded surface proteins were hypothesized to form a cell surface complex at the bacterial surface (7, 77). Proteins EF2685 to EF2682 encoded by the *elrA* operon could contribute to virulence independently of ElrA. Functional investigation of the complete *elrA* operon should help to answer this question. In addition, as reported for *ropB* of *S. pyogenes* (26) and *rovS* of *S. agalactiae* (25), *elrR* may regulate loci other than solely its adjacent *elrA* target locus, as suggested by *in vivo* data (Fig. 7).

In conclusion, we identified ElrR, an *E. faecalis* member of the Rgg regulator family, which activates *elrA* expression. We showed that the *elrR* gene is functionally linked to the contiguous *elrA*

operon and that ElrR is not expressed at detectable levels under laboratory conditions in strain OG1RF. Constitutive expression of *elrR* increases the RNA levels of *elrA*, and the ElrR protein binds specifically to the *elrA* promoter region, suggesting that ElrR acts directly as an activator of *elrA* transcription. Moreover, these data provide the first evidence for the involvement of ElrR in *E. faecalis* virulence. Identification of a signal that could trigger natural expression of *elrR* and/or *elrA* remains a challenging question in order to provide means to undertake mechanistic studies of ElrR effects and to delineate the ElrR regulon.

ACKNOWLEDGMENTS

We are very grateful to L. Rigottier-Gois for helpful discussions, to V. Juillard for *in silico* prediction of the short coding sequences, and to the reviewers for their constructive comments. We warmly thank A. L. Sonenshein for careful reading of the manuscript. We thank A. Salomon for technical assistance and M. Perego and S. Mesnage for providing p3TET and pET2818, respectively. We also thank C. Bevilacqua and C. Beauvallet from the ICE platform (INRA-GABI, Jouy-en-Josas) for their help in qRT-PCR experiments and the use of the Typhoon imager.

R.D. was supported by a fellowship from the Région Ile-de-France in the framework of the Dim Malfin-2008.

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