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RESEARCH ARTICLE

Examination of *Enterococcus faecalis* Toxin-Antitoxin System Toxin Fst Function Utilizing a Pheromone-Inducible Expression Vector with Tight Repression and Broad Dynamic Range

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ABSTRACT Tools for regulated gene expression in *Enterococcus faecalis* are extremely limited. In this report, we describe the construction of an expression vector for *E. faecalis*, designated pCIE, utilizing the P_Q pheromone-responsive promoter of plasmid pCF10. We demonstrate that this promoter is tightly repressed, responds to nanogram quantities of the peptide pheromone, and has a large dynamic range. To demonstrate its utility, the promoter was used to control expression of the toxic peptides of two *par* family toxin-antitoxin (TA) loci present in *E. faecalis*, par_{pAD1} of the pAD1 plasmid and par_{EF0409} located on the *E. faecalis* chromosome. The results demonstrated differences in the modes of regulation of toxin expression and in the effects of toxins of these two related systems. We anticipate that this vector will be useful for further investigation of *par* TA system function as well as the regulated expression of other genes in *E. faecalis*.

IMPORTANCE *E. faecalis* is an important nosocomial pathogen and a model organism for examination of the genetics and physiology of Gram-positive cocci. While numerous genetic tools have been generated for the manipulation of this organism, vectors for the regulated expression of cloned genes remain limited by high background expression and the use of inducers with undesirable effects on the cell. Here we demonstrate that the P_Q pheromone-responsive promoter is repressed tightly enough to allow cloning of TA system toxins and evaluate their effects at very low induction levels. This tool will allow us to more fully examine TA system function in *E. faecalis* and to further elucidate its potential roles in cell physiology.

KEYWORDS *Enterococcus*, expression vector, pheromone, toxin-antitoxin systems

Enterococci have emerged over the past few decades as major nosocomial pathogens. They are responsible for 15% of health care-associated urinary tract infections (1), cause 5 to 15% of all infectious endocarditis cases (2), and are currently the second leading cause of health care-associated bacteremia (1). Treatment is complicated by intrinsic resistance to a variety of antibiotics and acquired resistance to high-level aminoglycosides, vancomycin, and other antibiotics (3). A variety of mobile genetic elements (MGE) facilitate the transfer of antibiotic resistance determinants (4). *Enterococcus faecalis* and *Enterococcus faecium* cause the majority of infections, with *E. faecalis* generally being more pathogenic and *E. faecium* displaying greater antibiotic resistance, particularly to vancomycin. *E. faecalis* has also emerged as a model organism for

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the study of bacterial genetics in Gram-positive cocci, with an emphasis on the biology of MGE and the evolution and spread of antibiotic resistance. Therefore, numerous tools have been constructed to facilitate its genetic manipulation, including cloning vectors, transposon mutagenesis schemes, and allelic-replacement strategies (5–8). One specific tool that has eluded enterococcus researchers is an expression vector system that tightly represses expression of the cloned gene while allowing high-level expression when induced. The expression systems available tend toward leakiness under noninducing conditions and/or are inducible by toxic peptides (such as nisin [9]) or nutrients (10) that could alter the metabolism of the strain under investigation. Our previous attempt to study the mode of action of the Fst_{pAD1} toxin using the nisin-inducible expression was compromised by synergistic toxic effects of nisin and Fst_{pAD1} (11).

In this study, we utilized a new pheromone-inducible expression system to compare and contrast the effects of overproduction of the related toxin-antitoxin (TA) system members Fst_{pAD1} and Fst_{EF0409} on their native *E. faecalis* host. Fst_{pAD1} is the toxin of the pAD1 plasmid-encoded par_{pAD1} TA system that functions in plasmid stabilization via a postsegregational killing (PSK) mechanism (12, 13). par_{pAD1} is the founding member of a large family of type I TA systems on the plasmids and chromosomes of Gram-positive bacteria (14–16). All members of this family encode small peptide toxins with homology to Fst_{pAD1} and two convergently transcribed RNAs that share a bidirectional intrinsic transcriptional terminator. RNA I is the *fst* mRNA, and RNA II is a small RNA (sRNA) that suppresses toxin translation by complementary binding with RNA I (17, 18). Regulation of the par_{pAD1} system requires complementary regions in both the 5′ and terminator regions of the RNAs (18), though there is some indication that the details of RNA-RNA interaction may vary among family members (19, 20). Most, if not all, of the family members also encode an intramolecular stem-loop in RNA I that further modulates translation (17, 21). The toxins all encode conserved transmembrane domains and variable charged C-terminal tails (14–16). Overexpression of Fst_{pAD1} causes chromosomal condensation, interfering with segregation and cell division (22), but the mechanism of action of the toxin remains undetermined.

Fst_{EF0409} is the putative toxin of a *par* family member, par_{EF0409}, located on the chromosome of *E. faecalis* (16). (The EF0409 designation refers to the locus identifier [ID] of the Fst-like toxin in the chromosome of V583. The locus ID in OG1RF is RS01625, but we use the EF0409 designation for consistency with previous publications.) par_{EF0409} displays all the features of a canonical *par* family member but is not associated with any identifiable mobile genetic element. Therefore, it is unlikely to perform a PSK function and may possess features of regulation distinct from par_{pAD1}. While the overall structures of the two *par* loci are similar, there is little conservation in the putative RNA-RNA interaction regions, suggesting that both pairs of interacting RNAs should be able to coexist without interfering with one another's function. The par_{EF0409} locus is between two paralogous mannitol family phosphotransferase system (PTS) gene sets, suggesting that it may play a role in modulating sugar transport, though no evidence for such a function has yet been published. While no specific function of the par_{EF0409} locus has been identified, a mutation in the RNA II component has been observed to result in an increase in virulence, suggesting a possible role in pathogenesis (23).

In this report, we describe the construction and use of the pCIE *Escherichia coli-E. faecalis* shuttle/expression vector that uses the P_Q cCF10 pheromone-responsive promoter from the plasmid pCF10 (24, 25) for regulated transcription of cloned genes. Utilizing promoterless versions of the RNA I_{pAD1} and RNA I_{EF0409} genes, we demonstrated that this promoter (i) is tightly repressed in the absence of exogenous cCF10, (ii) is sensitive to added cCF10 at nanogram-per-milliliter concentrations, and (iii) possesses a large dynamic range. Comparison of the results of overexpression of the two toxin mRNAs suggests that each system possesses distinct modes of RNA-mediated regulation and targeted toxin effects that may be optimized for their different functions.

RESULTS

Expression from the pCIE P_Q promoter is tightly regulated, with a large dynamic range. The pCIE expression vector, constructed as detailed in Materials and Methods and shown in Fig. S1 and S2 in the supplemental material, is an *E. coli-E. faecalis* shuttle vector based on the lactococcal rolling-circle replicon from plasmid pCI372 encoding a chloramphenicol (Cm) resistance gene functional in both hosts. Regulated expression is provided by the pheromone cCF10-inducible P_Q promoter and its coresident repressor *prgX*, both from the *E. faecalis* plasmid pCF10 (24, 25). The *prgQ* transcript also encodes the iCF10 precursor whose mature peptide is a competitive inhibitor for cCF10 and should effectively counteract endogenous pheromone. Restriction sites for cloning of foreign DNA are approximately 400 nucleotides downstream of the P_Q transcriptional start site and downstream of the IRS1 terminator. Transcription from P_Q to IRS1 occurs constitutively at a low level. cCF10 induction increases transcription from P_Q, allowing IRS1 read-through into downstream genes (26); the additional level of posttranscriptional regulation of termination of transcripts from P_Q at IRS1 (27, 28) should result in nearly undetectable transcription of any DNA cloned into the pCIE multiple-cloning site (MCS) in the absence of pheromone.

In order to assess the ability of the pCIE-encoded P_Q promoter to adequately control production of toxic proteins in *E. faecalis*, promoterless versions of the RNA I_{EF0409} and RNA I_{pAD1} genes were cloned under its control to create pDAK1010E and pDAK1010A, respectively. These transcripts encode small peptide toxins, Fst_{EF0409} and Fst_{pAD1}, respectively, from related type I toxin-antitoxin (TA) systems (16). Neither RNA I_{pAD1} (18) nor RNA I_{EF0409} (data not shown) under the control of their own promoters can be introduced into strains lacking the cognate RNA II. Fragments cloned into pCIE contained the native RNA I intrinsic transcriptional terminator and a 5′ stem-loop structure that has been shown in the case of RNA I_{pAD1} to reduce ribosome binding and modulate Fst_{pAD1} toxin expression (29). Both constructs were introduced into the wild-type OG1RF strain and selected mutants to assess expression.

Northern blots of RNA purified from OG1RF(pDAK1010E) and probed with an RNA I_{EF0409}-specific oligonucleotide (Table 1) identified several bands that increased with increasing cCF10 concentration. The largest of the induced transcripts hybridized with both the RNA I_{EF0409}-specific probe (Fig. 1, left) and a probe complementary to sequences between P_Q and IRS1, prgQup (see Fig. S3), indicating that it is the full-length P_Q transcript. The smallest of the RNA I_{EF0409}-hybridizing bands did not hybridize with the prgQup probe and is slightly smaller than RNA I_{EF0409} produced from its native promoter on a multicopy plasmid, pDAK1001 (Fig. 1, right). These observations suggest that it is processed from the full-length transcript. The other RNA I_{EF0409}-hybridizing bands are most likely partial degradation products. A band migrating between the full-length and processed transcripts was identified that hybridized with the prgQup probe but not the RNA I_{EF0409}-specific probe in both induced and uninduced cells (Fig. S3). This is consistent with the known regulatory pattern of the P_Q promoter. Induction of OG1RF(pDAK1010A) resulted in production of a similar pattern of transcript production using an RNA I_{pAD1}-specific probe and prgQup, except that no processed product similar to the size of native RNA I_{pAD1} was observed (Fig. S3). Perhaps differences in the sequences of the two RNA I homologs result in differing sensitivities to RNases.

To determine the dynamics of induction, production of RNA I_{EF0409} was determined at intervals following maximal induction at 50 ng/ml of cCF10 (Fig. 2). Detectable transcript was observed within 30 min of induction, peaked at around 2 h after induction, and declined noticeably after 4 h. Interestingly, increasing RNA I_{EF0409} levels resulted in a decrease in RNA II_{EF0409} levels produced from the chromosome. In control experiments with induced empty vector, RNA II_{EF0409} levels actually increased during log phase before falling off again in stationary phase. Thus, induction of RNA I_{EF0409} reverses the natural dynamics of RNA II_{EF0409} expression. Similar effects can be observed in Fig. 1, where RNA II_{EF0409} levels dropped with increasing levels of cCF10, and hence RNA I_{EF0409}, induction, and in Fig. S3, where maximal RNA I_{EF0409} induction reduced RNA II_{EF0409} below the limit of detection.

TABLE 1 Oligonucleotide probes and primers used in this study[a]

Probe or primer	Sequence	Restriction enzyme recognition site(s)
Probes for Northern blots		
RNA I_{EF0409}	CAGGACAATCCCGACAAAAATCGGCACAAGAAT	
RNA II_{EF0409}	GCATCCATGCACGACTTTGTCACAAGTTACTTT	
RNA I_{pAD1}	ACCAATCCTACAAAGATTGGTGCGATAACC	
RNA II_{pAD1}	TGTGTTATCTGTACGATTTAATGTCG	
5S rRNA probe	AACAGGTGTATCCTTCTCGCTAT	
prgQup	TAGAGTGGTTTTCATTTACACCCCTCCTAT	
Primers for constructing EF0409 allelic-exchange deletion mutants		
EF0409 P1	GAGA**GAATTC**CGTGGATACCACCAAGGAAA	EcoRI
EF0409 P2	CTCT**GGATCC**GTACATGTGATGCACCTCCT	BamHI
EF0409 P4	CTCT**CTGCAG**TTGGGATTCCTCAGACGGAA	PstI
5′RNAIIdel	GCGCGC**GGATCC**GACGTAACTTTAGGCTTGA	BamHI
EF0409Up	GCACGTAAACCAGCATCTAA	
EF0409Down	CGATAGTGCAATCGTCTGTC	
Primers for plasmid construction and sequencing		
IRSX	GG**GGATCCGTCGACGCATGCAAGCTT**CCTAAAGAAGTAACCATGTATTATG	BamHI, SalI, SphI, HindIII[b]
IRSX-R	G**GAATTC**ATTTTGTTGGTGGCTTGGAAATG	EcoRI
EF0409RNAI-SalI	ATGT**GTCGAC**TGTATCCGCTTTCCAAATCTGGA	SalI
EF0409RNAI-BamHI	GAGAGA**GGATCC**GTGACAAAGTCGTGCATGGAT	BamHI
pACYC-seq	AGGAGCTGACTGGGTTGA	
RNAIpCIE5′	ATGT**GGATCC**TGGCGACTTGCCTCGAAAG	BamHI
RNAIpCIE3′	ATGT**AAGCTT**GACAAAGTCGTGCATGGAT	HindIII
Primers for qPCR		
EF0409 FWD	GCATCACATGTACGAGATTGTC	
EF0409 REV	GCAACTTGTTTATTCCTGGTTCT	
par-pAD1 FWD	GTTGGTTATCGCACCAATCTTT	
par-pAD1 REV	TTACTTTCGGCTATCGTCTTCC	
EF1017 FWD	GCAGGAATGAGCACAAGTTTATT	
EF1017 REV	TCAGAAGCCGATACTGCAAAG	
EF3110 FWD	GGACTTAGACGTTACCATTCA	
EF3110 REV	AGCCATTTCCGCAACTACA	
EF0417 FWD	GCACGATGTCTGGTGATGAT	
EF0417 REV	CTTCGCTCCTAAATCCGCTAAG	
EF1117 FWD	GTGGTGCGTAATCCAAACTTAT	
EF1117 REV	CAGCGGTGGTAAACACAGTA	
EF1304 FWD	AAAGGTGCGGTTGAAGAAATG	
EF1304 REV	TGACGCAGTGTCTCTGTTAAG	
EF0758 FWD	CAGATGACGGCTCAATTCAAAC	
EF0758 REV	CAGCGGTACTTCCTTCAATCA	
EF0938 FWD	CAATTGTCCGTGATGCCAAAG	
EF0938 REV	CGTTGGTGTAGTTTAGCGATTTC	
16S rRNA FWD	CAAGCGTTGTCCGGATTTATTG	
16S rRNA REV	GCACTCAAGTCTCCCAGTTT	

[a]All sequences are shown in the 5′ to 3′ direction. Added restriction sites are indicated in bold letters, and the restriction enzyme recognition site is given in the right column.

[b]Sites are present in the order indicated in tandem.

Induction of RNA I_{EF0409} and RNA I_{pAD1} resulted in cCF10 dose-dependent growth inhibition (Fig. 3). No consistent difference was observed between the growth inhibition of RNA I_{EF0409} and that of RNA I_{pAD1} (Fig. S4). The pheromone cCF10 is produced endogenously by all plasmid-free strains of *E. faecalis*, including OG1RF. To determine if endogenous cCF10 production affected P_Q expression, growth inhibition profiles and RNA I_{EF0409} expression levels were compared between OG1RF(pDAK1010E) and JRC101(pDAK1010E). JRC101 is a mutant of OG1RF unable to produce endogenous cCF10 (30). It was expected that a higher threshold of cCF10 might be required to induce RNA I_{EF0409}-mediated toxicity in JRC101 due to the absence of endogenous

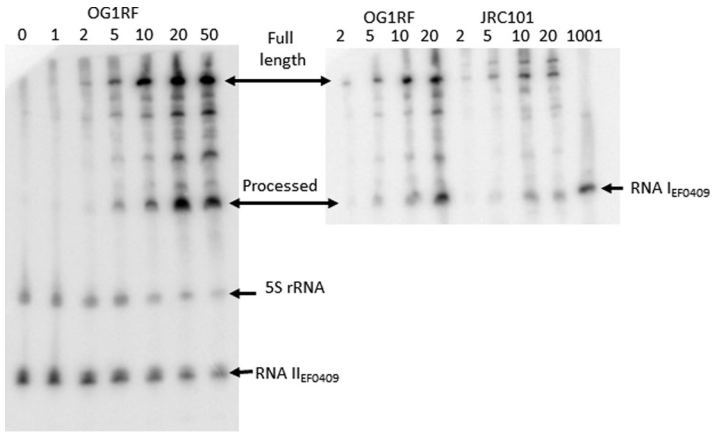

FIG 1 Inducible expression of RNA I_{EF0409} from pDAK1010E. OG1RF and JRC101 strains, the latter being unable to produce endogenous cCF10, containing pDAK1010E were induced with pheromone concentrations (in nanograms per milliliter) indicated above the blot for 1 h as described in Materials and Methods. The lane labeled 1001 is RNA purified from OG1RF(pDAK1001), which overproduces RNA I_{EF0409} from the multicopy vector pWM401. The blot on the left was also probed with 5S rRNA-specific and RNA II_{EF0409}-specific probes (Table 1) to provide internal size standards. RNA II_{EF0409} is produced from the par_{EF0409} locus on the OG1RF chromosome. RNA I_{EF0409} production from this locus is too low to be observed under these conditions.

cCF10 production. However, growth inhibition was apparent in both wild-type and mutant strains at 1 ng/ml of cCF10 (Fig. 3A and B), although JRC101(pDAK1010E) appeared to recover more quickly. Both Northern blotting (Fig. 1) and reverse transcriptase quantitative PCR (RT-qPCR) (Fig. 3C) demonstrated that there was very little difference in RNA I_{EF0409} levels between the two strains. Indeed, RT-qPCR (Fig. 3D) and transcriptome sequencing (RNA-seq; see below) data indicated that expression of RNA I_{EF0409} in OG1RF(pDAK1010E) was not significantly higher than that produced from the chromosomal par_{EF0409} locus alone. In fact, uninduced levels of RNA I_{EF0409} were consistently higher in JRC101 than in OG1RF for reasons that are not clear.

As previously observed with expression of RNA I_{pAD1} from a different vector (31), induction of pDAK1010E resulted in loss of viability of host cells (Fig. 4A). However, plates on which viable counts were determined showed an unusual pattern of growth that made the magnitude of the effect difficult to determine. After 24 h, OG1RF(pDAK1010E) cultures that had been maximally induced (50 ng/ml of cCF10) produced two distinct colony types. Relatively few large colonies were surrounded by a background "haze" of small-colony growth (Fig. 4B). By 48 h, the smaller colonies had grown to the size of normal 24-h colonies and counts indicated a drop in viability of 2 orders of magnitude. Further investigation revealed that the large colonies were no longer sensitive to cCF10 induction, suggesting that a mutation had occurred that either interfered with induction or resulted in toxin resistance. In contrast, the smaller colonies remained sensitive to cCF10, indicating that they had recovered from

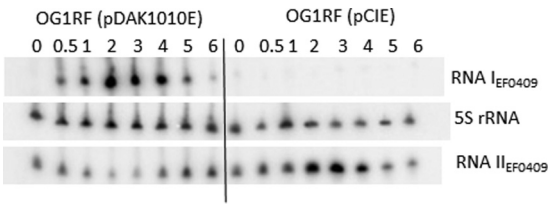

FIG 2 Time course of RNA I_{EF0409} expression from pDAK1010E. OG1RF(pDAK1010E) was induced with 50 ng of cCF10 per ml, and samples were harvested at the indicated times (in hours) above the blot. A parallel experiment was performed with OG1RF(pCIE) as an empty vector control. Bands hybridizing with specific probes are shown in labeled strips. For simplicity, only the processed RNA I_{EF0409} band is shown, but the full-length band showed a similar pattern of expression.

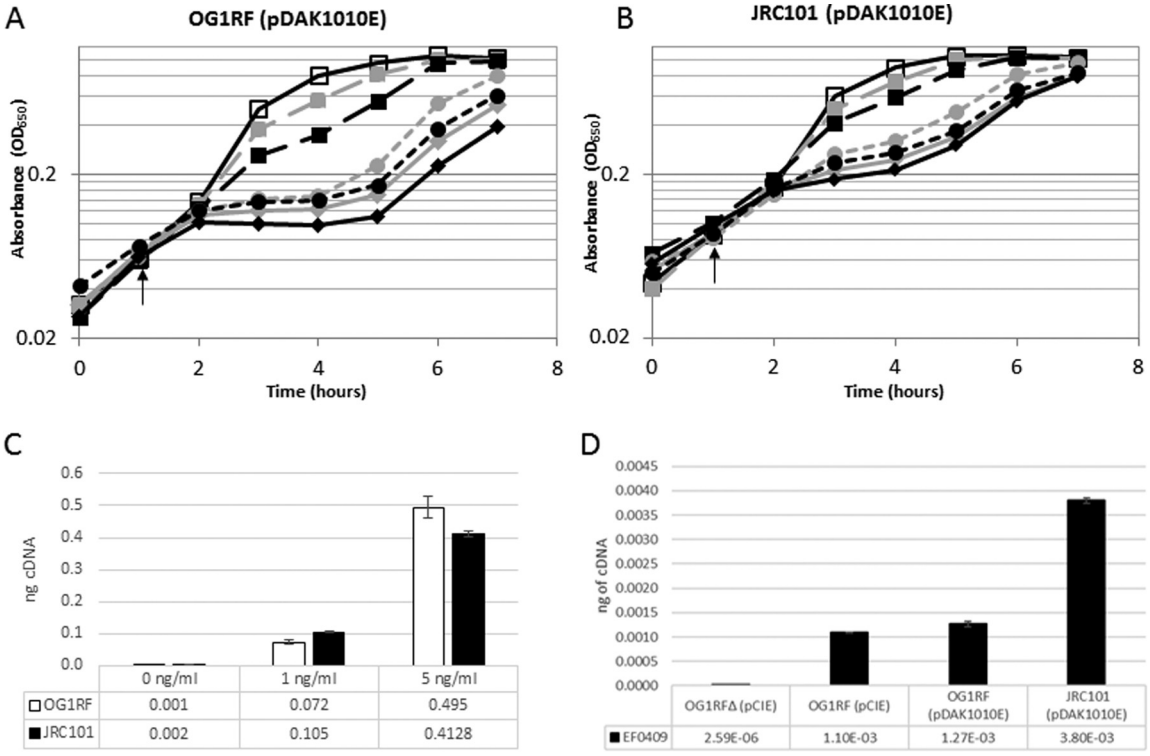

FIG 3 Growth inhibition and toxin expression in the presence and absence of endogenous pheromone. Induction profiles with various concentrations of cCF10 were compared between OG1RF(pDAK1010E) (A) and JRC101(pDAK1010E) (B). Growth of uninduced strains was indistinguishable from that of vector-only controls (data not shown). Cultures were diluted 1:100 from overnight growth, and cCF10 was added at the indicated concentrations after 1 h (arrow). White squares, 0 ng/ml; gray squares, 1 ng/ml; black squares, 2 ng/ml; gray circles, 5 ng/ml; black circles, 10 ng/ml; gray diamonds, 20 ng/ml; black diamonds, 50 ng/ml. (C) RT-qPCR comparison of RNA I_{EF0409} between OG1RF(pDAK1010E) and JRC101(pDAK1010E). RNA samples were prepared 1 h after induction with the indicated cCF10 concentrations and subjected to RT-qPCR as described in Materials and Methods. qPCR was performed in triplicate; transcript amounts (in nanograms) were determined by comparison to a standard curve and normalized to 16S rRNA as described in Materials and Methods. (D) RT-qPCR of uninduced cultures of OG1RF Δpar_{EF0409}(pCIE) [labeled OG1RFΔ(pCIE)] lacking the RNA I_{EF0409} gene, OG1RF(pCIE) carrying only the chromosomal RNA I_{EF0409} gene, and OG1RF(pDAK1010E) and JRC101(pDAK1010E) carrying both the chromosomal and uninduced plasmid genes. Independent RNA preparations were used for experiments whose results are depicted in panels C and D.

the effects of toxin induction (data not shown). Rapid recovery was dependent on chromosomal expression of RNA II_{EF0409}, since no background haze was observed in induced OG1RF Δpar_{EF0409}(pDAK1010E) cells (Fig. 4B). Slow-growing colonies did eventually appear in the absence of RNA II_{EF0409}, but their numbers declined with increasing time after exposure compared to the wild type (Fig. 4A). One possible explanation for this is that the RNA I_{EF0409} persists longer in the absence of RNA II_{EF0409}, perhaps due to coupled degradation of the RNA I_{EF0409}-RNA $II_{EEF0409}$ complex. In fact, at 6 h after induction at 50 ng cCF10 per ml, RNA I_{EF0409} levels were 4-fold higher in the mutant than in the wild type in two independent experiments (Fig. 4C). No 24-h haze of small colonies was seen after induction of pDAK1010A in either OG1RF or OG1RF Δpar_{EF0409}, consistent with the absence of RNA II_{pAD1} in both strains (data not shown). However, the presence of an ectopic copy of RNA II_{pAD1} on plasmid pDAK611 was demonstrated to partially protect OG1RF cells from induction of RNA I_{pAD1} but not RNA I_{EF0409} (Fig. 5). These results demonstrate the specificity of the RNA interactions of the two TA systems and also suggest that RNA II_{EF0409} can facilitate recovery of toxin-exposed cells. They also indicate that the RNAs can effectively interact in spite of the 5′ extension added to the RNA I's in the P_Q fusions.

Effects of Fst toxins on the transcriptome of host cells. To determine if the tight repression and high sensitivity of the P_Q promoter would make it possible to examine graded responses to toxic proteins, we performed RNA-seq on OG1RF(pDAK1010E) cells exposed to 1 and 5 ng of cCF10 per ml. Exposure of cells to 1 ng/ml resulted in a

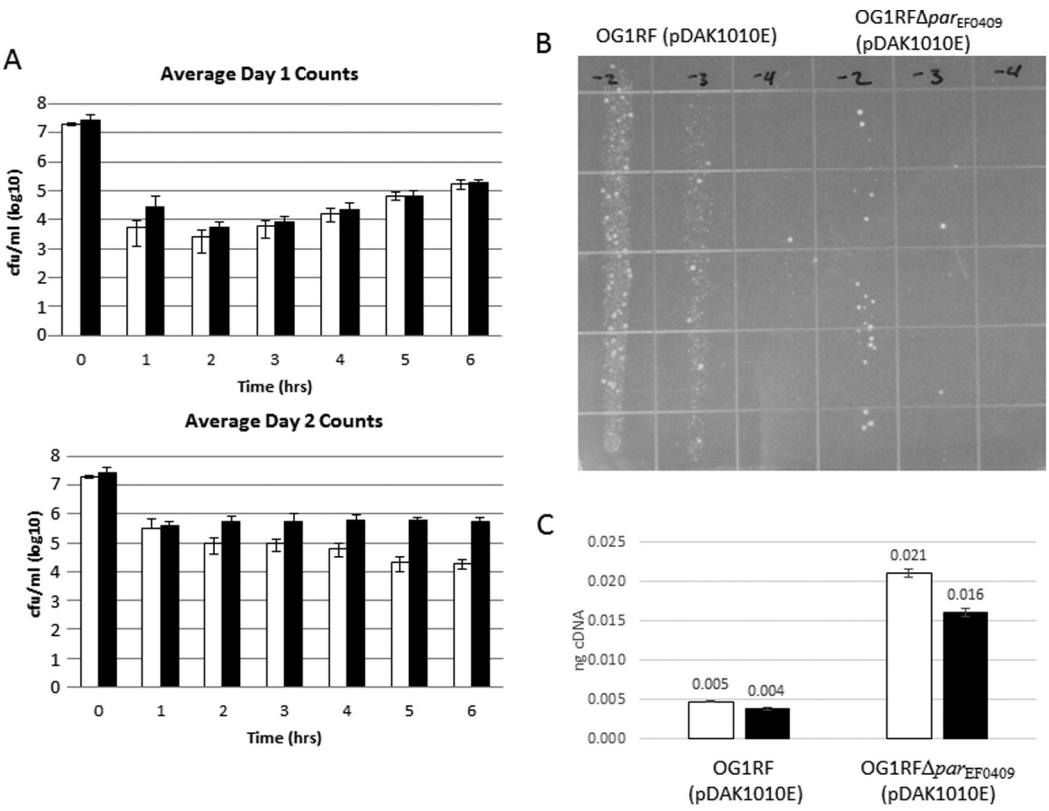

FIG 4 Effect of RNA I_{EF0409} expression on viability in the presence and absence of RNA II_{EF0409}. Strains OG1RF(pDAK1010E) and OG1RF Δpar_{EF0409}(pDAK1010E) were diluted 1:100 from overnight cultures and grown for 1 h before being maximally induced (50 ng of cCF10 per ml). Samples were taken at the indicated times after induction and colony counts were derived as described in Materials and Methods. Results shown in panel A are averages from three independent experiments. Day 1 counts were only of the large colonies observed with both strains after 24 h of growth. Day 2 counts are total colonies arising after 48 h of culture. White bars, OG1RF Δpar_{EF0409}(pDAK1010E); black bars, OG1RF(pDAK1010E). (B) A typical plate photographed after 24 h growth showing large and small colonies of OG1RF(pDAK1010E) but only large colonies of OG1RF Δpar_{EF0409}(pDAK1010E). (C) Residual levels of RNA I_{EF0409} in the presence and absence of RNA II_{EF0409}. OG1RF(pDAK1010E) and OG1RF Δpar_{EF0409}(pDAK1010E) were maximally induced as in viability experiments, and RNA was purified 6 h after induction. White and black bars represent results of two independent experiments.

modest inhibition of growth and no effect on viability, while 5 ng/ml resulted in a <2-fold drop in viability (data not shown). OG1RF(pDAK1010E) exposed to vehicle only (i.e., uninduced) and OG1RF(pCIE) induced with 5 ng of cCF10 per ml were used as controls. A sampling of genes of interest is shown in Table 2, with a complete listing of genes showing >2-fold differential expression in Tables S1 and S2. Complete data have been deposited in the GEO database. At 1 ng/ml, expression of 116 genes was altered more than 2-fold, with 90 induced and 26 repressed. The most highly induced and repressed genes are shown at the top and bottom of Table 2, respectively. Expression of all of the genes altered at 1 ng/ml was also altered at 5 ng/ml, most to a higher degree. Genes with altered expression at 5 ng/ml but not 1 ng/ml were generally affected less than those altered at both concentrations. Of the 81 genes induced >4-fold at 5 ng cCF10 per ml, all but 8 either were also induced or were adjacent to genes induced at 1 ng/ml. Therefore, a clear graded response was observed with induction.

Of the 13 most highly induced genes, 10 encode hypothetical proteins, 1 encodes a LemA family protein of unknown function, and 2, OG1RF_RS07140 and OG1RF_RS07145, together correspond to an ABC transporter homolog. Induction of pDAK1010E at 1 ng/ml affected expression of 30 genes in various transporter families, with 18 upregulated and 12 downregulated. Of particular interest was the OG1RF_RS01655/OG1RF_RS01660 ABC transporter gene closely linked to the Fst_{EF0409} toxin gene, two highly induced paralogous magnesium transporter genes (OG1RF_

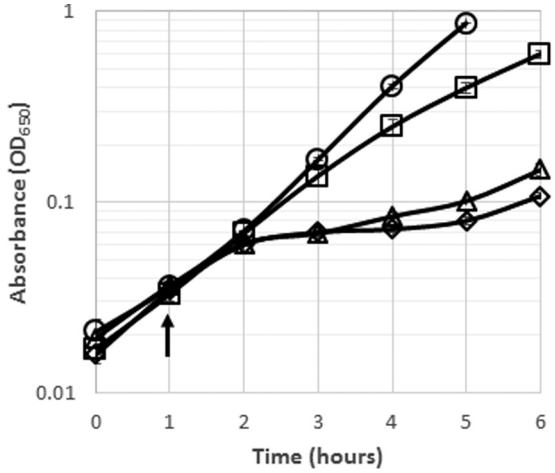

FIG 5 Specificity of protection from RNA I induction by RNA II_{pAD1}. The ability of RNA II_{pAD1} to protect cells from RNA I_{EF0409}- and RNA I_{pAD1}-mediated growth inhibition was determined. pDAK611, which contains a copy of the RNA II_{pAD1} gene under the control of its own promoter, or the empty pDL278 vector was introduced into OG1RF(pDAK1010E) and OG1RF(pDAK1010A). Cultures were diluted 1:100 in media selective for both plasmids and maximally induced (50 ng cCF10 per ml) 1 h after inoculation (arrow). Results shown are averages from three experiments. Error bars are shown but did not deviate from the average enough to transcend the symbol in most cases. Circles, OG1RF(pDAK1010A, pDAK611) uninduced control; squares, OG1RF(pDAK1010A, pDAK611), induced; triangles, OG1RF(pDAK1010A, pDL278), induced; diamonds, OG1RF(pDAK1010E, pDAK611), induced. OD_{650}, optical density at 650 nm.

RS05570 and OG1RF_RS059152), and two PTS system genes (OG1RF_RS03875-85 and OG1RF_RS12645-55) that were among the most highly repressed genes. The PTS systems are of interest because the par_{EF0409} locus is located between two paralogous mannitol PTS systems, the expression of which was not altered by toxin production. Altered expression of OG1RF_RS03875, OG1RF_RS01655, OG1RF_RS04680, and OG1RF_RS05570 was confirmed by RT-qPCR on independent RNA preparations (Table 3). The RNA-seq data also confirmed the low-basal-level transcription from the P_Q promoter, with no transcripts detected with the empty vector control and 330 at 1 ng/ml and 687 at 5 ng/ml with OG1RF(pDAK1010E) (see the supplemental material, OG1RF_RS01625).

To determine whether expression of the Fst_{EF0409} and Fst_{pAD1} toxins had similar or distinct effects on host cells, RNA-seq results with OG1RF strains expressing the two toxins with induction at 5 ng/ml of cCF10 were compared. Expression of 203 genes differed >2-fold between OG1RF(pDAK1010E) and OG1RF(pDAK1010A), 113 of which had higher expression and 90 of which had lower expression in OG1RF(pDAK1010A). However, expression of 36 of these genes was not significantly altered with respect to the vector-only control in either strain, and the expression of the majority of the rest was altered less than 4-fold. Table 4 shows the 29 genes with expression altered more than 4-fold; complete data are shown in the supplemental material and have been deposited in the GEO database. Of the 29 genes in Table 4, all but 7 are cell surface associated, including those for numerous transporters. The greatest difference between the effects of the two toxins was on a pair of adjacent genes annotated as corresponding to a copper-translocating P-type ATPase (OG1RF_RS02610-5) which were induced over 100-fold by RNA I_{pAD1} but not significantly induced by RNA I_{EF0409}. OG1RF_RS12135-45, encoding an oligopeptide permease system, was induced approximately 8-fold by RNA I_{pAD1} but not significantly by RNA I_{EF0409}. This difference is potentially functionally significant since Opp genes are important for the conjugative pheromone response (32). Conversely, the OG1RF_RS01655 ABC transporter closely linked to the EF0409 toxin gene was induced 16-fold by RNA I_{EF0409} but only 8-fold by RNA I_{pAD1}. OG1RF_RS03455, annotated as corresponding to a multiple-sugar ABC transporter, was induced approximately 3-fold by RNA I_{EF0409} but repressed approximately 3-fold by RNA I_{pAD1}. RNA-seq results with OG1RF_RS12145, OG1RF_RS01655, OG1RF_RS026108, and OG1RF_RS03455 were confirmed by RT-qPCR (Fig. 6). While

TABLE 2 Expression changes in selected genes of interest upon induction of OG1RF(pDAK1010E) with 1 and 5 ng of cCF10 per ml[a]

Gene[b]	Change[c] in expression with indicated concn of cCF10		*P* value	Function[d]
	1 ng/ml	5 ng/ml		
OG1RF_RS06480 (EF1533)	6.28	7.36	5.00E−05	Hypothetical protein
OG1RF_RS07140 (EF1672)	5.46	6.88	5.00E−05	Permease of ABC transporter
OG1RF_RS07145 (EF1673)	5.38	6.70	5.00E−05	ABC transporter
OG1RF_RS05570 (EF1304)	3.51	4.84	5.00E−05	Mg-translocating P-type ATPase (*mgtA*)
OG1RF_RS05915 (EF1352)	3.25	4.73	5.00E−05	Mg-translocating P-type ATPase (*mgtA2*)
OG1RF_RS01655 (EF0417)	3.14	4.06	5.00E−05	Efflux transporter
OG1RF_RS10570 (EF2698)	3.04	4.32	5.00E−05	Tellurite resistance protein
OG1RF_RS01660 (EF0418)	2.94	4.14	5.00E−05	ABC superfamily ATP binding cassette
OG1RF_RS07525 (EF1751)	2.76	4.33	5.00E−05	Membrane protein (putative)
OG1RF_RS07305 (EF1707)	2.53	4.14	5.00E−05	Glycosyl hydrolase, family 38
OG1RF_RS12755 (EF3245)	2.45	3.21	5.00E−05	Cell envelope-associated acid phosphatase
OG1RF_RS05670 (EF1323)	2.21	3.56	5.00E−05	Flotillin
OG1RF_RS08050 (EF1903)	2.18	3.01	5.00E−05	Integral membrane protein
OG1RF_RS09175 (EF2355)	1.94	3.32	5.00E−05	Chaperone protein ClpB
OG1RF_RS07135 (EF1671)	1.84	3.31	5.00E−05	NADPH:quinone reductase
OG1RF_RS02235 (EF0680)	1.74	2.45	5.00E−05	Penicillin-binding protein 2A
OG1RF_RS11615 (EF2986)	1.65	2.62	0.0041	ABC superfamily ATP binding cassette
OG1RF_RS10800 (EF2749)	1.62	2.90	5.00E−05	D-Alanine–D-alanine ligase[e]
OG1RF_RS08055 (EF1904)	1.61	2.16	5.00E−05	Glycerophosphoryl diester phosphodiesterase
OG1RF_RS11610 (EF2985)	1.50	2.58	0.0002	Permease protein
OG1RF_RS06270 (EF1495)	1.48	2.61	5.00E−05	V-type ATPase (V-ATPase), subunit E[e]
OG1RF_RS08495 (EF2050)	1.48	2.29	0.0004	ABC superfamily ATP binding cassette
OG1RF_RS00450 (EF0095)	1.47	2.24	5.00E−05	Putative lipoprotein
OG1RF_RS07315 (EF1709)	1.39	2.27	5.00E−05	Sugar-binding transcriptional regulator
OG1RF_RS08490 (EF2049)	1.39	2.42	5.00E−05	ABC superfamily ATP binding cassette
OG1RF_RS05195 (EF1227)	1.25	2.00	5.00E−05	NADPH-dependent FMN reductase domain
OG1RF_RS04690 (EF1119)	1.10	2.09	5.00E−05	Glutamine ABC transporter
OG1RF_RS13100 (EF3314)	1.03	3.00	0.0001	Cell wall surface anchor family protein
OG1RF_RS03880 (EF1018)	−1.20	−3.05	0.00075	PTS lactose/cellobiose component IIA
OG1RF_RS00400 (EF0082)	−1.49	−2.28	5.00E−05	Major facilitator family transporter
OG1RF_RS12645 (EF3210)	−1.60	−2.76	5.00E−05	PTS fructose/mannitol (Fru) component IIA
OG1RF_RS03875 (EF1017)	−2.10	−3.01	5.00E−05	PTS system transporter subunit I

[a]Genes were selected to demonstrate the variety of genes encoding membrane proteins affected. Except for OG1RF_RS06480 (EF1533), which was the most highly induced gene, genes for hypothetical proteins were left off. The complete sets of genes with expression affected >2-fold at induction with 1 ng/ml and 5 ng/ml of cCF10 are shown in Tables S1 and S2, respectively.

[b]Both OG1RF locus designations and V583 locus designations (in parentheses) are provided.

[c]Fold changes in induced OG1RF(pDAK1010E) are presented as $\log_2$ changes relative to the induced (5 ng/ml) OG1RF(pCIE) empty vector strain.

[d]Putative function based on sequence homology and conserved domains as determined using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

[e]In several cases multiple adjacent genes with related putative functions were induced. In those cases only the gene most altered in expression is listed. For example, genes EF2746 to EF2749, involved in D-alanine transfer, and EF1493 to EF1500, encoding components of the V-type ATPase, were all induced greater than 2-fold at 1 ng/ml, with higher induction at 5 ng/ml.

significant differences were observed, expression of many genes showed similar patterns in response to both toxins. For example, the magnesium transporters OG1RF_RS05570 and OG1RF_RS05915 were highly induced by both toxins and the OG1RF_RS03875 PTS component was repressed 8-fold by RNA I_{EF0409} and 26-fold by

TABLE 3 RT-qPCR of selected genes altered by RNA I_{EF0409} expression[a]

Gene	Amt of RNA after induction with indicated concn (ng/ml) of cCF10		
	0	1	5
OG1RF_RS03875 (EF1017)	0.601 ± 0.019	0.356 ± 0.036	0.048 ± 0.001
OG1RF_RS01655 (EF0417)	0.060 ± 0.003	0.146 ± 0.005	0.871 ± 0.018
OG1RF_RS04680 (EF1117)	0.063 ± 0.001	0.119 ± 0.003	0.793 ± 0.010
OG1RF_RS05570 (EF1304)	0.032 ± 0.001	0.084 ± 0.003	0.585 ± 0.016

[a]RNA samples were prepared and subjected to RT-qPCR as described in Materials and Methods. RNA amounts are depicted as nanograms of RNA as determined by comparison to a standard curve and normalized to 16S rRNA as described in the text. Cultures were induced with the indicated concentration of cCF10 as described in Materials and Methods and harvested 1 h after induction.

TABLE 4 Expression differences greater than 4-fold between OG1RF(pDAK1010E) and OG1RF(pDAK1010A) induced with 5 ng/ml of cCF10

Gene[a]	Fold change (log_2)	*P* value	Function[b]
OG1RF_RS02610 (EF0758)[c]	7.04	5.00E−05	Copper-translocating P-type ATPase
OG1RF_RS02615 (EF0759)[c]	6.38	5.00E−05	SapB protein, putative
OG1RF_RS06160 (EF1400)	5.48	5.00E−05	Cadmium-translocating P-type ATPase
OG1RF_RS12145 (EF3110)[c]	3.09	5.00E−05	ABC superfamily ATP binding cassette
OG1RF_RS12140 (EF3109)[c]	2.89	5.00E−05	ABC superfamily ATP binding cassette
OG1RF_RS12135 (EF3108)[c]	2.73	5.00E−05	Oligopeptide permease OppB
OG1RF_RS08565 (EF2067)[c]	2.59	5.00E−05	Phospholipid-binding protein
OG1RF_RS08570 (EF2068)[c]	2.53	5.00E−05	Major facilitator family transporter
OG1RF_RS12130 (EF3107)[c]	2.46	5.00E−05	Peptide ABC transporter, permease protein
OG1RF_RS02900 (EF0820)[c]	2.42	5.00E−05	50S ribosomal protein L25
OG1RF_RS04695 (EF1120)	2.29	5.00E−05	Amino acid ABC transporter, ATP-binding protein
OG1RF_RS08220 (EF1938)[c]	2.26	5.00E−05	Calcium-transporting ATPase
OG1RF_RS05580 (EF1306)[c]	2.22	5.00E−05	Heat-inducible repressor HrcA
OG1RF_RS01455 (EF0375)	2.20	5.00E−05	S-layer protein
OG1RF_RS07260 (EF1698)	2.13	5.00E−05	NADPH-dependent flavin mononucleotide reductase
OG1RF_RS02695 (EF0778)[c]	2.10	5.00E−05	Hypothetical protein
OG1RF_RS02640 (EF0764/5)[c]	2.08	5.00E−05	Hypothetical protein
OG1RF_RS10230 (EF2623)[c]	2.04	5.00E−05	P-ATPase cadmium transporter
OG1RF_RS05585 (EF1307)[c]	2.02	5.00E−05	Cochaperone GrpE
OG1RF_RS10300 (EF2641)[c]	2.02	5.00E−05	Glycine betaine/L-proline ABC protein
OG1RF_RS04690 (EF1119)	2.01	5.00E−05	Glutamine ABC cassette transporter
OG1RF_RS10795 (EF2748)[d]	−2.09	5.00E−05	D-Alanine transfer protein DltB
OG1RF_RS10785 (EF2746)[d]	−2.12	5.00E−05	D-Alanyl-LTA biosynthesis protein DltD
OG1RF_RS12755 (EF3245)[d]	−2.22	5.00E−05	Cell envelope-associated acid phosphatase
OG1RF_RS00745 (EF0202)[d]	−2.24	5.00E−05	Phosphomethylpyrimidine kinase
OG1RF_RS00555 (EF0118)[c]	−2.45	0.0037	Ornithine cyclodeaminase family protein
OG1RF_RS11615 (EF2986)[d]	−2.63	0.0001	ABC superfamily ATP binding cassette
OG1RF_RS03455 (EF0938)[e]	−2.73	5.00E−05	Multiple-sugar ABC transporter
OG1RF_RS11610 (EF2985)[d]	−2.74	5.00E−05	Permease protein

[a]Both OG1RF locus designations and V583 locus designations (in parentheses) are provided.
[b]Putative function based on sequence homology and conserved domains as determined using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
[c]Change was <2-fold for RNA I_{Ef0409}.
[d]Change was <2-fold for RNA I_{pAD1}.
[e]Expression changed downward for RNA I_{pAD1} and upward for RNA I_{EF0409}.

RNA I_{pAD1}.So while both toxins appear to predominantly affect the expression of genes for surface proteins, the suite of surface proteins is different for each.

DISCUSSION

Expression vectors are essential tools for the genetic and physiological investigation of all cell types. Currently available vectors for gene expression in enterococci are plagued by leakiness, limited dynamic range, and the use of toxic inducer molecules. The pheromone-responsive P_Q promoter offers a unique solution to these problems. As demonstrated above, in the presence of the coresident gene for the PrgX negative regulator, the P_Q promoter is tightly repressed, responds to nanogram-per-milliliter concentrations of the innocuous cCF10 peptide pheromone, and has a large expression range over a fairly narrow inducer concentration range (roughly 1 to 20 ng of cCF10 per ml). While the experiments reported here all employed M9YEG medium, cCF10 pheromone-inducible heterologous gene expression also can be used in more complex media such as brain heart infusion or tryptic soy broth, with appropriate adjustments of inducing concentrations of cCF10 (33). Adjustments may also be required for induction under conditions other than exponential phase.

We demonstrated the effectiveness of the pCIE expression vector by controlling the expression of the toxic peptides Fst_{pAD1} and Fst_{EF0409} from the par_{pAD1} and par_{EF0409} TA systems, respectively. In the process of evaluating the features of the P_Q expression system, we uncovered several interesting characteristics of these two TA systems. Previous work demonstrated that RNA I_{pAD1} and RNA II_{pAD1} are more stable together, presumably in complex, than either is alone (34). This makes sense given the PSK function of the par_{pAD1} locus because targeted degradation would leave no toxin mRNA following plasmid loss to be translated. The function of par_{EF0409} is unknown,

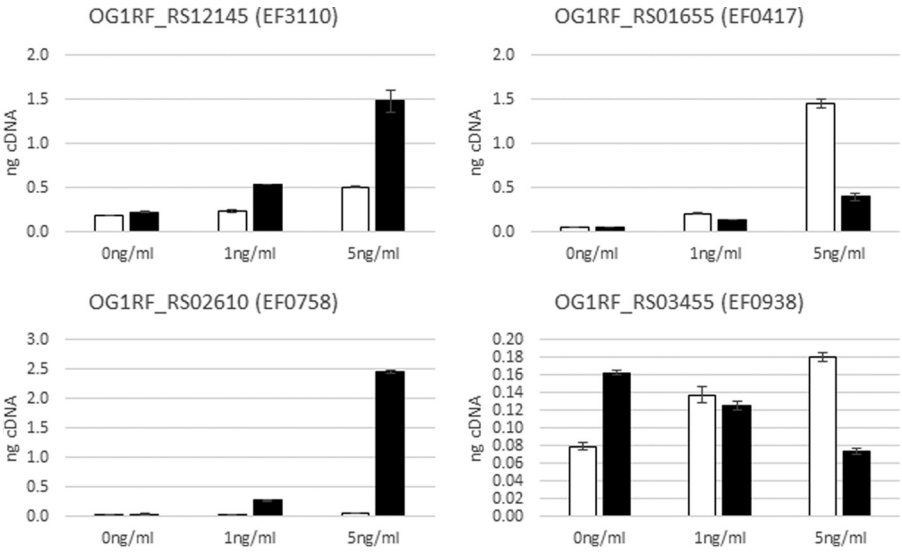

FIG 6 RT-qPCR of selected genes showing differences between the effects of RNA I_{EF0409} (white bars) and RNA I_{pAD1} (black bars) by RNA-seq. RNA samples were prepared and subjected to RT-qPCR as described in Materials and Methods. Transcript levels are depicted as nanograms of cDNA as determined by comparison to a standard curve and normalized to 16S rRNA as described in the text. cCF10 concentrations to which cells were exposed are shown on the *x* axis.

but since a PSK function is unlikely, a different mode of regulation might be expected. Indeed, the fact that RNA I_{EF0409} induction results in a drop in RNA II_{EF0409} suggests that interaction of the par_{EF0409} RNAs has a destabilizing effect (Fig. 2; see also Fig. S3 in the supplemental material). While it is possible that the 5′ extension introduced by expression from the P_Q promoter might have subtle effects on RNA I_{EF0409} structure that alter its interaction with RNA II_{EF0409}, two observations argue against this possibility. First, expression of RNA I_{pAD1} has no detectable effect on the amount of RNA II_{pAD1} expressed from pDAK611 (Fig. S3). Second, we have observed that excess expression of RNA I_{EF0409} from its native promoter on pDAK1001 also results in a decrease in the levels of RNA II_{EF0409} produced from the chromosome (data not shown). The fact that RNA I_{EF0409} seems to persist longer in the absence of RNA II_{EF0409} (Fig. 4C) also suggests that the destabilization effects are reciprocal. It is likely that this regulation pattern is relevant for par_{EF0409} function. Further work will be required to determine what structural features of the RNAs and their complexes account for the differences between the results of interaction of par_{pAD1} and par_{EF0409} RNAs.

Examination of host cell viability after RNA I_{EF0409} expression revealed that while maximal induction has a significantly detrimental effect, a large proportion of cells eventually recover from toxin exposure (Fig. 4A). Recovery is accelerated by the presence of the cognate RNA II, presumably due to a more rapid clearance of induced RNA I. But even without RNA II_{EF0409}, recovery is observed. At lower levels of induction, even though significant changes in the transcriptome occur, little cell viability loss is observed. It seems likely that natural expression of RNA I_{EF0409} never reaches that attainable by cCF10 induction from pCIE and that the Fst_{EF0409} "toxin" exerts its effects without ever killing cells. This is consistent with observations with other TA toxins (35, 36) which have been shown to be involved in the production of persister cells. Whether par_{EF0409} performs a similar function is currently unclear. It is worth noting in this regard that a previous study reported the isolation of an RNA II_{EF0409} mutant in the EryS strain of *E. faecalis* (23). Given the apparent toxicity of RNA I_{EF0409} expression shown in this study, one might expect such a strain to be nonviable due to unregulated translation of RNA I_{EF0409}. However, deletion of the antitoxin gene in spite of demonstrated lethal toxin activity has also been demonstrated in the *Bacillus subtilis bsrE*/SR5 system (37). Possible explanations for this discrepancy include strain and medium differences, exceedingly low expression of the toxin in its native context under standard laboratory

growth conditions, or selection for a suppressor mutation resistant to the effects of toxin expression in the mutant. In spite of the lack of effect on viability, numerous proteomic differences were observed between the wild-type and RNA II_{EF0409} mutant strain (23). There was surprisingly little overlap between the list of protein expression differences in that study and the transcriptomic differences in this study, perhaps suggesting the presence of a suppressor mutation.

par-related loci have been identified throughout the *Firmicutes* through homology of the Fst toxins and similar organizations of the toxin and antitoxin genes (14, 15). However, it has been noted that the putative interaction sites, so far confirmed in only two systems, par_{pAD1} and the *Staphylococcus aureus sprA* locus (19), are not well conserved, possibly allowing specificity of interaction. Results presented here clearly show that RNA II_{EF0409} does not accelerate recovery of cells exposed to RNA I_{pAD1} and that ectopic expression of RNA II_{pAD1} at high levels does not protect cells from expression of RNA I_{EF0409} (Fig. 5), confirming that the two *par* systems do not interfere with one another. Thus, it appears that the plasmid-encoded PSK systems have evolved to avoid interference with the function of chromosomal systems. Other enterococcal plasmids also encode *par* homologs (16), suggesting that multiple related systems may occupy the same cell simultaneously, apparently without interference, though this has not been formally tested.

Type I TA system toxins, including those not related to Fst, are generally small peptides with apparent transmembrane domains. In some cases, a pore-forming function has been indicated (38, 39), but in most others the mechanism of action is unclear and the phenotypic effects do not necessarily require pore formation (22, 40). In the *Bacillus subtilis bsrG*/SR4 system, pore formation has been explicitly ruled out (41). The similarity of the Fst_{EF0409} and Fst_{pAD1} toxins but apparent different functions provided a unique opportunity to determine whether these peptide toxins were simply general pore-forming toxins or had some specificity of action. If the two toxins acted in the same manner, it would be expected that their expression would lead to the induction of identical sets of genes in the cellular response. If they had specific functions, differences in the spectrum of induced genes would be expected. The RNA-seq data presented here, confirmed by RT-qPCR, showed mixed results, with expression of some genes affected by both toxins and others affected only by one or the other. In at least one case, expression was affected in opposite directions by the two toxins. We propose that expression of the two toxins has both general effects, perhaps due to perturbation of membrane organization, and specific effects, perhaps due to interaction of variant amino acid residues with specific target membrane proteins. Further experiments will be required to tease out the details of these interactions.

In summary, we have developed an expression vector system that tightly regulates gene expression in *E. faecalis*, can be induced with nanogram-per-milliliter concentrations of an innocuous peptide, and has a large dynamic range. With modifications of the replicon it may be possible to extend the range of species that this vector will be useful in. We have used this system to examine the regulation and function of two related type I TA toxins. Our results indicate that specific differences in modes of interaction and toxin mechanism of action may relate to differences in function of the two toxins. The pCIE vector system, with its tightly regulated P_Q promoter, will be a useful tool in further examination of these issues.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All bacterial strains and plasmids used in this study are shown in Table 5. All primers used for construction of plasmids and strains are shown in Table 1.

All *E. faecalis* cultures for growth curves and RNA preparation were grown in M9YEG medium (42). Cultures were routinely diluted to 1 to 2% from overnight culture and grown with appropriate antibiotics for plasmid selection. Luria-Bertani (43) medium was used for growth of *E. coli*. All cultures were grown at 37°C with rotary shaking at either 200 rpm (*E. coli*) or 50 rpm (*E. faecalis*). Antibiotics (Sigma-Aldrich) were added at the following concentrations: chloramphenicol, 10 μg ml^{-1} (Cm10) or 25 μg ml^{-1} (Cm25); tetracycline (Tc), 10 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}; erythromycin (Em), 10 μg ml^{-1} for *E. faecalis* and 100 μg ml^{-1} for *E. coli*; streptomycin (Sm), 1,000 μg ml^{-1}; rifampin (Rif), 100 μg ml^{-1}; fusidic acid

TABLE 5 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype	Source or reference
Strains		
DH5α	*E. coli* intermediate host for cloning	Invitrogen
JRC101	OG1RF strain defective in endogenous cCF10 production	30
OG1RF	Spontaneous Rif^r Fus^r derivative of OG1 (56)	57
OG1RF Δpar_{EF0409}	OG1RF with deletion of the complete par_{EF0409} locus	This work
Plasmids		
pWM401	Cm^r *E. coli-E. faecalis* shuttle vector	58
pCIE	Cm^r *E. coli-E. faecalis* shuttle expression vector with cCF10-regulatable promoter *prgQ*	This work
pDAK1010E	pCIE containing a promoterless RNA I_{EF0409} gene downstream of *prgQ*	This work
pDAK1010A	pCIE containing a promoterless RNA I_{pAD1} gene downstream of *prgQ*	This work
pDAK611	pDL278 clone containing the RNA II_{pAD1} gene under the control of its native promoter	12
pDAK1001	pWM401 containing the RNA I_{EF0409} gene	This work
pGEM-T Easy	*E. coli* cloning vector for PCR products	Promega

(Fus), 25 μg ml^{-1}; and spectinomycin (Sp), 100 μg ml^{-1}. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.033 mM; Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg ml^{-1}; Gold Biotechnology, St. Louis, MO) were used for screening of pGEM-T Easy clone and allelic-exchange experiments. Solid medium was produced by adding 1.7% agar (Fisher Scientific, Pittsburgh, PA). Peptide pheromone cCF10 (H-LVTLVFV-OH) was obtained from Mimotopes (Clayton, Australia), dissolved in dimethylformamide, and stored at −20°C.

For pheromone induction experiments, cultures were grown overnight in M9YEG-Cm25 to ensure maximum retention of the pCIE constructs. Cultures were diluted at 1 to 2% in M9YEG-Cm10. cCF10 was added 1 h after inoculation to ensure that the cells were out of lag phase. In viability experiments, samples were removed each hour after pheromone induction and appropriate dilutions were plated on M9YEG or M9YEG-Cm10. All viability experiments were performed in triplicate.

DNA purification and manipulation. Genomic DNA was purified from OG1RF as described previously (44). Plasmid DNA was purified from *E. coli* strains utilizing the Quantum Prep plasmid miniprep kit (Bio-Rad, Hercules, CA) or using the Qiagen midiprep kit (Qiagen, Valencia, CA). DNA sequencing was performed at the DNA sequencing facility at Iowa State University (Ames, IA). Electroporation of *E. faecalis* cells was performed using glycine-treated electrocompetent cells prepared and electroporated as previously described (44, 45) or using lysozyme-treated cells as previously described (46). DNA was introduced into competent *E. coli* DH5α cells according to the manufacturer's instructions (Invitrogen). PCR was performed using either PCR-HiFi Supermix or Platinum PCR kits from Invitrogen as previously described (44). PCR products were prepared for cloning or sequencing using Wizard Plus SV miniprep columns (Promega) according to the manufacturer's instructions. Restriction digests and ligations were performed with enzymes acquired from either Promega or New England BioLabs (Ipswich, MA) according to the manufacturer's instructions.

Strain and plasmid construction. OG1RF Δpar_{EF0409}, with a deletion of the chromosomal copy of the par_{EF0409} locus, was constructed utilizing the protocol of Kristich et al. (5), modified as in the work of Brinkman et al. (44). Primers used to construct deletions of alleles and verify deletions are shown in Table 1. PCR was performed with primers P1 and P2 and primers 5′RNAIIdel and P4, with OG1RF genomic DNA as the template. PCR products were cleaned, cloned into pGEM-T Easy, and transformed into DH5α competent cells. Plasmid DNA was purified from white colonies and inserts were sequenced using pGEM-T Easy-specific primers to ensure that no extraneous mutations were introduced. Deletions of alleles were constructed by fusing the relevant clones at their introduced BamHI sites by cutting the pGEM clones with this enzyme and then religating. The desired allele was then amplified by using the P1 and P4 primers, recloned into pGEM-T Easy, and resequenced. The fusion fragments were then subcloned into the allelic-exchange vector pCJK47 utilizing the introduced restriction sites on the ends of P1 and P4 and utilized for allelic-exchange experiments. Appropriate deletions were confirmed by colony PCR (44) using primers EF0409Up and EF0409Down, followed by sequencing. Failure to produce the par_{EF0409} RNAs was confirmed by Northern blotting. OG1RF Δpar_{EF0409} has a deletion of DNA from OG1RF genomic sequence nucleotide 309585, 100 nucleotides upstream of the RNA II_{EF0409} promoter, to 309936.

pDAK1001 contains the RNA I_{EF0409} gene under the control of its native promoter cloned into shuttle vector pWM401 (Table 5). For its construction, the RNA I_{EF0409} gene was amplified by PCR using primers EF0409RNAI-SalI and EF0409RNAI-BamHI (Table 1). The PCR product was cleaned as described above, cloned directly into pWM401 utilizing the introduced restriction sites, and transformed into competent DH5α cells. Transformants were selected on Cm-containing plates and screened for Tc sensitivity. The presence of an insert of the proper size was determined by agarose gel electrophoresis, and insert DNA was sequenced using the pACYC184-seq primer (Table 1). DNA was then purified from the *E. coli* strains for introduction into *E. faecalis* strains by electroporation.

pCIE is an *E. coli-E. faecalis* shuttle cloning vector in which cloned genes can be placed under the control of the pheromone-responsive promoter P_Q. The vector includes a copy of the PrgX regulator that represses the P_Q promoter and responds to the cCF10 pheromone. Also included is the *prgQ* gene, which includes iCF10, a competitive inhibitor of cCF10 that further tightens repression. The plasmid was

constructed by cloning *prgXQ* through the IRS1 region from pBK2 (47) digested with BamHI and PstI into pCI372 (48) digested with the same enzymes. A fragment containing a multiple-cloning site (MCS) with recognition sites for BamHI, SalI, SphI, and HindIII and an additional transcriptional terminator, IRSX (the native *prgX* terminator), was amplified from pBK2 using primers IRSX and IRSX-R (Table 1) and added using restriction enzymes BamHI and EcoRI. The IRSX terminator is located downstream of the MCS to prevent read-through into the rest of the plasmid from the induced P_Q promoter. A map and the sequence of pCIE are shown in Fig. S1 and S2 in the supplemental material.

pDAK1010E (pCIE::RNA I_{EF0409}) was constructed by amplifying a promoterless version of the RNA I_{EF0409} gene using PCR primers RNAIpCIE5′ and RNAIpCIE3′ (Table 1). This DNA fragment contains OG1RF nucleotides 309752 and 309972 (complement) and includes the putative 5′ stem-loop structure that modulates Fst expression in the analogous par_{pAD1} system (17) and the transcription terminator. The PCR fragment was cleaned and cloned into pGEM-T Easy and transformed into DH5α cells. Plasmid DNA was purified, and samples showing the correct restriction profile were sequenced. The fragment was then subcloned into pCIE using the primer-introduced BamHI site and the pGEM-T Easy SphI site and transformed into DH5α. Plasmid DNA purified with the Bio-Rad Quantum Prep miniprep kit and confirmed to have the proper restriction pattern was then introduced into relevant *E. faecalis* strains by electroporation (45). Plasmid DNA was then purified and sequenced again to make sure no unexpected base changes had occurred.

pDAK1010A (pCIE::RNA I_{pAD1}) was constructed by transferring a commercially synthesized fragment (Blue Heron Biotechnology, Bothell, WA) from the provided pUCminus MCS vector to pCIE. The fragment contained DNA sequence from the pAD1 plasmid from nucleotide 4083 to 4317 (complement), using the sequence with GenBank accession number L01794.1 with BamHI and SphI flanking for cloning into pCIE in the appropriate orientation. As for pDAK1010E, the fragment contains the translational modifying 5′ stem-loop structure and terminator. DNA was purified and introduced into *E. faecalis* as described above.

RNA purification and manipulation. Cultures for RNA preparation were inoculated at 2% from cultures grown overnight in M9YEG and the appropriate antibiotic, allowed to grow for 1 h to ensure that they had entered log phase, and then induced with the desired concentration of pheromone.

RNA isolation for Northern blotting was performed using the Fast RNA Pro system (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. In time course experiments, volumes of cells harvested at each time point were adjusted to ensure that similar cell amounts were obtained. Samples were harvested in a refrigerated Beckman J2-21 centrifuge at 15,000 $\times$ *g* at 4°C, resuspended in RNA Pro solution, and stored at 4°C overnight or until all samples from a single experiment were acquired. RNA samples were fractionated on polyacrylamide gels, transferred to a membrane, and hybridized to end-labeled oligonucleotide probes as previously described (34). Probe sequences are given in Table 1.

For RT-qPCR experiments, strains were induced with the desired cCF10 concentration for 1 h before harvest. Cells were harvested as described above. Cell pellets were resuspended in Bacteria RNA Protect (Qiagen) and incubated at room temperature for 5 min. Cells were then pelleted and stored at −80°C. Once all samples were collected, pellets were thawed, resuspended in 200 μl of Tris/EDTA-mutanolysin-lysozyme solution (10 mM Tris [pH 8.0], 1.0 mM EDTA [pH 8.0], mutanolysin [500 U ml^{-1}], lysozyme [30 mg ml^{-1}]), and incubated at 37°C for 10 min. To the resuspensions, 700 μl of buffer RLT (Qiagen RNeasy minikit) with 10 μl of β-mercaptoethanol (β-ME) was added, with vortexing to mix. RNA was then purified using the Qiagen RNeasy minikit according to the manufacturer's instructions. Total RNA from biological replicates was treated with TURBO DNase (Ambion/Thermo Scientific, Waltham, MA) using the rigorous protocol according to the manufacturer's instructions. DNase was removed from treated samples using the RNA Clean & Concentrator-25 (Zymo Research). One microgram of RNA was reverse transcribed with random nonamers (New England BioLabs, Ipswich, MA) using SuperScript III RT (Invitrogen/Life Technologies) according to the manufacturer's instructions. The RT reaction mixtures were diluted 10-fold with water and aliquoted and stored at −80°C. Equal volumes of cDNA were used in 25-μl qPCR mixtures with SYBR green (Quanta Biosciences, Gaithersburg, MD) and measured on an ABI 7300 system (Applied Biosystems [Life Technologies]) using the standard amplification cycle with a melting-curve analysis. Results were compared to a standard curve generated against purified OG1RF genomic DNA. RT-qPCR results were corrected for efficiency (typically above 90%, with r^2 values above 0.999). Primer sequences are listed in Table 1 and were designed using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA). To normalize for variation between samples, qPCR was also performed with primers specific for *E. faecalis* 16S rRNA. rRNA quantities from each sample used in an experiment were averaged, and then quantities of the gene of interest were adjusted based on the ratio of the rRNA quantity to that average.

For RNA-seq, RNA was purified as described above, with the further step of rRNA removal using the MICROB*Express* bacterial mRNA purification kit (Ambion/Thermo Scientific) according to the manufacturer's instructions. Illumina RNA-seq library creation and sequencing, using the HiSeq 2500 (paired-end, 125 bp) platform, were performed at the University of Minnesota Genomics Center (UMGC). Sequence analysis on paired end reads was carried out using the Galaxy server (49–51), maintained by the Minnesota Supercomputing Institute (MSI; University of Minnesota). FASTQ Quality Trimmer by sliding window (49) was used to remove the 3′ ends of raw sequencing reads that had a Phred quality score of <20. FASTQ Interlacer and FASTQ de-interlacer were used to remove any unmatched paired end reads, followed by mapping to the OG1RF reference genome (NC_017316.1/CP002621.1) (52) using Bowtie for Illumina (53). The Bowtie SAM files were sorted by chromosome and start position using Picard Sortsam (54). For each culture condition, Cufflinks (55) was used to assemble the transcripts and determine expression levels (FPKM). The generated gtf files created for biological replicates, for the two conditions

to be compared, were assembled using Cuffmerge (55). Cuffdiff (55) was used to determine significant differences in transcriptional expression between culture conditions with the false-discovery rate at 0.05. The percentage of mapped reads (~93% for all samples) compared to total reads (~20 million for each sample) was determined using Flagstat (54).

Accession number(s). RNA-seq raw data files as well as the processed files on which the conclusions of this report are based are available in the GEO database under accession number GSE96072. The sequence of pCIE was deposited in GenBank with the accession number KY774666.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00065-17.

SUPPLEMENTAL FILE 1, XLSX file, 1.2 MB.
SUPPLEMENTAL FILE 2, XLSX file, 1.2 MB.
SUPPLEMENTAL FILE 3, XLSX file, 1.2 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.5 MB.

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