PrgU: A Suppressor of Sex Pheromone Toxicity in *Enterococcus faecalis*

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SUMMARY

Upon sensing of the peptide pheromone cCF10, *Enterococcus faecalis* cells carrying pCF10 produce three surface adhesins (PrgA, PrgB or Aggregation Substance, PrgC) and the Prg/Pcf type IV secretion system and, in turn, conjugatively transfer the plasmid at high frequencies to recipient cells. We report that cCF10 induction is highly toxic to cells sustaining a deletion of *prgU*, a small *orf* located immediately downstream of *prgB* on pCF10. Upon pheromone exposure, these cells overproduce the Prg adhesins and display impaired envelope integrity, as evidenced by antibiotic susceptibility, misplaced division septa, and cell lysis. Compensatory mutations in regulatory loci controlling expression of pCF10-encoded *prg/pcf* genes, or constitutive PrgU overproduction, block production of the Prg adhesins and render cells insensitive to pheromone. Cells engineered to overproduce PrgB, even independently of other pCF10-encoded proteins, have severely compromised cell envelopes and strong growth defects. PrgU has an RNA-binding fold, and *prgB-prgU* gene pairs are widely distributed among *E. faecalis* isolates and other enterococcal and staphylococcal species. Together, our findings support a model in which PrgU proteins represent a novel class of RNA-binding regulators that act to mitigate toxicity accompanying overproduction of PrgB-like adhesins in *E. faecalis* and other clinically-important Gram-positive species.

Graphical Abstract

Upon sensing of sex pheromone, *Enterococcus faecalis* cells carrying pCF10 produce PrgB (Aggregation Substance, AS) and a type IV secretion system responsible for high-frequency plasmid transfer. We show PrgB overproduction is highly toxic to *E. faecalis* cells, and that PrgU mitigates toxicity by downregulating PrgB synthesis. PrgU is a predicted RNA binding protein, and *prgB-prgU* gene pairs are widely dispersed among enterococci suggestive of a conserved mechanism of feedback regulation of a major surface adhesin.

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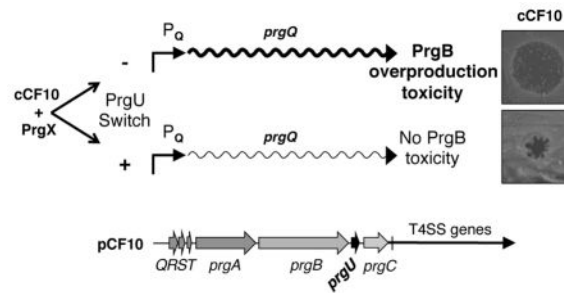
Keywords

Gram-positive cell surface adhesins; cell death; PUA domain; sex pheromone; Enterococcus; gene regulation

INTRODUCTION

Enterococcus faecalis infections are increasingly recognized as serious clinical threats due to the acquisition of multiple antibiotic resistance and the capacity of these organisms to rapidly disseminate resistance and virulence traits by lateral gene transfer (Lebreton *et al.*, 2013, Van Tyne & Gilmore, 2014). Many *E. faecalis* clinical isolates harbor members of a large family of conjugative plasmids whose transmission is induced by sensing of peptide pheromones (Dunny, 2013, Dunny & Berntsson, 2016). These plasmids typically code for antibiotic resistance as well as surface proteins such as bacteriocins (cytolysin) or adhesins (PrgB, Esp) of established importance for tissue attachment and biofilm formation (Clewell *et al.*, 2014). Many *E. faecalis* clinical isolates also carry fragments of the pheromone responsive plasmids encoding virulence traits in their chromosomes, underscoring both the selective advantages of the plasmid-borne elements and the plasticity of enterococcal genomes (McBride *et al.*, 2007, Palmer *et al.*, 2010).

One of the best-characterized representatives of the family of pheromone responsive plasmids is the tetracycline-resistance plasmid pCF10 (Dunny, 2013, Dunny & Berntsson, 2016). Detailed studies have unveiled a complex regulatory circuitry that operates to regulate pheromone-dependent expression of the plasmid-borne *prgQ* operon. The *prgQ* operon has three cassettes of genes encoding proteins of importance for plasmid transfer in natural settings such as biofilms (Bhatty *et al.*, 2015). One cassette codes for three surface adhesins (PrgA, PrgB, PrgC) and an uncharacterized protein (PrgU), a second for the Prg/Pcf type IV secretion system (T4SS), and a third for DNA transfer and replication (Dtr) factors required for processing of pCF10 prior to its delivery through the T4SS (Fig. 1A) (Dunny, 2013). The *prgQ* operon is expressed from the P_Q promoter, which is repressed by binding of the transcriptional regulator PrgX (Nakayama *et al.*, 1994, Kozlowicz *et al.*, 2006b). When cells import the cCF10 sex pheromone (LVTLVFV), secreted by plasmid-free enterococci in the vicinity, PrgX interacts with the pheromone and undergoes a structural transition resulting in P_Q activation (Kozlowicz *et al.*, 2006a, Kozlowicz *et al.*, 2006b). Within 15 – 30 min of exposure to pheromone, donor cells undergo a burst of transcriptional activity and Prg/Pcf protein synthesis. They form intercellular aggregates primarily as a result of synthesis of

PrgB (also known as Aggregation Substance or AS) and conjugatively transfer pCF10 at high frequencies approaching 1 transconjugant per donor (Hirt *et al.*, 2005, Bhatty *et al.*, 2015). Then, within the next 1 – 2 h, donor cells enter a shut-down phase characterized by a return of *prgQ* transcription to preinduction levels (Hirt *et al.*, 2005, Chatterjee *et al.*, 2013).

A number of transcriptional and posttranscriptional mechanisms control expression of the *prgQ* operon, presumptively to ensure tight regulation of the energetically expensive processes associated with Prg/Pcf T4SS assembly and plasmid transfer (Fig. 1A) (Johnson *et al.*, 2010, Chatterjee *et al.*, 2011, Dunny & Johnson, 2011). Despite this complex regulatory circuitry, we recently gained evidence that a subpopulation of pheromone induced pCF10-carrying cells undergo lysis during early stages of biofilm development as a result of overproduction of the Prg surface proteins (Bhatty *et al.*, 2015). When exposed to pheromone, OG1RF cells carrying pCF10 form considerably thicker biofilms than plasmid-free OG1RF, and these biofilms possess abundant amounts eDNA, polysaccharides, and other matrix materials. Confirming the importance of the Prg surface adhesins, pheromone-treated OG1RF cells engineered to carry a plasmid expressing only *prgA-C* genes among the *prgQ*-regulated *prg/pcf* genes similarly develop thick biofilms with a pronounced accumulation of lysed cells and matrix components. In view of these findings, we hypothesized that the stochastic overproduction of Prg surface proteins in a subpopulation of pheromone-induced cells has lethal consequences (Bhatty *et al.*, 2015).

Here, we tested this model by evaluating the contributions of proteins encoded within the *prgA-C* cassette to pheromone-mediated toxicity. Strikingly, we discovered that deletion of a small *orf* termed *prgU* located immediately downstream of *prgB* (Fig. 1A) confers strong growth defects when cells are exposed to cCF10 pheromone. We present several lines of evidence that PrgU functions as a suppressor of sex pheromone-mediated cell toxicity by blocking overproduction of the Prg surface adhesins. Indeed, overproduction specifically of PrgB in the presence or absence of other pCF10-encoded proteins severely compromises cell envelope integrity, as evidenced by sensitivity to antibiotics, misplaced division septa, and cell lysis. *prgB-prgU* gene pairs are widely distributed on plasmids or chromosomes of *E. faecalis* clinical isolates and other enterococci. Our cumulative findings thus suggest that feedback control by PrgU serves to maintain cell envelope homeostasis by blocking overproduction of PrgB-like adhesins in *E. faecalis* and related species.

RESULTS

PrgU regulates production of Prg surface adhesins and the Prg/Pcf T4SS

The P_Q promoter and flanking regulatory region controls expression of the ~28-kb *prgQ* operon (Hirt *et al.*, 2005). Our recent studies of the Prg adhesins encoded by the *prgA-C* gene cassette established their importance for intercellular aggregation and biofilm development, but surprisingly not for high-frequency transfer of pCF10 (Bhatty et al., 2015). Immediately downstream of *prgB* in the *prgA-C* cassette is an uncharacterized *orf* termed *prgU* (Fig. 1A), which is predicted to encode a 106-residue, cytosolic protein. We deleted *prgU* from pCF10, and determined that OG1RF cells transferred the pCF10 *prgU* mutant plasmid at frequencies comparable to wild-type (WT) pCF10 in short-term (1 h) matings and at slightly lower frequencies in longer (2 h) matings on solid surfaces (Fig. 1B).

Interestingly, however, within 1 h of exposure to exogenous cCF10 pheromone, OG1RF(pCF10 $prgU$) cells accumulated the PrgA, PrgB, and PrgC surface adhesins at levels 2-fold higher than comparably-treated OG1RF(pCF10) cells (Fig. 1C). No corresponding increases in protein levels were detected for the downstream-encoded T4SS subunits, PrgJ or PcfC.

Within 30 min of exposure to cCF10 pheromone, OG1RF(pCF10) cells accumulate abundant levels of P_Q transcripts spanning the length of the $prgQ$ operon, and then over the next 1 – 2 hours transcript levels return to pre-induction levels (Hirt *et al.*, 2005). OG1RF harboring pCF10 $prgU$ displayed a similar transcription profile, with the exception that at 1 h following pheromone induction, transcript levels remained high, suggestive of a block or delay in P_Q promoter shutdown (Fig. S1). It is interesting to note that we did not detect elevated levels of the T4SS subunits (Fig. 1C) even though levels of the corresponding transcripts remained high (Fig. S1). We suspect this is because these Prg/Pcf subunits assemble in specific stoichiometries as stable type IV machines, as shown for other T4SSs (Low *et al.*, 2012), and that excess amounts of machine subunits are shunted to a degradative pathway.

OG1RF(pCF10 $prgU$) cells carrying pMB11, which expresses $prgU$ from the constitutive P_{23} promoter, accumulated PrgU at levels ~4–5 fold relative to levels detected in pheromone-induced OG1RF(pCF10) cells (Fig. 1C). Strikingly, the $prgU$ mutant strain harboring pMB11 accumulated only a very low level of PrgA and undetectable amounts of PrgB and downstream-encoded Prg/Pcf proteins (Fig. 1C). Similarly, OG1RF(pCF10) cells carrying pMB11 overproduced PrgU and accumulated low or undetectable levels of the Prg/Pcf proteins. As expected, PrgU overproduction strongly suppressed transfer of both pCF10 and pCF10 $prgU$ to undetectable levels in short-term (1 h) matings, and by 3- to 4-orders of magnitude in 2 h or overnight matings (Fig. 1B). The phenotypes associated with the $prgU$ mutation and PrgU overproduction suggested that PrgU negatively regulates Prg protein synthesis in response to pheromone induction.

The $prgU$ mutation confers pheromone toxicity

During these initial studies, we discovered that the $prgU$ mutation also confers striking growth defects upon exposure of cells to cCF10. Pheromone toxicity was shown with complementary serial dilution (Fig. 2A), pheromone spot (Fig. 2B), and growth curve (Fig. 2C) assays. As shown with each assay, pheromone treatment strongly inhibited growth of OG1RF(pCF10 $prgU$) without affecting growth of isogenic OG1RF(pCF10) cells or the $prgU$ mutant strain harboring the PrgU-overproducing plasmid pMB11 (Figs. 2A, B & C). The $prgU$ mutation was unique among our collection of prg or pcf mutations ($prgA$, $prgB$, $prgU$, $prgC$, $pcfC$, $prgK$, $prgJ$) in conferring pheromone growth suppression (Fig. S2) (Chen *et al.*, 2008, Li *et al.*, 2012, Laverde Gomez *et al.*, 2014, Bhatty *et al.*, 2015). Furthermore, pheromone toxicity of the $prgU$ mutant required active cell growth, as shown by the inhibitory effects of pheromone on cells in early-exponential but not stationary phases of growth (Fig. S2).

U-Res mutants accumulate compensatory mutations conferring pheromone insensitivity

Variants of OG1RF(pCF10 *prgU*), designated as U-Res, arose as colonies within the zone of inhibition in the pheromone spot assay (Fig. 2B) and when undiluted cell cultures were plated on pheromone-containing media (Fig. 2A). The proliferation of U-Res variants also accounted for the eventual increase in cell density of the *prgU* mutant when grown in the presence of pheromone (Fig. 2C), as deduced from findings that all tested isolates from a stationary-phase (16-h) culture exhibited pheromone-insensitive growth (data not shown). We sequenced the genome of one U-Res variant, designated *R1*, and identified a single frame-shift mutation at basepair 271 of *prgR*, which is a putative regulator of *prgB* expression (Fig. 3A) (Chung & Dunny, 1992). The U-Res *R1* strain accumulated detectable levels of the PrgA surface adhesin, but not of PrgB or PrgC or other Prg or Pcf proteins encoded by the distal region of the *prgQ* operon (Fig. 3B). Correspondingly, this variant accumulated detectable amounts of transcript for *prgA* but not for *prgB* or other downstream *prg* or *pcf* genes (Fig. S1). Previous studies established that pheromone induction of the P$_Q$ promoter generates a long *prgQ* transcript spanning the entire *prgQ* operon, and that independently of pheromone induction a distinct promoter within the *prgQ* regulatory region generates a transcript that spans only *prgA* (see Fig. 1A) (Bensing & Dunny, 1997). Our data thus suggest that the *prgR* 271 mutation blocks production of the long *prgQ* transcript from the pheromone-inducible P$_Q$ promoter without affecting synthesis of the short *prgA* transcript.

We confirmed that the *prgR* 271 mutant allele was responsible for the observed block in production of the Prg and Pcf proteins by *trans*-expression of *prgR* alone (data not shown) or together with *prgS* from plasmid pMC003 (Fig. 3). Reminiscent of the *prgU* parental strain, the complemented *R1* strain exhibited pheromone sensitivity and abundant production of PrgB (Figs. 3B, C). Furthermore, whereas the *R1* strain was completely defective for plasmid transfer, *trans*-expression of *prgR,prgS* in the *R1* strain supported transfer at levels approaching the *prgU* parental strain (Fig. 3C). We also analyzed the effects of *prgR,prgS* *trans*-expression in OG1RF(pCF10). OG1RF(pCF10, pMC003) did not display pheromone toxicity (Fig. 3C), but interestingly accumulated PrgA and PrgB at reduced levels relative to OG1RF(pCF10) (Fig. 3B). These findings extend earlier work implicating the *prgR/S* loci as regulators of *prgB* expression (Chung & Dunny, 1992, Bensing & Dunny, 1997). It is interesting to note, however, that the PrgR/S regulators act positively in a *prgU* mutant and negatively in pCF10-carrying cells with respect to production of PrgB and other downstream-encoded Prg/Pcf proteins. These strains differ only in their capacity to produce PrgU, raising the possibility that PrgR and PrgS coordinate with PrgU in some way to regulate pheromone-inducible expression of the *prgQ* operon.

We analyzed 9 other U-Res strains (designated *R2 – R10*) recovered from a pheromone spot assay for Prg protein production and conjugative transfer (Fig. S3A). Reminiscent of the *R1* variant, these strains produced undetectable or very low levels of the PrgB or PrgC surface adhesins and of the downstream-encoded PrgJ or PcfC T4SS subunits (Fig. S3B; data not shown). In matings, only two of the U-Res variants were capable of low-frequency plasmid transfer (Fig. S3C). Sequence analyses confirmed that nearly all of the *R2–R10* mutant strains accumulated mutations in *prgR* or the adjacent putative regulator *prgS*,

although several strains also had mutations elsewhere in the *prgQ* regulatory region (Fig. S3C). Together with our studies of the *R1* variant, these findings suggest that compensatory mutations accumulate in *prgR* or *prgS*, or elsewhere in the *prgQ* regulatory region, to mitigate pheromone toxicity associated with production of one or more of the Prg/Pcf proteins..

The *prgU* mutation confers strong growth defects

We gained four lines of evidence that pheromone induction impairs viability of the *prgU* mutant through disruption of cell envelope integrity. First, OG1RF(pCF10 *prgU*) cells exhibited profound sensitivity to bile salts (Fig. S4). The *prgU* mutant grew poorly upon exposure to pheromone and even more poorly with additional exposure to sodium cholate or sodium deoxycholate. By contrast, OG1RF(pCF10), OG1RF(pCF10 *prgU*) in the absence of pheromone, or OG1RF(pCF10 *prgU*) expressing P$_{23}$::*prgU* from pMB11 were not growth suppressed in the presence of bile salts (Fig. S4).

The *prgU* mutant also exhibited exquisite sensitivity to antibiotics (Fig 4A, B). OG1RF(pCF10) cells showed no growth impairment when exposed to antibiotics to which they encode resistance (Rif[200], Fus[25], Tet[10]) or to subinhibitory concentrations of antibiotics commonly used to treat *E. faecalis* infections (Dap[6], Lin[0.25]). OG1RF(pCF10 *prgU*) also grew in these antibiotic-containing media in the absence of pheromone exposure, as did this strain when engineered to overproduce P$_{23}$::*prgU* from pMB11 regardless of pheromone treatment. When exposed to pheromone, however, OG1RF(pCF10 *prgU*) failed to grow in the presence of all of the tested antibiotics over a 16-h incubation period (Fig. 4A). These findings suggested not only that OG1RF(pCF10 *prgU*) is highly sensitive to antibiotics, but further that the combination of pheromone and antibiotic treatments strongly suppressed even the appearance and outgrowth of the U-Res variant subpopulation. The synergistic effects of pheromone and antibiotic treatments also were evident with a disc diffusion assay (Fig. 4B).

We next used a quantitative β-galactosidase (β-Gal) release assay to test for pheromone-dependent permeabolization of OG1RF(pCF10 *prgU*) cells (Fig. 4C). OG1RF(pCF10) and OG1RF(pCF10 *prgU*) lacking or containing the P$_{23}$::*prgU* expression plasmid were engineered to constitutively express a *lacZ* reporter. Cells were grown in the absence or presence of pheromone in media containing chlorophenyl red-β-D-galactopyanoside (CPRG), a membrane-impermeable substrate of β-Gal (Paradis-Bleau *et al.*, 2014). Intact cells producing cytoplasmic β-Gal do not degrade extracellular CPRG, whereas cells with elevated permeability due to cell envelope alterations exhibit higher β-Gal activities as a result of CPRG entry or β-Gal release to the milieu. We monitored β-Gal activity in culture supernatants over 6-hr following pheromone induction, a period during which the *prgU* mutant accumulates abundant amounts of the Prg proteins without proliferation of a U-Res variant population. In the absence of pheromone induction, OG1RF(pCF10) did not appreciably hydrolyze CPRG, in line with previous findings (Djoric & Kristich, 2015, Dale *et al.*, 2015). Within 2 h of pheromone treatment, OG1RF(pCF10) showed a slight increase in CPRG hydrolysis compared with the uninduced culture, but this increase was not statistically significant and it also was transient as shown by a reduction in CPRG hydrolysis

to basal levels at 4 h and 6 h post-induction. In striking contrast, within 2 h and increasingly at 4 h and 6 h following pheromone induction, OG1RF(pCF10 *prgU*) showed statistically-significant increases in CPRG hydrolysis that were not observed in the absence of pheromone (*P*<0.05). OG1RF(pCF10 *prgU*) overproducing P$_{23}$::*prgU* from pMB11 hydrolyzed CPRG at low levels regardless of pheromone exposure throughout the duration of the experiment. Pheromone induction thus strongly perturbs the *prgU* mutant cell envelope during a period of robust *prgQ* expression and Prg/Pcf protein synthesis.

Finally, ultrastructural studies showed that the pheromone-exposed OG1RF(pCF10 *prgU*) cells were morphologically aberrant (Fig. 4D). OG1RF(pCF10) and OG1RF(pCF10 *prgU*) cells expressing P$_{23}$::*prgU* from pMB11 grew as typical ovoid diplococci in short chains and clumps regardless of pheromone treatment. In striking contrast, pheromone-exposed OG1RF(pCF10 *prgU*) cells exhibited defects in placement of division septa, resulting in aberrantly large and small cells. Cells also were elongated and some possessed partial invaginations, suggestive of incomplete formation of septa. Membrane blebbing and the apparent release of cytoplasmic contents also were evident, suggestive of partial or complete disruption of the cell envelope.

PrgB (Aggregation Substance) contributes to pheromone toxicity

Having established that the *prgU* mutation correlates with enhanced levels of the Prg surface proteins (Fig. 1C) and with a disruption of cell envelope integrity (Figs. S4 and 4), we asked whether pheromone toxicity could be recapitulated in an OG1RF strain producing only the Prg surface proteins. To this end, we constructed a miniature version of pCF10, designated p10-mini, which carries: i) *prgP-prgY* encoding the pheromone sensing and uptake system, ii) the *prgQ* regulatory region, and iii) the *prgA-C* gene cassette from pCF10 with or without *prgU*. Interestingly, OG1RF(p10-mini *prgU*) displayed phenotypes similar to those of OG1RF(pCF10 *prgU*), including pheromone-dependent overproduction of the Prg surface adhesins (Fig. S5A), growth inhibition (Fig. S5B), exquisite sensitivity to subinhibitory concentrations of antibiotics (Fig. S5C), and morphological aberrations (Fig. S5D). OG1RF(p10-mini) or OG1RF(p10-mini *prgU*) expressing P$_{23}$::*prgU in trans* showed no discernible pheromone-mediated growth defects (Figs. S5B, C, D), strongly supporting the notion that overproduction of one or more of the Prg surface adhesins is responsible for pheromone toxicity.

Next, we constitutively expressed each of the *prgA*, *prgB* or *prgC* genes from the P$_{23}$ promoter in otherwise plasmid-free OG1RF. At the outset, we noted that OG1RF electroporated with the P$_{23}$::*prgB* expression plasmid formed only a few small colonies on the transformation plates, whereas transformation with the P$_{23}$ vector and P$_{23}$::*prgA* and P$_{23}$::*prgC* expression plasmids yielded many hundreds of colonies. OG1RF cells also were rapidly cured of the P$_{23}$::*prgB* expression plasmid but not of the P$_{23}$::*prgA* or P$_{23}$::*prgC* expression plasmids (Fig. 5A). In this plasmid curing assay, cultures were grown overnight in the absence of spectinomycin antibiotic selection for maintenance of the P$_{23}$ expression plasmids, and then serially diluted on plates lacking or containing spectinomycin. Strikingly, OG1RF transformed with the P$_{23}$::*prgB* expression plasmids grew well on antibiotic-free media, but formed only a few small colonies on the spectinomycin-containing media (Fig.

5A). We were unable to isolate the P$_{23}$::*prgB* expression plasmid from colonies appearing on antibiotic-free plates, suggesting that the bulk population of cells had lost the plasmid in the absence of antibiotic selection (data not shown). In contrast to these findings, strains carrying the P$_{23}$ vector or the P$_{23}$::*prgA* or P$_{23}$::*prgC* expression plasmids retained their plasmids following overnight cultivation in the absence of antibiotics, as shown by comparable growth on media with and without spectinomycin (Fig. 5A).

We attempted to assay the OG1RF cells carrying the P$_{23}$::*prgB* expression plasmid for sensitivity to bile salts and antibiotics, but these experiments were complicated by the apparent rapid accumulation of compensatory mutations by cells grown under conditions selective for maintenance of the P$_{23}$::*prgB* expression plasmid. However, when cells from colonies just emerging upon transformation with the P$_{23}$::*prgB* expression plasmid were examined by transmission electron microscopy, they exhibited abundant morphological aberrations highly similar to those of pheromone-exposed cells harboring pCF10 *prgU* (compare Figs. 4D, 5B). By contrast, cells freshly transformed with the *prgA* or *prgC* expression plasmids showed very few ultrastructural abnormalities (Fig. 5B). We obtained similar results to those described above when a strain of OG1RF harboring pCF10 *prgA-C*, a plasmid deleted of the *prgA-C* cassette, was transformed with the P$_{23}$::*prgA*, P$_{23}$::*prgB*, and P$_{23}$::*prgC* expression plasmids (Fig. S6A–C).

Interestingly, however, in a pheromone spot assay, OG1RF(pCF10 *prgA-C*) showed a slight growth suppression despite the fact that this strain does not produce PrgB or the other surface proteins (Fig. S6D). Although introduction of the P$_{23}$::*prgB* expression plasmid disrupted growth of OG1RF(pCF10 *prgA-C*) cells even in the absence of pheromone induction, the additional exposure to pheromone imposed an additional stress that strongly suppressed further growth (Fig. S6D). The PrgB-overproducing strain formed considerably more U-Res-type variants than the *prgU* mutant in the zone of pheromone growth inhibition. We suspect that most of these variants arose through loss of the P$_{23}$::*prgB* expression plasmid, although compensatory U-Res mutations also might have arisen that enabled growth in the presence of PrgB overproduction. Overall, these data corroborate with our other findings indicating that PrgB overproduction is highly toxic to *E. faecalis* cells, but further suggest that one or more additional pheromone-induced factors probably encoded by pCF10 imposes an additional stress that exacerbates PrgB overproduction toxicity.

DISCUSSION

Extensive studies of the cCF10 pheromone sensing pathway of plasmid pCF10, and other pheromone plasmids, have unveiled a complex regulatory circuitry that controls expression of genes encoding surface adhesins and conjugation functions (Clewell, 2007, Dunny, 2013, Clewell *et al.*, 2014, Hirt *et al.*, 2005). Assembly of T4SSs is an energetically expensive process, and tight control of the sex pheromone response might have arisen to mitigate the large fitness costs associated with high-frequency plasmid transfer (Dunny & Berntsson, 2016). Here, we discovered another reason for controlling the sex pheromone response in *E. faecalis*. A novel pCF10-encoded regulatory factor, PrgU, appears to have evolved to specifically control production of the PrgB surface adhesin, more widely known as Aggregation Substance. Controlled synthesis of PrgB is critical because its overproduction is

highly toxic, manifesting as a severe disruption of cell envelope integrity and defective cell growth. Below, we discuss the broad biological importance of PrgU regulatory control and of PrgB overproduction toxicity in enterococci and, possibly, other bacterial pathogens.

PrgU, a novel regulatory factor

Several repression mechanisms operate to control transcription of the *prgQ* operon in the absence of sex pheromone sensing (see Fig. 1A) (Bae *et al.*, 2004, Kozlowicz *et al.*, 2006b). These include: i) PrgX repression of transcription from the P_Q promoter, ii) enhanced PrgX repression through binding of iCF10, a small inhibitor peptide produced from the *prgQ* gene located immediately downstream of the P_Q promoter, and iii) production of a small antisense RNA (anti-Q) from the convergent P_X promoter that binds nascent *prgQ* transcripts and causes them to fold into a terminator structure that blocks extension of *prgQ* transcription (Shokeen *et al.*, 2010, Nakayama *et al.*, 1994, Chatterjee *et al.*, 2013). Upon binding and import of cCF10 pheromone produced by recipient cells in the vicinity, the pheromone binds PrgX and induces a structural change that impairs PrgX repression of P_Q. This leads to increased levels of nascent short *prgQ* transcripts (Q_S) that overwhelm the anti-Q termination mechanism, and result in production of longer transcripts (Q_L) that can ultimately extend through the entire operon. Within ~30–60 min following pheromone induction of the P_Q promoter, iCF10 levels increase to a threshold sufficient to initiate a rapid shut-down of transcription to pre-induction levels. There is also extensive genetic and biochemical evidence suggesting that polypeptides and regulatory RNAs produced from the region between *prgQ* and *prgA*, e.g., *prgR* and *prgS*, encode functions that modulate transcription elongation and translation of the downstream genes (see Figs. 3 & S3) Chung & Dunny, 1992, Bensing & Dunny, 1997, Bensing *et al.*, 1997).

Here, we discovered that PrgU confers another layer of negative feedback regulation. One model under investigation is that PrgU coordinates with one of the P_Q repression systems specifically to achieve controlled synthesis of the Prg surface adhesins in response to pheromone induction. Consistent with such a model, we found that the *prgU* mutation correlated with enhanced accumulation of the Prg adhesins and also with a failure to undergo a shut-down phase in transcription from the P_Q promoter at 60 min postinduction (Fig. S1). Conceivably, upon pheromone induction, PrgU accumulates to a threshold level necessary for activating the switch to the repression mode. This could be achieved through establishment of stabilizing or activating interactions between Q_S and anti-Q RNA, by inhibiting production of extended Q_L transcripts, or by inhibiting positive regulatory functions of the *prgRS* region required for expression of the downstream conjugation genes. Alternatively, PrgU might impact the formation, stability or function of PrgX/cCF10 or PrgX/iCF10 complexes, but we view this as less likely given the locations and phenotypes of the U-Res compensatory mutations.

Studies of these U-Res variants supplied insights into the function of PrgU and identified possible coregulators. Our detailed genome sequencing and complementation studies of one variant, U-Res *R1*, confirmed that a single *prgR* frame-shift mutation was responsible for the U-Res phenotype (Fig. 3). Among 9 other U-Res variants analyzed, nearly all had mutations in *prgR* or the downstream gene *prgS*, although some variants acquired additional

mutations elsewhere in the *prgQ* regulatory region. Most importantly, however, all U-Res mutants were blocked for production of PrgB and the downstream-encoded Prg and Pcf proteins, establishing a correlation between Prg protein production and toxicity (Fig. S3). Interestingly, *trans*-expression of the putative regulators *prgR* and *prgS* exerted opposite effects on production of the Prg proteins in isogenic strains lacking or carrying *prgU*. PrgU thus might physically coordinate with the PrgR/PrgS regulators to fine-tune the pheromone response, although it remains formally possible that PrgU and PrgR/PrgS act at distinct points in the regulatory circuitry.

We gained further insight into the mechanism of action of PrgU through structural modeling of a crystal structure solved for a PrgU homolog (EFA0046) from the clinical isolate V583. The crystal structure presents PrgU as a tetramer where each monomer forms a six β-strand barrel with three accompanying α helices (Fig. S7A,B) (Chang C, 2006). By threading PrgU through the Phyre2 algorithm (Kelley & Sternberg, 2009), we determined that PrgU adopts a PUA (pseudouridine synthase and archaeosine transglycosylase) fold (Fig. S7). PUA domains are widely distributed among diverse types of proteins within prokaryotes, eukaryotes and archaea (Perez-Arellano *et al.*, 2007), and typically bind RNA substrates (Sabina & Soll, 2006, Zhang *et al.*, 2012, Tempel *et al.*, 2013). Other characterized PUA domains invariably are physically joined to other motifs that typically catalyze posttranscriptional modifications of tRNA and rRNA, although other biochemical functions have been identified (Perez-Arellano *et al.*, 2007). Strikingly, the PUA fold encompasses the entire PrgU sequence, making this protein unique among members of the PUA family characterized to date. It is enticing to suggest that PrgU controls Prg protein production through binding of an RNA target, potentially a regulatory *trans*-acting sRNA or *prgQ* transcripts subject to posttranscriptional control. In fact, such a regulatory function was postulated for another PUA-domain containing protein, *Bacillus subtilis* ProB. Although ProB is a glutamate kinase, ProB also downregulates the expression of transcription factor sigma D (σ^D)-dependent genes (Ogura & Tanaka, 1996). It was suggested that ProB disrupts synthesis of σ^D through binding of its PUA domain to σ^D mRNA, resulting in premature transcription termination or a block in translation (Perez-Arellano *et al.*, 2007). By analogy, the PUA domain of PrgU might bind an mRNA target to block transcription or translation of *prgB* and downstream *prg* genes.

In this context, it is of interest to note that the *prgB - prgU* genetic linkage is not restricted to pCF10. We found that *prgU* genes are widely distributed on plasmids and chromosomes of *E. faecalis* and other enterococci, e.g., *Enterococcus faecium*, *Enterococcus raffinosus*, as well as in the genomes of *Streptococcus agalactiae* and *Staphylococcus aureus* (Fig. S8). In all cases we have examined thus far, *prgU*-like genes are genetically linked to *prgB*-like genes. Most often, this gene pair is part of a larger *prgA*-*C*-like cassette, but other genetic contexts exist in which *prgA* or *prgC* are absent or nearby genes instead encode cell wall hydrolases. We therefore suggest that the *prgB*-*prgU* genetic linkages might have evolved to ensure the controlled synthesis of PrgB-like adhesins or, possibly, cell wall metabolizing enzymes in various Gram-positive pathogens. If so, it is reasonable to predict that PrgU exerts its control by binding regulatory DNA or mRNA motifs positioned upstream or within *prgB* or, possibly, other target genes.

The biological importance and mechanism of PrgB overproduction toxicity

Sex pheromone-mediated toxicity in *E. faecalis* was first reported by M. Gilmore and his colleagues (Gilmore *et al.*, 2015). In that study, commensal isolates of enterococci were shown to effectively kill the clinical strain V583 through release of an inducing sex pheromone, cOB1. cOB1-mediated killing of V583 requires two genetic elements, the pheromone-dependent plasmid pTEF2, which is highly similar in gene composition to pCF10, and an integrated chromosomal (IS-like) element. The mechanism of killing was not defined, but the following observations suggest cOB1-mediated killing might result from the dysregulated overproduction of one or more PrgB adhesins. V583 carries four copies of *prgB*-like genes, one on each of the resident pTEF1 (*EFA0047*) and pTEF2 (*EFB0011*) plasmids, one in the pathogenicity island (PAI, *EF0485*) and one in the IS-like element (*EF0149*). *prgU* genes are linked to three *prgB* genes (pTEF1, *EFA0046*; pTEF2, unannotated; PAI, *EF0486*), but, interestingly, not EF0149 in the IS-like element. These observations and results of a microarray analysis showing that cOB1 induces expression of *prg*- and *pcf*-like genes on pTEF2 (Gilmore et al., 2015) suggest the possibility that V583 accumulates one or more of the PrgB adhesins at toxic levels when exposed to cOB1 sex pheromone. Conceivably, pTEF2 encodes a regulatory factor that activates expression of chromosomal-borne *EF0149*, which in the absence *prgU* regulator, leads to excess accumulation of the PrgB adhesin. Alternatively, V583 cells might produce EF0149 endogenously at subinhibitory levels, but cOB1 induction of pTEF2-encoded *EFB0011* confers PrgB overproduction toxicity due to the combined production of EF0149 and EFB0011.

PrgB is a well-characterized adhesin that mediates extensive cellular clumping and attachment to abiotic and biotic surfaces as a critical early step in establishment of robust biofilms (Olmsted *et al.*, 1991, Chuang *et al.*, 2009). Our present findings add to results of our recent study in which we showed that OG1RF cells carrying a miniaturized version of pCF10 that expresses only the *prgA-C* cassette form robust biofilms with an accumulation of dead or permeabolized cells (Bhatty *et al.*, 2015). Although chromosomally-encoded autolytic or fratricidal mechanisms might account for the observed cell death (Thomas & Hancock, 2009, Thomas *et al.*, 2009), we suggest instead that a subset of pCF10-carrying cells might stochastically overproduce PrgB with lethal consequences. In natural settings composed of mixed communities of plasmid-carrying and -free enterococci as well as other species, PrgB-mediated cell lysis could provide a source of extracellular matrix components, e.g., lipoteichoic acids, polysaccharides, eDNA, of importance for establishment of robust biofilms (Dunny *et al.*, 2014). Consistent with this proposal, single cell analyses (Cook *et al.*, 2011) and our additional unpublished findings have shown that *prgB* expression is highly variable among individual cells in cCF10-exposed populations.

By scanning electron microscopy, PrgA and PrgB were shown to colocalize on the *E. faecalis* cell surface, initially at the equatorial region of dividing cells, and then more uniformly around the polar caps of older cells (Olmsted *et al.*, 1993). Both proteins were associated with extended filaments that form a fibrous mesh around the cell periphery. More recently, it was shown that the general secretion (Sec) system and the sortase machinery colocalize as discrete foci in the equatorial regions of *E. faecalis* cells, suggestive of a

coordination of cell envelope biogenesis and cell division (Kline *et al.*, 2009, Kandaswamy *et al.*, 2013). Abundant production of PrgB, possibly with contributions by PrgA at or near the growing septum might perturb deposition of the cell wall, septal placement, and cell division. It is also noteworthy that eDNA is localized around the equatorial regions of dividing cells (Barnes *et al.*, 2012), and recently we reported that PrgB induces extensive clumping of cells and biofilm formation by an eDNA-dependent mechanism (Bhatty *et al.*, 2015). Interestingly, we have observed that DNase I treatment diminishes the toxic effect of pheromone on growth of PrgB-overproducing strains (data not shown). These findings suggest that the association of eDNA with abundant PrgB fibers at or near the equatorial plane of actively dividing cells might disrupt cell wall biogenesis and septal placement. Such complexes might impede cell growth through steric effects or alterations in surface hydrophobicity or charge.

Although PrgA or PrgC overproduction did not confer toxicity, arguing against the notion that overproduction of surface proteins is generally toxic to enterococci, it remains possible that excessive accumulation of PrgB disrupts export of other surface proteins through the general secretory (Sec) or their sorting at the cell surface. In *E. faecalis*, the Fst toxin consists of a single transmembrane (TM) domain, and its overproduction yields growth defects similar to those shown here to be associated with PrgB overproduction (Weaver *et al.*, 2003, Patel & Weaver, 2006). It is conceivable that insertion of the C-terminal TM domains of abundantly produced PrgB, either cleaved or not by sortase, has toxic consequences similar to those accompanying Fst toxin overproduction. Recent studies have shown that excessive accumulation of certain surface proteins, e.g., *Streptococcus pyogenes* M protein, *Actinomyces oralis* AcaC surface glycoprotein, in sortase mutants results in loss of cell envelope integrity and defects in cell growth (Raz *et al.*, 2015, Nobbs *et al.*, 2007, Wu *et al.*, 2014). We have confirmed that PrgB overproduction toxicity occurs in the strain OG1RF (Figs. 5 & S6) and is not affected by sortase mutations (data not shown). To our knowledge, this is the first example of surface protein overproduction toxicity in a sortase-proficient strain, although further studies are needed to define the mechanism by which PrgB confers toxicity.

In summary, we identified a novel regulator, PrgU, that controls sex pheromone toxicity, and we determined that excessive accumulation of PrgB adhesin, also termed Aggregation Substance (AS) (Dunny *et al.*, 1985), is responsible for toxicity. In the streptococci, it is well established that small peptide quorum signals induce death of a subpopulation of cells during development of genetic competence, an activity thought to provide a source of DNA for the competent cell population (Cook and Federle, 2014, Leung *et al.*, 2015). Our findings expand the biological functions of peptide-mediated cell death among Gram-positive species, and further establish the potential for dysregulated synthesis of a surface adhesin, as opposed to induced synthesis of an autolytic murein hydrolase (Claverys *et al.*, 2007, Dufour & Levesque, 2013), as a basis for toxicity. Finally, despite the fact that PrgB is a well-established virulence factor (Schlievert *et al.*, 1998, Rakita *et al.*, 1999), our results suggest the interesting possibility that intervention strategies aimed at transient overproduction of at least certain types of cell surface adhesins early during a treatment regimen might prove efficacious in sensitizing enterococci and, possibly other Gram-positive species, to antibiotics.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains, plasmids, and oligonucleotides used in the study are listed in Tables S1, S2, and S3. For plasmid construction, *E. coli* DH5α and EC1000, a strain that produces the pWV01 RepA protein (Leenhouts *et al.*, 1996), were used as hosts. *E. coli* strains were grown at 37°C with shaking in Lysogeny Broth (LB) Broth (Difco Laboratories). Brain heart infusion broth (BHI; Difco Laboratories) was used to grow *E. faecalis* strains at 37°C without shaking. *E. coli* strains were grown with the following antibiotics as needed: chloramphenicol (20 μg ml^{-1}), erythromycin (100 μg ml^{-1}), spectinomycin (50 μg ml^{-1}). The following antibiotics were used as needed for *E. faecalis*: erythromycin (100 μg ml^{-1} final concentration for plasmid markers, 10 μg ml^{-1} for chromosomal markers), fusidic acid (25 μg ml^{-1}), rifampin (200 μg ml^{-1}), spectinomycin (1,000 μg ml^{-1} for plasmid markers, 250 μg ml^{-1} for chromosomal markers), streptomycin (1,000 μg ml^{-1}), tetracycline (10 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), daptomycin (6 μg ml^{-1}) in media supplemented with 50 μg ml^{-1} CaCl$_2$, linezolid (0.25 μg ml^{-1}). All antibiotics were obtained from Sigma-Aldrich.

Construction of *prgU* deletion mutants

For the construction of pCF10 *prgU*, ~500–600 base-pair (bp) regions immediately upstream and downstream of *prgU* were amplified with forward and reverse (F/R) primers designated prgUup-XbaI or -XmaI and prgUdown-XmaI or -NcoI. These fragments were joined by overlapping PCR using the primers F-prgUup-XbaI and R-prgU down-NcoI, and the resulting fragment was digested with XbaI and NcoI for introduction into similarly-digested pCJK47, resulting in pMCM3. *prgU* was deleted from pCF10 using a marker-less recombination strategy (Kristich *et al.*, 2007), resulting in plasmid pCF10 *prgU. prgU* also was deleted from plasmid p10-mini, which contains the *prgQ* regulatory region and the downstream *prgA* - *prgC* gene cassette on shuttle vector pCI372 (see below). p10-mini *prgU* was constructed by inverse PCR using 5′ phosphorylated primers F-prgUdown5′phos and R-prgUup5′phos. The amplified product was ligated and introduced into *E. faecalis* strain OG1RF by electroporation (Dunny *et al.*, 1991). *prgU* deletions in pCF10 and p10-mini were confirmed by PCR and nucleotide sequence analysis.

prg expression plasmids

pMB11 constitutively expresses *prgU* from the P$_{23}$ promoter. It was constructed by amplifying *prgU* from pCF10 using gene specific F/R primers containing BamHI/SphI restriction sites. The PCR product was digested using the specified restriction enzymes and introduced into similarly-digested plasmid pDL278p23 downstream of the constitutive promoter P$_{23}$ from *Lactococcus lactis* (Chen *et al.*, 2007). This plasmid was introduced into *E. faecalis* strain OG1RF by electroporation (Dunny *et al.*, 1991). Plasmids pMC001, pMC002, and pMB4 constitutively expressing *prgA*, *prgB*, and *prgC* from the P$_{23}$ promoter were constructed similarly by use of pCF10 as a template and F/R primers listed in Table S3 for the gene amplifications. Plasmid MC003 constitutively coexpresses *prgR* and *prgS* from the P$_{23}$ promoter. It was constructed as above using pCF10 as a template and F/R primers listed in Table S3.

Conjugation assays

Overnight liquid cultures of *E. faecalis* donor and recipient strains were diluted 1:10 in BHI and incubated for 1 h at 37°C. Donor and recipient cells were then mixed in a ratio of 1:10 and allowed to mate for the specified time periods at 37°C in liquid or on filters placed on BHI plates. Serial dilutions of the mating mixture plated on selective BHI agar plates were used to obtain the donor and transconjugant counts. The plasmid transfer frequencies were calculated as the number of transconjugants per donor cell (Chen *et al.*, 2008). The results are reported as an average of at least three replicates of each experiment.

Detection of Prg/Pcf proteins by immunoblotting

Overnight liquid cultures of *E. faecalis* strains were diluted 1:20 in fresh BHI and grown for 1 h at 37°C. Cultures (5–10 ml) were normalized to an OD_{600} of 0.3 and induced with 10 ng ml^{-1} of peptide cCF10 for 1 h at 37°C. Cells were pelleted by centrifugation at $13,200 \times g$ for 15 min at 4°C and washed once with cold 1X physiological buffer saline (PBS). The pellet was resuspended in 250 μl of SMM buffer (0.5M sucrose, 0.02 M $MgCl_2$, 0.02 M maleate, pH 6.5) containing 500 U ml^{-1} of mutanolysin (Sigma-Aldrich) and 10 mg ml^{-1} of lysozyme (Sigma-Aldrich), and the resulting mix was incubated for 1 h at 37°C with shaking. The mix was centrifuged at $13,200 \times g$ for 15 min at 4°C to separate the cell wall (supernatant) and cellular (pellet) fractions. The pellet fraction was suspended in 1 X PBS, and both fractions were analyzed on a per cell equivalent basis for the presence of Prg/Pcf proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, western blot transfer and immunostaining with anti-Prg/Pcf antibodies (Christie *et al.*, 1988, Chen *et al.*, 2008, Li *et al.*, 2012, Bhatty *et al.*, 2015). As a loading control, blots were developed with antibodies to the RNA polymerase β subunit (Santa Cruz Biotechnology).

Whole genome sequencing and analysis

E. faecalis DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following the pre-treatment for Gram-positive bacteria protocol. Purified DNA was submitted to the University of Minnesota Genomics Center for whole genome sequencing using the MiSeqV2 platform (Illumina, Inc.) and 150-bp paired-end reads. Sequencing data from the OG1RF(pCF10 *prgU*) and OG1RF(pCF10 U-Res *R1*) were compared to the OG1RF(pCF10) reference genome (NCBI accession numbers NC_017316 and NC_006827) using breseq version 0.24rc6 (Deatherage & Barrick, 2014).

qRT-PCR

Three colonies of each strain were inoculated into 10 ml M9-YE (Dunny & Clewell, 1975) and incubated at 37°C for 14.5–16 h. Cultures were diluted 1:10 into 10 ml fresh M9-YE, further incubated for 1 h, and then induced with 5 ng ml^{-1} cCF10. Aliquots of cells collected at 0, 30 and 60 min postinduction were treated with RNAprotect Bacteria Reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Cell pellets were resuspended in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) containing 500 U ml^{-1} mutanolysin and 30 mg ml^{-1} lysozyme and incubated at 37°C for 10 min. RNA was extracted with the RNeasy Mini Kit (Qiagen, Inc.) according to the manufacturer's instructions. Contaminating DNA was removed with the Turbo-DNA free kit (Ambion).

cDNA was synthesized with random hexamers using the Superscript III First-Strand Synthesis System (Invitrogen Corp.) according to the manufacturer's instructions. Quantitative PCR was carried out as previously described (Frank *et al.*, 2012). *gyrB* was used as a reference gene. Two biological replicates were performed with similar results.

Bile susceptibility assays

Overnight liquid cultures of *E. faecalis* strains were diluted 1:100 and incubated for 1 h at 37 °C. Cultures were serially diluted and spotted (5 μl) onto BHI or BHI containing cCF10 (2 ng/ml) with or without sodium cholate (4% w/v) or sodium deoxycholate (0.06% w/v) (Dale *et al.*, 2015). Colony forming units (CFUs) per ml were determined after overnight incubation at 37°C.

Pheromone spot assay

Overnight liquid cultures of *E. faecalis* strains were diluted 1:10 in BHI and incubated for 1 h at 37°C. Cultures (200 μl) were spread on BHI plates and cCF10 (10 ng ml^{-1} final concentration) was added to the center of the plates. To monitor the effect of DNase I on pheromone toxicity, DNase I was added (500 U ml^{-1} final concentration) to a BHI plate prior to spreading of the cultures and addition of pheromone to the center of the plates. Pheromone toxicity was evidenced by a zone of clearance after overnight incubation at 37°C.

Growth curve determinations

Overnight liquid cultures of *E. faecalis* strains were diluted 1:50 in BHI and incubated for 1 h at 37°C. The cells were normalized to an OD_{600} of 0.1 using fresh BHI, and then incubated in the absence or presence of cCF10 (10 ng ml^{-1}) at 37°C. Aliquots were removed at specified time points, 10 μl of 0.5 M EDTA was added to disaggregate the cells, and the OD_{600} was measured. All experiments were performed in duplicate and results represent the average of three experiments.

CPRG (chlorophenol red-β-D-galactopyranoside) β galactosidase assay

CPRG hydrolysis was measured as described previously (Dale *et al.*, 2015, Djoric & Kristich, 2015). Briefly, overnight cultures of *E. faecalis* strains were diluted to a starting OD_{600} of 0.05 in BHI supplemented with erythromycin and 25 μg ml^{-1} CPRG in the presence or absence of cCF10 (10 ng ml^{-1}). Cultures were incubated statically at 37°C for 0, 2, 4 or 6 h. At each time point, 1 ml culture aliquots were removed for determinations of viable bacteria (CFUs) by serial dilution. CPRG hydrolysis was quantitated by measuring the absorbance at 570 nm (OD_{570}) following removal of bacteria by centrifugation.

Antibiotic susceptibility assays

E. faecalis strains were inoculated from frozen glycerol stocks stored at −80°C. Each strain was inoculated into BHI containing antibiotics with or without cCF10 (10ng ml^{-1}). Cultures were incubated overnight, 10 μl of 0.5 M EDTA was added to disaggregate the cells, and OD_{600} values were determined. All the experiments were performed in duplicate and the results represent the average of two different experiments.

Transmission Electron microscopy

Overnight liquid cultures of *E. faecalis* strains grown in BHI were diluted 1:10 in BHI lacking or containing 10 ng ml^{-1} of cCF10 and incubated statically for 1 h at 37°C. To prepare the samples for visualization, 10 μl aliquots of cultures were placed on carbon-coated nickel or copper grids (Electron Microscopy Sciences) for 5 min, washed 3 times in sterile H$_2$0, and stained with 0.2% ammonium molybdate for 1 min. The grids were imaged using a JEOL JEM-1400 transmission electron microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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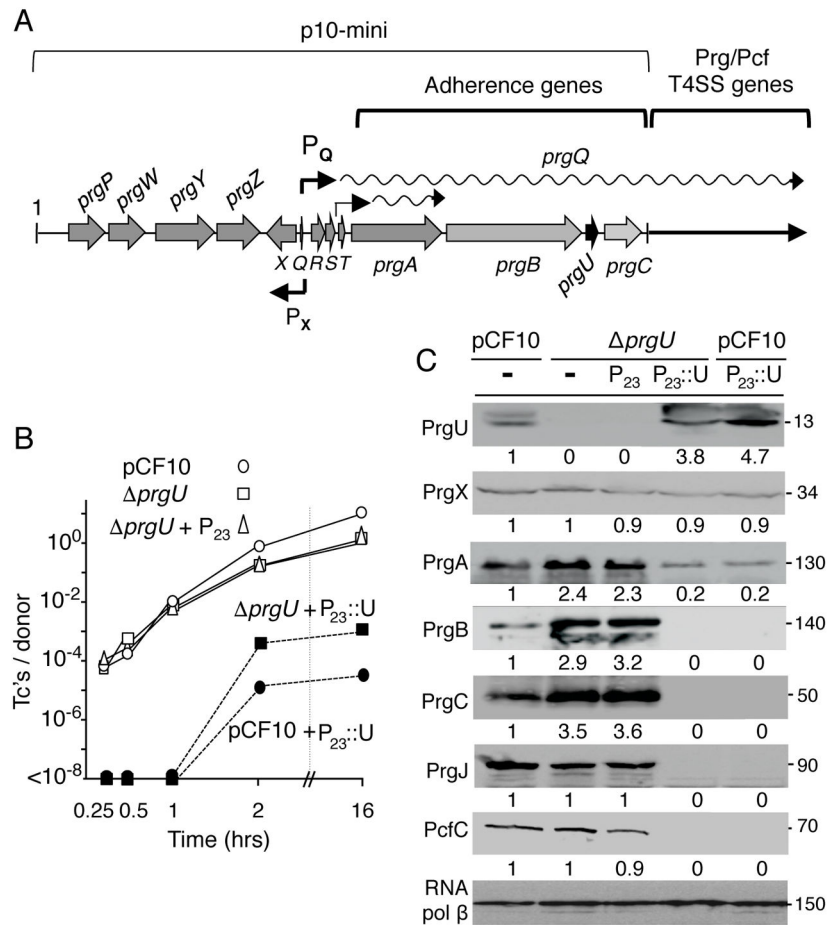
FIG. 1.

Effects of PrgU deletion and overproduction on Prg/Pcf protein synthesis and plasmid transfer. **A)** Schematic of the P_Q regulatory region and *prgQ* operon carried by pCF10. The pheromone-responsive P_Q promoter is regulated by flanking regulatory functions and directs expression of the downstream-encoded adherence and plasmid transfer functions. The P_X promoter controls expression of *prgX*, which encodes the PrgX transcriptional regulator. Another promoter in the *prgR/S* region constitutively expresses a short *prgA* transcript at low levels. Plasmid p10-mini carries the ~17-kb fragment shown. **B)** Transfer frequencies of pCF10 plasmids in filter matings carried out for the durations indicated. Strains: OG1RF with **pCF10** or pCF10 *prgU* (denoted *prgU*) plasmids alone or additionally with the vector plasmid (**P$_{23}$**; pDL278p23) or the P_{23}:*prgU* expression plasmid (denoted **P$_{23}$::U**; pMB11). Transfer frequencies are presented as the number of transconjugants per donor cell (Tc's/Donor). Experiments were repeated at least three times in duplicate, and results from a representative experiment are shown. **C)** Steady-state levels of Prg/Pcf proteins in strains induced for 1 h with cCF10 pheromone (10 ng ml^{-1}). OG1RF with plasmids indicated. Upper line: **pCF10,** *prgU* (pCF10 *prgU*). Lower line: **P$_{23}$** (pDL278p23), **P$_{23}$::U** (pMB11), **-** (no P$_{23}$ plasmid). Immunoblots were developed with antibodies to the Prg/Pcf proteins shown or to RNA polymerase β subunit as a loading control. Protein sizes (in kilodaltons, kDa) are listed at the right. Protein extracts were loaded on a per-cell equivalent
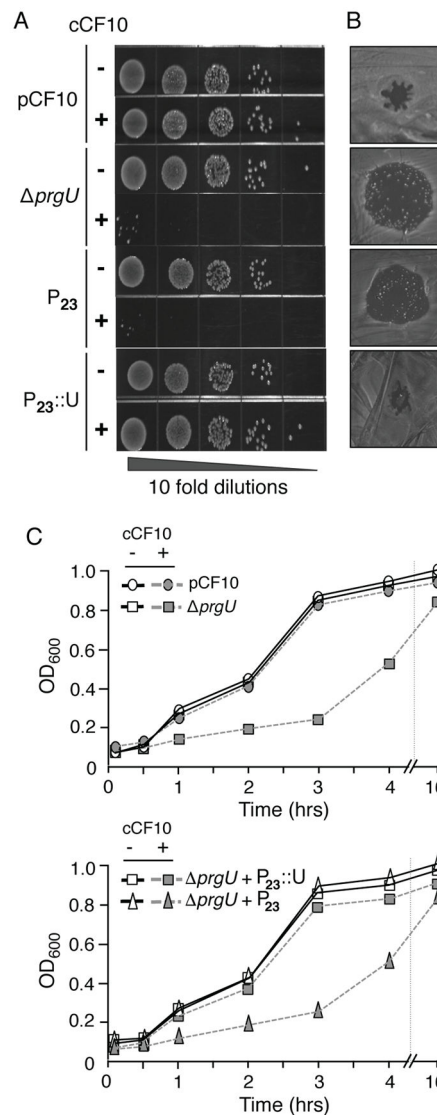
basis. Numbers below each panel correspond to relative protein abundance compared to levels detected in OG1RF(pCF10) cell extracts (set to 1), as determined by densitometry tracing using Image J software.

FIG. 2.
cCF10 pheromone suppresses growth of *E. faecalis* cells carrying the pCF10 *prgU* plasmid.
A) Serial dilution plate assay. *E. faecalis* overnight cultures were diluted 1:100 in fresh BHI
and incubated for 1 h at 37°C in the absence (−) or presence (+) of cCF10 (10 ng ml^{-1}).
Tenfold serial dilutions were inoculated onto BHI medium and assessed for growth. Strains:
OG1RF with **pCF10** or *prgU* (pCF10 *prgU*) alone, or additionally with the **P$_{23}$** vector
plasmid (pDL278P$_{23}$) or the P$_{23}$::*prgU* expression plasmid (denoted **P$_{23}$::U**; pMB11). **B)**
Pheromone spot assay. Overnight cultures listed at the left in Panel A were diluted 1:100 in
fresh BHI and incubated for 1 h at 37°C in the absence of pheromone. Cultures were spread
on BHI media, allowed to dry, and cCF10 pheromone (10 ng ml^{-1}) was added to the center
of the plate. Plates were incubated overnight at 37 °C and assessed for growth. cCF10
pheromone is solubilized in DMSO, which inhibits *E. faecalis* growth and causes small
zones of clearance independently of cCF10-induced toxicity; for example, see spot assay for
OG1RF(pCF10). **C)** Growth curve assay. Overnight cultures were diluted 1:50 in BHI and

incubated for 1 h at 37 °C. The cells were normalized to an OD_{600} of 0.1 and cultures were incubated in the absence or presence of cCF10 (10 ng ml^{-1}) at 37°C. Aliquots were removed at specified time points, 10 μl of 0.5 M EDTA was added to disaggregate the cells, and the OD_{600} was measured.
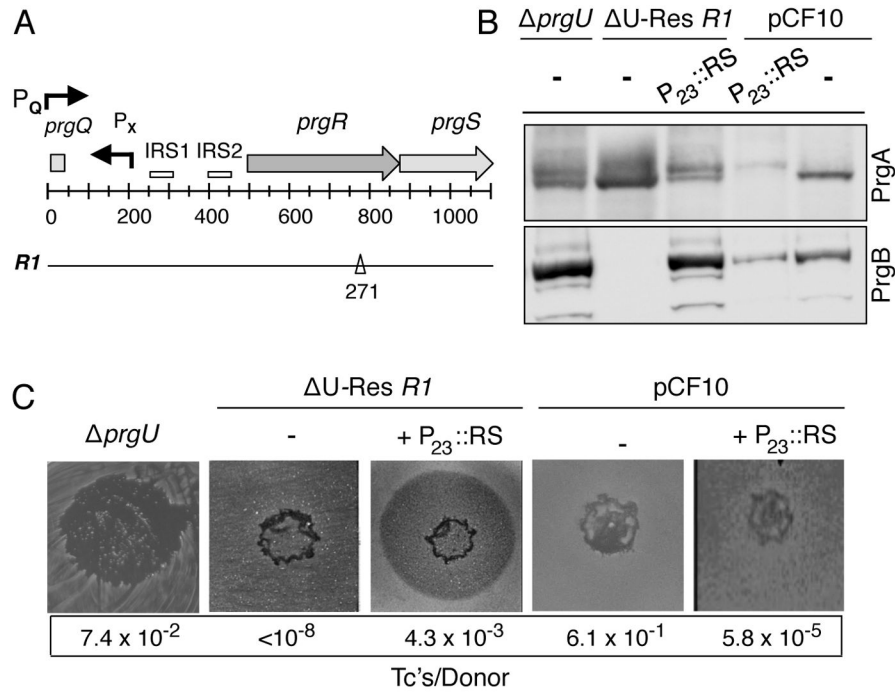
FIG. 3.
The ΔU-Res *R1* compensatory mutation maps to the putative regulatory gene *prgR*.. **A)** Schematic of the *prgQ* regulatory region showing the locations of the P_Q and P_X promoters, inverted repeats IRS1 and IRS2 predicted to form stem-loop transcription terminators, and the putative regulatory genes *prgR* and *prgS*. Numbers refer to distances (in base pairs, bp's) from the P_Q promoter start-site. Below: The ΔU-Res *R1* mutation is a 1 bp deletion at position 271 in the 318-bp *prgR* sequence. **B)** Modulation of PrgA and PrgB levels by the P_{23}::*prgR,prgS* expression plasmid. Steady-state levels of PrgA and PrgB in strains induced for 1 h with cCF10 pheromone (10 ng ml^{-1}). OG1RF with plasmids indicated. Upper line: **pCF10**, **Δ*prgU*** (pCF10 Δ*prgU*), **ΔU-Res *R1*** (pCF10 Δ*prgU,prgR* Δ271). Lower line: **-** (no P_{23} plasmid), **P_{23}::RS** (P_{23}::*prgR,prgS* expression plasmid, pMC003). Immunoblots were developed with antibodies to PrgA or PrgB. Protein extracts were loaded on a per-cell equivalent basis with RNA polymerase β subunit as a loading control. **C)** Pheromone spot and mating assays. Strains analyzed are the same as in panel B. Transfer frequencies of pCF10 and its derivatives are presented as the number of transconjugants per donor cell (Tc's/Donor).
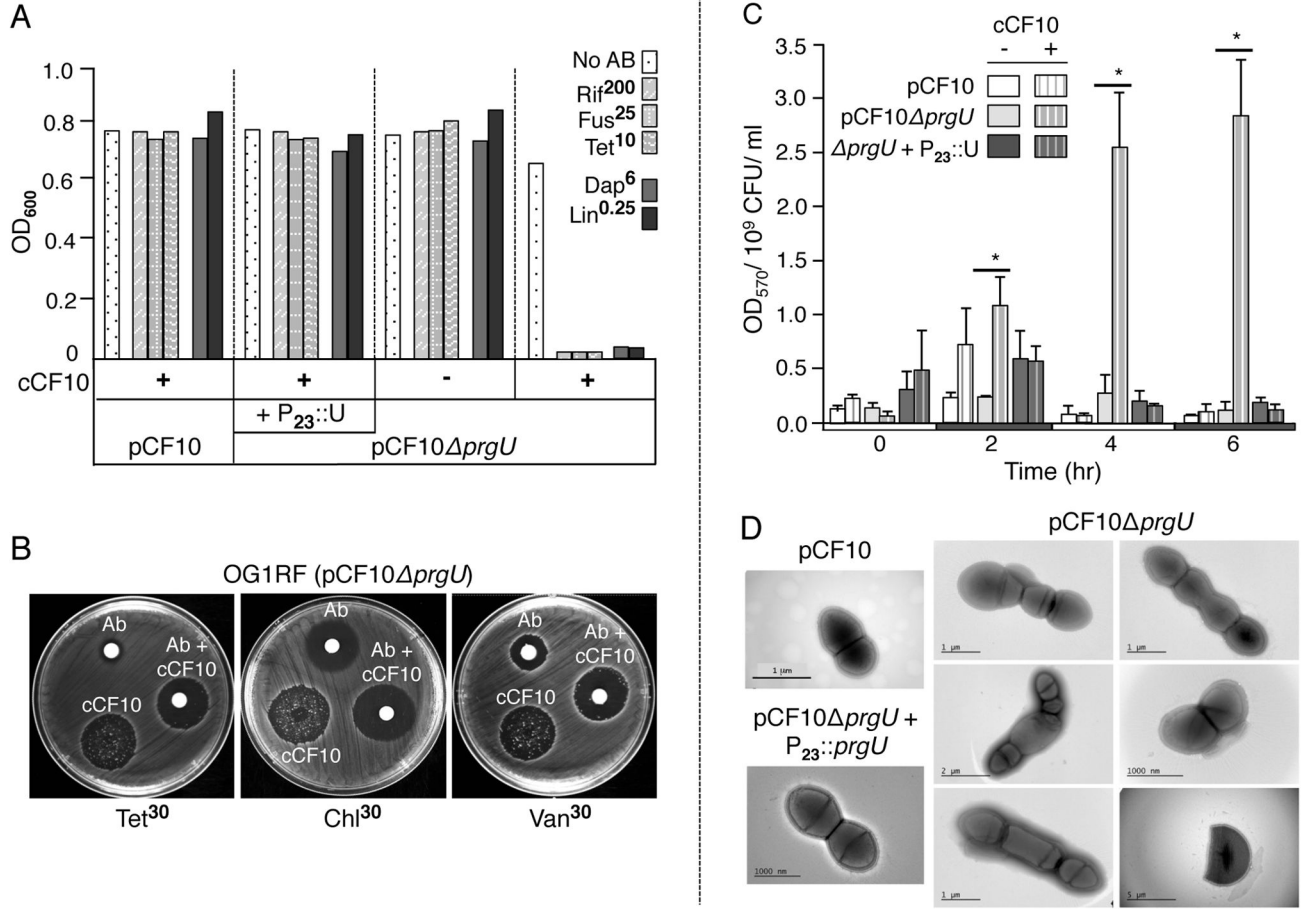
FIG. 4.

prgU mutation confers defects in cell envelope integrity and cell growth. **A)** Growth defect of the *prgU* mutant in the presence of subinhibitory concentrations of antibiotics. Strains: OG1RF with **pCF10**, *prgU* (pCF10 *prgU*) alone or with the P_{23}::*prgU* expression plasmid (**P_{23}::U**, pMB11). Strains were inoculated from glycerol stocks into BHI lacking (−) or containing (+) cCF10 and in the absence (**No AB**) or presence of the antibiotics at final concentrations (in µg ml^{-1}) listed. Cultures were incubated overnight at 37°C without shaking and culture densities (OD_{600}) were measured. **B)** Disc diffusion assay showing that a combination of pheromone (10 ng ml^{-1}) and antibiotics at the indicated concentrations suppressed the appearance of U-Res variants. **C)** *E. faecalis* strains containing the constitutive *lacZ* reporter construct pCJK205 were cultured in BHI broth supplemented with erythromycin and 40 µg ml^{-1} CPRG, and incubated in the absence or presence of cCF10 at 37°C. Samples were removed at the times indicated, and CPRG hydrolysis was quantitated by measuring the absorbance (OD_{570}) of the bacterium-free culture supernatant and normalizing to CFU. The experiment was performed at least two times, and the data are represented as the mean +/− standard deviation. Statistical significance was evaluated by *t* test, *, *P<0.05* versus cCF10-untreated culture. **D)** Morphological aberrations of pheromone-exposed *prgU* mutant cells. Overnight cultures were diluted 1:100 in fresh BHI

supplemented with pheromone (10 ng ml^{-1}) and incubated without shaking for 1 h at 37°C. Cells were processed for imaging as described in the Experimental procedures.
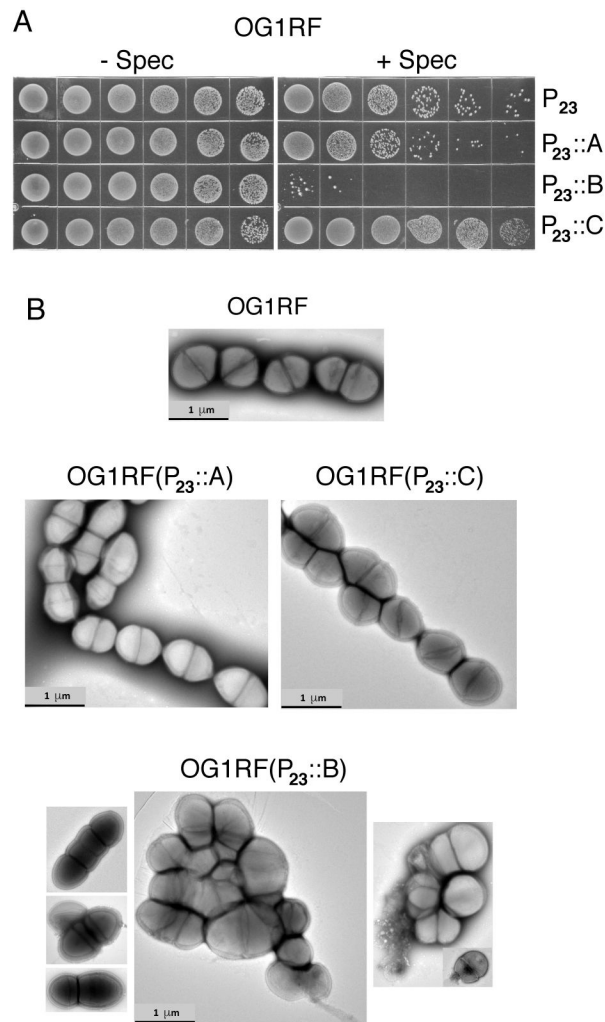
FIG. 5.
Constitutive overproduction of PrgB confers toxicity in the absence of other pCF10-encoded factors. **A)** Plasmid curing assay. Colonies from transformation plates were inoculated into antibiotic-free BHI and incubated without shaking overnight at 37°C. Overnight cultures were serially diluted and spotted onto BHI agar plates containing or lacking spectinomycin (500 μg ml^{-1}) to which the P$_{23}$ plasmid confers resistance. **B)** PrgB overproduction confers severe growth defects. Freshly transformed cells were inoculated in fresh BHI supplemented with pheromone (10 ng ml^{-1}) and incubated without shaking for 1 h at 37°C. Cells were processed for imaging as described in the Experimental procedures. Strains: OG1RF alone or harboring the P$_{23}$::*prgA*, P$_{23}$::*prgB*, or P$_{23}$::*prgC* expression plasmids.