


# *Enterococcus faecalis* 6-Phosphogluconolactonase Is Required for Both Commensal and Pathogenic Interactions with *Manduca sexta*

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*Enterococcus faecalis* is a commensal and pathogen of humans and insects. In *Manduca sexta*, *E. faecalis* is an infrequent member of the commensal gut community, but its translocation to the hemocoel results in a commensal-to-pathogen switch. To investigate *E. faecalis* factors required for commensalism, we identified *E. faecalis* genes that are upregulated in the gut of *M. sexta* using recombinase-based *in vivo* expression technology (RIVET). The RIVET screen produced 113 clones, from which we identified 50 genes that are more highly expressed in the insect gut than in culture. The most frequently recovered gene was locus OG1RF\_11582, which encodes a 6-phosphogluconolactonase that we designated *pglA*. A *pglA* deletion mutant was impaired in both pathogenesis and gut persistence in *M. sexta* and produced enhanced biofilms compared with the wild type in an *in vitro* polystyrene plate assay. Mutation of four other genes identified by RIVET did not affect persistence in caterpillar guts but led to impaired pathogenesis. This is the first identification of genetic determinants for *E. faecalis* commensal and pathogenic interactions with *M. sexta*. Bacterial factors identified in this model system may provide insight into colonization or persistence in other host-associated microbial communities and represent potential targets for interventions to prevent *E. faecalis* infections.

Many bacteria influence their hosts in multiple ways, acting as mutualists, commensals, and pathogens in the same host, with the outcome often dependent upon the location of the association (1, 2). Each of the three genera most commonly associated with nosocomial infections, *Staphylococcus*, *Enterococcus*, and *Candida*, contains members that often live as commensals (3–6). These commensal species comprise a reservoir of potential pathogens capable of causing disease when translocated to normally sterile sites, such as the blood or heart tissue, particularly in immunocompromised hosts. Although there has been a nationwide effort to reduce pathogen reservoirs in hospitals (3–6), little work has addressed the mechanisms by which commensals become pathogens, thereby ignoring an important reservoir of pathogens in community settings. Improved understanding of the commensal lifestyle and the mechanisms by which commensals become pathogens may suggest new strategies to prevent and manage opportunistic infectious diseases.

*Enterococcus faecalis* is a cosmopolitan bacterium found in most vertebrate and invertebrate guts (7, 8) and has both commensal and pathogenic roles in diverse hosts, including humans (9, 10). We previously developed a lepidopteran insect model, *Manduca sexta*, in which to study factors that determine the success of *E. faecalis* as both a commensal and pathogen (11, 12). When fed to *M. sexta* larvae, *E. faecalis* OG1RF (13), a human isolate, persists in the gut without causing detectable changes in the host (12). In contrast, it is a virulent pathogen when injected directly into the hemocoel or released from the gut by the action of the pore-forming toxin Bt Cry1Ac (12). Enterococci are core members of the lepidopteran gut microbial community (7, 14), and while many bacterial phyla are found in guts of lepidopteran larvae, stable-isotope probing of larvae fed <sup>13</sup>C-labeled tobacco showed that only enterococci are metabolically active in *M. sexta* (15). Previous studies have validated invertebrate models as useful systems for the evaluation of *Enterococcus* pathogenicity (16, 17). Yet little is known about *E. faecalis* factors that contribute to its

persistence in the lepidopteran alimentary canal, an important prerequisite for both commensal and pathogenic lifestyles. Understanding the factors required for colonization of insects may identify factors that are important for *E. faecalis* invasion of existing microbial communities and persistence in mammalian guts.

We used recombinase-based *in vivo* expression technology (RIVET) to identify genes from *E. faecalis* that contribute to persistence in *M. sexta*. The RIVET screen identified genes in *E. faecalis* OG1RF that were transcriptionally activated in the *M. sexta* gut. We constructed mutations in several candidate genes identified in the screen and assessed their role in commensal and pathogenic interactions with the insect host.

## MATERIALS AND METHODS

**Insects and rearing conditions.** *M. sexta* eggs, obtained from Carolina Biological Supply (Burlington, North Carolina), were surface sterilized for 3 min in a solution of 1% bleach and 2% Tween 80 and then rinsed

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twice for 1 min in sterile deionized water. Larvae were reared on a gypsy moth artificial diet, USDA Hamden formula (12), amended with 250  $\mu\text{g ml}^{-1}$  each rifampin and gentamicin, in groups of 10 larvae per 100-mm-diameter petri dish. For the RIVET screen, larvae were maintained at 26°C on a 16:8-h light:dark cycle. In subsequent bioassays, larvae were reared at room temperature in standard laboratory lighting. For persistence assays, larvae were transferred to an unamended diet within 8 h after molting to the third instar. After 24 h of exposure to an unamended diet, individual larvae were placed in the wells of 12- or 24-well polystyrene microtiter dishes and starved for 12 to 24 h before experimental feeding. For pathogenesis assays, larvae were maintained on rifampin/gentamicin through the fifth instar. After molting to the fourth instar, larvae were individually housed in 2- or 5-oz SD5 plastic cups (Solo Cup, Lake Forest, IL) and then transferred to an unamended diet for the remainder of the experiment and allowed to feed 24 h before exposure to *E. faecalis*. Larvae were injected in the right first proleg with a sterile BD PrecisionGlide 30 gauge 0.5-in. needle 2 to 3 days after molting to the fifth instar. Hemolymph for *in vitro* growth experiments was collected from an incision in the right first proleg of surface-sterilized 5th-instar larvae and amended with 0.43 mM phenylthiocyanate; larvae were chilled at 4°C for 30 min before bleeding. Hemocytes and debris were removed by centrifugation at  $5,000 \times g$  for 10 min at 4°C. Hemolymph was stored at  $-20^{\circ}\text{C}$ .

**RIVET screen for *E. faecalis* genes expressed during colonization of the *M. sexta* gut.** We screened the RIVET library described by Ballering et al. (18) in the digestive tract of third-instar *M. sexta* larvae. The inoculum was prepared by diluting a 175- $\mu\text{l}$  aliquot of the RIVET library into 3 ml of M9YECAA medium (19) amended with 2,000  $\mu\text{g ml}^{-1}$  kanamycin and 200  $\mu\text{g ml}^{-1}$  chloramphenicol. After incubation for 16 h at 37°C without shaking, 1 ml of culture was added to 11 ml M9YECAA medium containing the same antibiotics and incubated at 37°C without shaking. After 15 h, 2 ml of this culture was diluted 1:10 with fresh M9YECAA medium without antibiotics, grown for 5 h, and concentrated by centrifugation to a final volume of 500  $\mu\text{l}$ . Each larva was offered 2  $\mu\text{l}$  ( $\sim 2.5 \times 10^7$  CFU) of the final concentrate, incubated at 26°C for 24 h, and then sacrificed. Homogenized larval guts were plated on brain heart infusion (BHI; Sigma-Aldrich, St. Louis, MO) agar containing 520  $\mu\text{g ml}^{-1}$  5-fluorouracil. Colonies were picked after 24 h and patched onto agar plates containing BHI, BHI plus 520  $\mu\text{g ml}^{-1}$  5-fluorouracil, and BHI plus 2,000  $\mu\text{g ml}^{-1}$  kanamycin. Plasmid DNA was purified from clones resistant to 5-fluorouracil but sensitive to kanamycin and grown overnight at 37°C, without shaking, in M9YECAA medium with 20  $\mu\text{g ml}^{-1}$  chloramphenicol and 200  $\mu\text{g ml}^{-1}$  rifampin. Plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen) with the following modifications. After addition of buffer N3, 100  $\mu\text{l}$  of chloroform was added, and samples were inverted five times before centrifugation at  $14,000 \times g$  for 10 min. Aqueous phases were collected and mixed with 1/10 vol 0.3 M sodium acetate and 1 vol cold ( $-20^{\circ}\text{C}$ ) isopropanol and then centrifuged at  $14,000 \times g$  for 25 min at 4°C. Supernatants were discarded, and pellets were gently washed once with 500  $\mu\text{l}$  buffer PB (Qiagen) and twice with 70% ethanol. Final pellets were air dried and resuspended in Tris (50 mM)-EDTA (10 mM) (pH 8.0). Plasmid DNA was sequenced using primers RIVET-forward and RIVET-reverse (F and R) (18) at the Yale University DNA Analysis Facility (New Haven, CT).

**Bioinformatic analysis.** Genomic insertion sequences were trimmed for quality and aligned to the *E. faecalis* OG1RF genome (GenBank accession number CP002621; October 2011) (20) using Lasergene Seqman software (DNASTAR, Madison, WI). Alignments were inspected visually to determine locations of putative promoters, which were expected to initiate transcription in the same orientation as the F sequencing primer. Clones for which the F and R reads aligned to locations on the chromosome too distant to represent a single contiguous cloned chromosomal segment were designated chimeras. For chimeric clones, the R sequence (proximal on the plasmid to the reporter gene) was assumed to correspond to the location of the putative promoter. Corresponding open reading frames (ORFs) in *E. faecalis* V583 (21) were identified using Seq-

builder software (DNASTAR, Madison, WI) by searching the V583 genome using partial (20- to 70-bp) sequences from OG1RF ORFs as search queries and then confirming the matches using metadata and visual inspection, with additional search queries as needed. Categories of cellular roles of the V583 genes were found at the JCVI Comprehensive Microbial Resource web site (<http://cmr.jcvi.org/in>; January 2012).

**Construction of deletion strains.** All strains and plasmids used in this study are listed in Table 1. The published genomes of *E. faecalis* strains OG1RF (20) and V583 (21) were used to obtain DNA sequences. All restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Platinum Taq DNA Polymerase High Fidelity (Life Technologies, Carlsbad, CA) was used for all PCRs. The manufacturer's recommendations were followed for all amplification and digestion steps. For all deletion strains, upstream and downstream flanking regions of each locus were PCR amplified from the OG1RF chromosome and then combined using overlap PCR. Primers are listed by ORF genome locus in Table S1 in the supplemental material. The combined amplicons used to construct deletion strains OG1RF $\Delta$ pglA and OG1RF $\Delta$ radA were ligated into vector pCR4TOPO following the manufacturer's instructions (Life Technologies, Carlsbad, CA). Plasmids were digested with NotI and PstI. Insertion DNA was gel purified and then ligated into plasmid pCJ47 (19) predigested with NotI and PstI. A similar strategy was used to construct strains OG1RF $\Delta$ rhoN, OG1RF $\Delta$ EF1144, and OG1RF $\Delta$ menB, with the exception that outside primers (see Table S1) contained restriction sites to bypass TOPO cloning. Resulting products were digested with EcoRI (strain OG1RF $\Delta$ rhoN) or PstI (strains OG1RF $\Delta$ EF1144 and OG1RF $\Delta$ menB) and ligated into pCJ47 predigested with the same enzymes. The resulting plasmids were propagated in *Escherichia coli* EC1000 in BHI broth with 100  $\mu\text{g ml}^{-1}$  erythromycin and used for allelic exchange (19). OG1RF $\Delta$ arc2 was constructed previously (22).

**Construction of complemented strain OG1RF $\Delta$ pglA\_TCDR\_97.** A region 239 bp upstream and 161 bp downstream of the pglA ORF (OG1RF\_11582) was PCR amplified using primers EF1918pCJ41BamHI\_F and EF1918pCJ41EcoRI\_R, digested with BamHI and EcoRI, and ligated with plasmid pCJ41 (23). The resulting plasmid was propagated in *E. coli* EC1000 on BHI medium with erythromycin at 100  $\mu\text{g ml}^{-1}$  and used for allelic exchange (19) into strain OG1RF $\Delta$ pglA. Plasmid pCJ41 is designed to facilitate allelic integration of the cloned insertion at an ectopic site in the *E. faecalis* chromosome (23). However, despite our making several attempts to obtain the intended ectopic integrant, integration of this construct into the  $\Delta$ pglA genetic background instead consistently led to repair of the pglA deletion at the native locus. Strain OG1RF $\Delta$ pglA\_TCDR\_97 was chosen from among the recombinants with the repaired pglA locus.

**Persistence, invasion, and competition assays in *M. sexta*.** To measure *E. faecalis* persistence in the *M. sexta* gut, larvae were starved overnight after molting to the third instar. The next morning, larvae were fed unamended diet discs (2.5-mm radius, 1-mm thickness), followed by experimental treatments 6 h later. Bacterial cultures were grown for 24 h in BHI broth with 200  $\mu\text{g ml}^{-1}$  rifampin, concentrated by centrifugation, and washed three times in sterile phosphate-buffered saline (PBS), and 2  $\mu\text{l}$  was then applied to diet discs and offered to each larva ( $10^7$  CFU per larva). Larvae were maintained at room temperature in sterile 24- or 12-well polystyrene microtiter plates to which fresh diet was added twice daily. At experimental time points, larvae were rinsed in 70% ethanol, and guts were dissected using featherweight forceps (Bioquip, Rancho Dominguez, CA) and placed into 1 ml sterile PBS. Guts were manually disrupted by pipetting for 30 s, sonicating for 45 s in an ultrasonic cleaning bath (Branson, Danbury, CT), and mixing using a vortex device for 30 s before plating on BHI solid media amended with 200  $\mu\text{g ml}^{-1}$  rifampin was performed. Plates were inoculated using an Autoplate (Advanced Instruments, Norwood, MA) and incubated 24 to 48 h at 37°C, and the CFU/gut were counted using Q-count (Advanced Instruments). Recovered bacterial population sizes were compared in Prism (GraphPad, La Jolla, CA) using analysis of variance (ANOVA) with Dunnett's posttest for

TABLE 1 List of strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>Enterococcus faecalis</i> OG1RF	Spontaneous mutant of OG1, resistant to rifampin and fusidic acid	20
<i>Enterococcus faecalis</i> OG1RFΔ <i>radA</i>	Alternative names: OG1RFΔOG1RF_10039, OG1RFΔEF0040	This work
<i>Enterococcus faecalis</i> OG1RFΔ <i>pglA</i>	Alternative names: OG1RFΔOG1RF_11582, OG1RFΔEF1918	This work
<i>Enterococcus faecalis</i> OG1RFΔ <i>rpoN</i>	Alternative names: OG1RFΔOG1RF_10514, OG1RFΔEF0782	This work
<i>Enterococcus faecalis</i> OG1RFΔEF1144	Alternative names: OG1RFΔOG1RF_10921, OG1RFΔEF1144	This work
<i>Enterococcus faecalis</i> OG1RFΔ <i>menB</i>	Alternative names: OG1RFΔOG1RF_10330, OG1RFΔEF0445	This work
<i>Enterococcus faecalis</i> OG1RFΔ <i>rnc2</i>	Alternative names: OG1RFΔOG1RF_12365, OG1RFΔEF3097	22
<i>Enterococcus faecalis</i> OG1RFΔ <i>pglA</i> _TCDR_97	Complemented <i>pglA</i> mutant with wild-type copy of <i>pglA</i> gene on the chromosome	This work
<i>Enterococcus faecalis</i> OG1Sp	Spontaneous mutant of OG1; resistant to spectinomycin	19
<i>Escherichia coli</i> DH5α	General cloning host	New England Biolabs
<i>Escherichia coli</i> EC1000	Carries <i>repA</i> allele for replication of pCJ47 and pCJ141; resistant to kanamycin	37
<i>Escherichia coli</i> K-12 MG1655	Laboratory strain encoding a 6-PGL gene	30
<i>Escherichia coli</i> BL21(DE3)	Host strain for pET28b	Life Technologies
<i>Escherichia coli</i> BW25113	Keio collection parent strain	29
<i>Escherichia coli</i> JW0750-3	BW25113 <i>pgl</i> deletion strain	29
Plasmids		
pCJ47	Vector for general allelic exchange in <i>E. faecalis</i> : <i>repA</i> dependent, <i>p</i> -cl-Phe sensitive, <i>erm</i> resistant, <i>lacZ</i>	19
pCJ141	Vector for allelic exchange in <i>E. faecalis</i> : <i>repA</i> dependent, <i>p</i> -cl-Phe sensitive, <i>erm</i> resistant, <i>lacZ</i>	23
pBluescript KS+	High-copy-number ColE1-based phagemid with <i>f1</i> origin in positive orientation and Kpn-Sac polylinker	Agilent Genomics
pBluescript/ <i>pglA</i>	pBluescript with promoterless copy of <i>pglA</i> from <i>E. faecalis</i> OG1RF	This work
pBluescript/ <i>pgl</i>	pBluescript with promoterless copy of <i>pgl</i> from <i>E. coli</i> K-12 MG1655	This work
pET28b/EF1918-C-His	pET28b with <i>pglA</i> from <i>E. faecalis</i> OG1RF	This work

multiple comparisons. All treatments were compared with strain OG1RF results, and strains were considered defective for persistence if they differed from the wild type with  $P < 0.05$ . Strain OG1RFΔ*pglA* was tested in seven independent experimental feedings, and strain OG1RFΔ*pglA*\_TCDR\_97 was included in two of these experiments. Additional *E. faecalis* strains were tested in at least two independent experiments, with the exception of strain OG1RFΔ*rnc2*, which was tested once.

Invasion and competition assays were performed similarly, with the following modifications. For invasion assays, larvae were reared on unamended diet only, and bacteria were plated on plates containing 1/10-strength tryptic soy agar (to estimate the abundance of culturable bacteria from the native microbiota) as well as on BHI agar plates amended with rifampin (200 μg ml<sup>-1</sup>) (to estimate the abundance of the *E. faecalis* strain). For competition assays, larvae were fed equal doses of *E. faecalis* OG1Sp mixed with the strain to be tested (estimated by turbidity and microscopy, with a combined dose of 10<sup>7</sup> CFU/larva), and bacteria were plated on BHI agar amended with spectinomycin at 1000 μg ml<sup>-1</sup> as well as on BHI agar plates containing rifampin at 200 μg ml<sup>-1</sup>. Both the wild-type OG1RF and OG1Sp strains were confirmed to have gelatinase activity *in vitro* by growing on agar plates containing gelatin and observing a zone of clearing (24). Mutant phenotypes were confirmed by at least three independent experiments using distinct cohorts of larvae unless otherwise noted. Wild-type phenotypes were confirmed twice in the same manner unless otherwise noted. Student's *t* test was used to compare each mutant strain to the wild type.

**Pathogenesis assays in *M. sexta*.** Early fifth-instar larvae were fed unamended insect diet for 24 h prior to experimental injections. The 24-h-old bacterial cultures were concentrated by centrifugation and washed 3 times in sterile PBS prior to injection. Cultures were diluted and plated to determine retrospectively the viable injected dose. Larvae were washed with 70% ethanol and blotted dry and then injected with 10 μl

into the right first proleg and placed in a new container with fresh unamended diet. Mortality was assessed daily, and diet was replenished as needed. Larvae were scored dead when they ceased to respond to prodding and lost turgor. Multiple wild-type control groups receiving injections of various doses were used in each experiment, and experimental treatments were later compared only to a single control treatment based on the similarity of the viable injected dose as determined by plating inocula. Survival curves were analyzed in Prism using the Mantel-Cox log-rank test, and strains were considered defective for pathogenesis if they differed from the wild type with  $P < 0.05$  in every experiment. All mutant data were compared only to control data from the same cohort of insects. Strains that produced inconsistent results (defective in at least one experiment and indistinguishable from the wild type in at least one experiment) were designated “inconsistent” for pathogenesis. Strains OG1RF, OG1RFΔ*pglA*, OG1RFΔ*rpoN*, and OG1RFΔEF1144 were tested four times, strain OG1RFΔ*radA* was tested three times, strain OG1RFΔ*rnc2* was tested twice, and strain OG1RFΔ*menB* was tested once. Average injected doses were 10<sup>7</sup> CFU per larva.

***E. faecalis* microtiter plate biofilm assays.** Each *E. faecalis* colony was inoculated into 5 ml TSB supplemented with 0.2% glucose (TSB+G) in 15-ml screw-cap Falcon tubes and grown statically for 24 h at 37°C. These cultures were then diluted 1:100 in fresh TSB+G, and 200 μl was transferred to each well of a 96-well flat-bottom tissue culture treated microtiter plate (Corning, Tewksbury, MA). Plates were sealed with Parafilm, and biofilms were grown statically for 24 h at 37°C. To assess biofilm growth, planktonic and nonadherent cells were removed by vacuum filtration, wells were washed with 200 μl of sterile water, and the plate was inverted onto paper towels, tapped to remove excess liquid, and allowed to dry for 10 min. Wells were then stained for 10 min with 1 vol 0.1% crystal violet. The crystal violet solution was removed by vacuum filtration, and wells were washed and dried as described above and destained for 1 h using 1



volume of 80:20 ethanol:acetone solution per well. Destain solution was then pipetted into a new Corning no. 3595 microtiter plate, and absorbance at 630 nm was measured on a Synergy HT plate reader (Biotek, Winooski, VT). Experiments were repeated in triplicate with six replicate wells per strain evaluated per experiment. Data from replicate experiments were pooled, and the mean stainable biomass for each strain was compared to the wild-type level by ANOVA with Dunnett's posttest for multiple comparisons.

**Growth curves and determination of doubling times.** Log-phase broth cultures grown in BHI medium with 200  $\mu\text{g ml}^{-1}$  rifampin were diluted in fresh medium to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.005 and then dispensed into wells of 96-well microtiter plates. Plates were incubated for 16 h in a temperature-controlled microtiter plate reader (SynergyHT or ELx808) at 37°C or 30°C for BHI broth or hemolymph, respectively, and the  $\text{OD}_{630}$  was read every 15 or 10 min (growth in BHI broth or hemolymph, respectively) after 5 s of shaking. Each experimental condition was represented by at least four replicate wells. Growth curves in hemolymph were collected twice. Minimum doubling times in BHI broth were measured for each culture on three occasions, and replicate wells were used to calculate an average doubling time for each experiment. Doubling times were calculated from at least eight sequential data points representing exponential growth. Triplicate doubling times for each culture were then compared using one-way ANOVA.

**Purification of *E. faecalis* PglA.** To construct a PglA overexpression vector, OG1RF\_11582 was PCR amplified using primers pgl-NcoI-f and pgl-XhoI-C-His-r. The vector and amplified PCR product were cut with restriction enzymes NcoI and XhoI and ligated with T4 DNA ligase (New England BioLabs), resulting in pET28b/EF1918-C-His. The recombinant vector, which inserts a His<sub>6</sub> tag at the C terminus of PglA, was transformed into *E. coli* BL21(DE3) and induced with IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside; 250  $\mu\text{M}$ ) at 30°C for 4.5 h. The cells were resuspended in binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) supplemented with lysozyme (1 mg/ml), DNase I (19  $\mu\text{g/ml}$ ), and RNase A (60  $\mu\text{g/ml}$ ). Cells were lysed by seven cycles of sonication (VWR Sonifier; VWR, Radnor, PA) and then centrifuged to remove cellular debris. Supernatant was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Venlo, Netherlands) column and washed with washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Finally, PglA was eluted using elution buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and concentrated using an Amicon 10K spin filter (Millipore, Billerica, MA). Glycerol was added to reach a final concentration of 20%, and the mixture was stored at  $-80^{\circ}\text{C}$  for later use in enzymatic assays.

**Complementation of *pgl* in *E. coli* JW0750-3.** Gene OG1RF\_11582 was amplified from strain OG1RF genomic DNA using primers EF1918\_XhoI\_EcoRBS\_pBlu\_F and EF1918\_SacI\_pBlu\_R, digested with restriction enzymes XhoI and SacI, and ligated into pBluescript KS+ (Agilent Genomics, Santa Clara, CA) predigested with the same enzymes to generate plasmid pBluescript/*pglA*. *pgl* from *E. coli* K-12 MG1655 was amplified from genomic DNA using primers EcoliPgl\_SacI\_pBlu\_R and EcoliPgl\_XhoI\_pBlu\_F and cloned into pBluescript using the same restriction enzymes as those described above to generate plasmid pBluescript/*pgl*. Ligation mixtures were transformed into the *E. coli* *pgl* insertion mutant strain JW0750-3 by electroporation and propagated on LB agar with 50  $\mu\text{g ml}^{-1}$  carbenicillin. For complementation assays, gene expression was induced by addition of 50  $\mu\text{M}$  IPTG.

**Maltose Blue assay for 6-phosphogluconolactonase (6-PGL) activity.** *E. coli* strains were grown at 37°C on modified MM9YEG agar (19) consisting of M9 salts amended with 0.25% yeast extract and 0.25% maltose. Maltose Blue assays were performed according to the procedure of Kupor and Fraenkel (25), with Lugol solution (Sigma) as the source of iodine. Plates flooded with Lugol solution were incubated at room temperature for 30 s before reading was performed. Colony color was compared to that of freshly grown, freshly stained control colonies. Phenotype scores were based on the color of the majority of colonies.

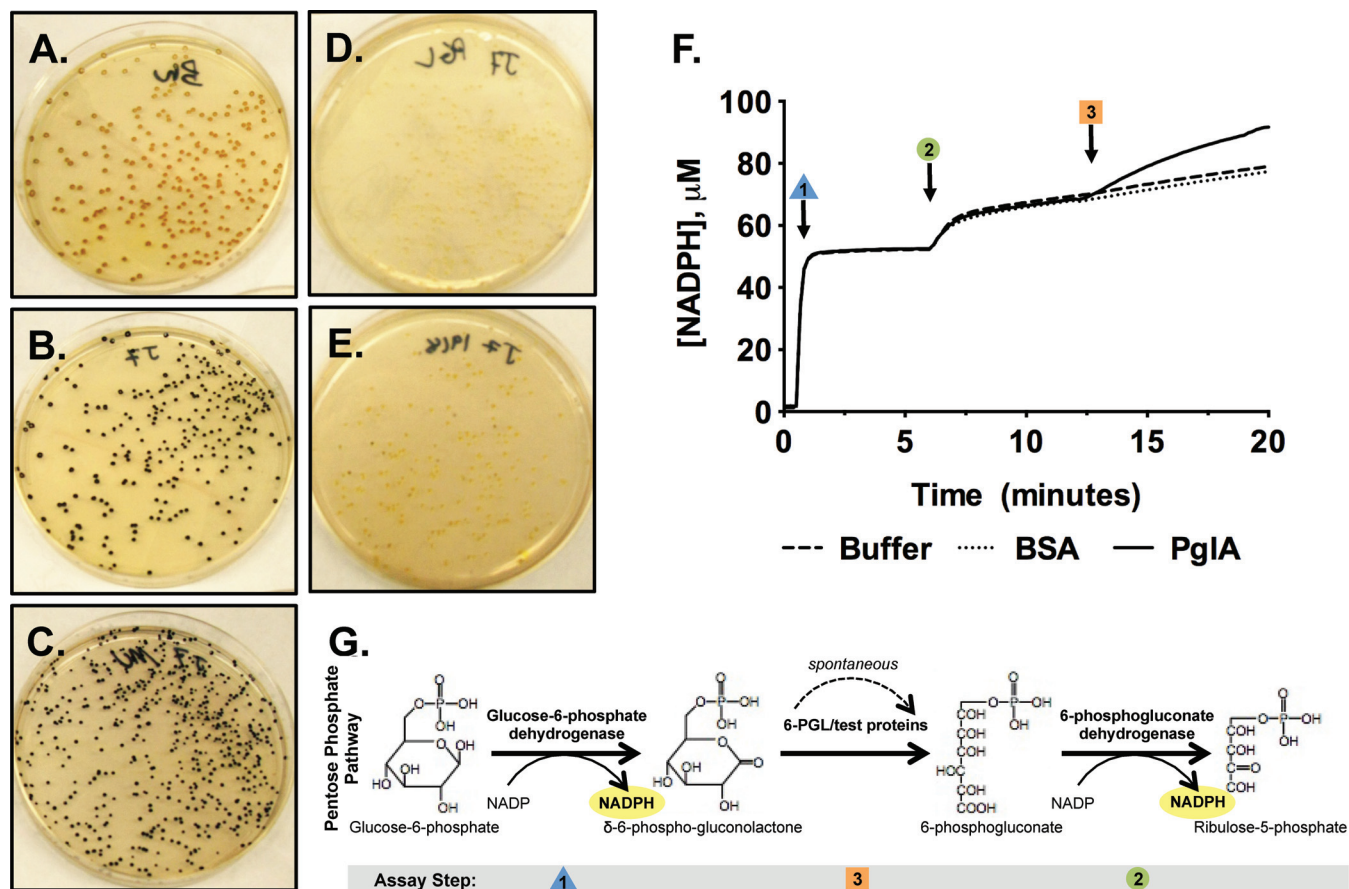
**Measurement of 6-phosphogluconolactonase activity.** We reconstituted the first three steps of the pentose phosphate pathway in 25 mM HEPES–2 mM  $\text{MgCl}_2$ –0.2 mM NADP following the procedure described by Collard et al. as modified by Roy et al. (26, 27). NADPH was quantified by absorbance at 340 nm (Fig. 1F). At time zero, with no enzymes present, 50  $\mu\text{M}$  glucose-6-phosphate was added. Glucose-6-phosphate dehydrogenase (Sigma G7877) (1.75 U  $\text{ml}^{-1}$ ) was added at 30 s. 6-Phosphogluconate dehydrogenase (Sigma P4553) (0.5 U  $\text{ml}^{-1}$ ) was added at 6 min, followed by test extracts (6.0  $\text{pg ml}^{-1}$  PglA or controls) at 12 min. Reaction rates were measured from all readings taken over 1 min beginning 30 s after addition of test extract, with the rate measured between 11 and 12 min subtracted. Bovine serum albumin (BSA; 8.0  $\text{pg ml}^{-1}$ ) served as a nonspecific protein control. An extinction coefficient of 6,224  $\text{mol}^{-1} \text{cm}^{-1}$  was used for quantification of NADPH.

## RESULTS

**A RIVET screen identified *E. faecalis* genes activated in the *M. sexta* gut.** We isolated and sequenced DNA inserts from 113 clones recovered after feeding a library of randomly constructed RIVET clones (18) to *M. sexta* larvae. Insertion DNA from each *in vivo* upregulated clone was expected to contain a promoter that drives expression of an *in vivo*-activated gene in *E. faecalis* while in the gut of *M. sexta*. Of the recovered clones, 76 contained insertion DNA readily traceable to a putative promoter region upstream of an ORF in the OG1RF genome (see Dataset S1 in the supplemental material). We identified 27 clones as chimeras whose proximal portion could be assigned to likely ORFs (see Dataset S1), while 9 other chimeric clones could not be assigned and were not studied further. The orientation of one clone suggested that its putative promoter drives transcription of an antisense transcript and was also not characterized further in this work. The *in vivo*-activated promoters from the 103 clones with assigned promoters (see Dataset S1) were well distributed around the OG1RF chromosome (see Fig. S1 in the supplemental material), with the upregulated genes serving a diverse set of functional roles in the cell (see Table S2).

Six regions of the genome were represented in two or more clones, and in five cases, these clones represented siblings with identical insertion sequences (Table 2; see also Dataset S1 in the supplemental material). The region upstream of locus OG1RF\_11582 (EF1918 in *E. faecalis* V583) was identified in 34 clones, including two nonsibling clones, one recovered 31 times and the second, a chimeric clone, recovered three times. The five remaining overrepresented regions were each identified from apparent sibling clones recovered more than once. The overrepresented clones were distinct from those observed in previous RIVET studies using the same library under different selective conditions (18, 28); therefore, the overrepresented regions were among those targeted for downstream analysis (Table 2).

**OG1RF\_11582 encodes a 6-phosphogluconolactonase.** As it was the most frequently identified locus among the RIVET clones, we initially focused on ORF OG1RF\_11582 for further characterization. The deduced protein contained high similarity to 6-phosphogluconolactonases (EC 3.1.1.31), or 6-PGLs. We verified the function in a Maltose Blue assay (25) in which wild-type *E. coli* strain BW25113 was positive for 6-PGL activity (Fig. 1A). An *E. coli* *pgl* mutant, JW0750-3 (29), with or without the empty vector pBluescript II KS+ gave a negative result (Fig. 1B and C), whereas 6-PGL activity was restored by *pgl* from either *E. coli* MG1655 (30) or *E. faecalis* OG1RF\_11582 (Fig. 1D and E). We purified the gene product from locus OG1RF\_11582 and demonstrated 6-PGL ac-



**FIG 1** 6-PGL activity of OG1RF\_11582 when expressed in *E. coli*. (A to E) Maltose Blue phenotype of *E. coli* strains BW25113 and JW0750-3 and derivatives. Plates contain M9 salts amended with 0.25% maltose as the primary carbon source and 50  $\mu\text{M}$  IPTG. Plates were flooded with Lugol solution and photographed after 30 s. (A) *E. coli* BW25113. (B) *E. coli* JW0750-3 ( $\Delta\text{pgl}$ ). (C) *E. coli* JW0750-3 (pBluescript II KS+). (D) *E. coli* JW0750-3 (pBluescript/pglA), which expresses *E. coli* pgl. (E) *E. coli* JW0750-3 (pBluescript/pglA), which expresses *E. faecalis* pglA. (F) Direct enzyme activity assay using purified PglA from *E. faecalis* OG1RF\_11582. Arrows indicate addition of glucose-6-phosphate dehydrogenase (arrow 1), 6-phosphogluconate dehydrogenase (arrow 2), and test proteins (arrow 3). The experimental protein used was 8  $\text{pg ml}^{-1}$  BSA or 6  $\text{pg ml}^{-1}$  PglA, which was purified as described in Materials and Methods. Solid line, PglA; dotted line, BSA; dashed line, buffer. Lines indicate the averages of the results of four trials. Standard errors for all points were less than 1.7  $\mu\text{M}$ ; thus, error bars have been omitted for clarity. (G) Schematic of 6-PGL enzyme activity assay (modeled after the pentose phosphate pathway diagram by Zimenkov et al. [31]), with steps labeled 1 to 3 corresponding to the times of addition of the enzymes specified in panel F.

tivity of the purified protein (Fig. 1F) by reconstituting the first three steps of the pentose phosphate pathway *in vitro* (diagrammed in Fig. 1G). The purified recombinant PglA had activity of  $520 \pm 20 \text{ U mg}^{-1}$ , which is similar to previously reported activity of YbhE from *E. coli* (780  $\text{U mg}^{-1}$  protein) [31] and a human 6-PGL (710  $\text{U mg}^{-1}$ ) [27].

**A  $\Delta\text{pglA}$  mutant has a small-colony growth phenotype on agar plates.** Single-gene-deletion mutants for each of the six over-represented ORFs from the RIVET screen were constructed in the OG1RF genetic background (Table 2). In addition, a complemented strain that repaired the *pglA* deletion was constructed in the OG1RF $\Delta\text{pglA}$  background (Table 2). Doubling times of the deletion and complemented strains in BHI broth did not differ from that of the wild type (Fig. 2A). OG1RF $\Delta\text{pglA}$  produced smaller colonies on BHI and M9 agar amended with glucose. The phenotype was rescued by complementation (Fig. 2B). All other strains used in this study formed colonies similar in size to those seen with wild-type OG1RF (data not shown).

***E. faecalis* requires *pglA* to persist in the guts of *M. sexta* larvae.** We defined persistence in *M. sexta* larvae guts by recov-

ery of viable cells at abundance similar to that of the wild-type OG1RF strain at 48 h after feeding. Among the six *E. faecalis* strains with deletions in genes identified in the RIVET screen, only OG1RF $\Delta\text{pglA}$  persisted at lower levels than wild-type strain OG1RF (Table 2 and Fig. 3A). Persistence was restored by reintroduction of *pglA* into the deletion strain (Fig. 3A).

Given the small-colony phenotype of strain OG1RF $\Delta\text{pglA}$  and that all measurements in the persistence assays were made by enumerating colonies on agar plates, the observed persistence defect could alternatively be explained by decreased plating efficiency of strain OG1RF $\Delta\text{pglA}$ . Low plating efficiency could lead to underestimation of OG1RF $\Delta\text{pglA}$  population sizes. If this were the sole explanation for the observed persistence defect, we would expect to see a constant ratio of recovered mutant and wild type at all time points after feeding. Alternatively, if strain OG1RF $\Delta\text{pglA}$  were killed or otherwise removed from the gut faster than OG1RF, we would expect similar recovered population sizes immediately after feeding and increasingly different population sizes over time. To investigate this possibility, we collected time course data showing persistence over several days. The population dynamics of strains OG1RF and

TABLE 2 Phenotypes of *E. faecalis* strains<sup>a</sup>

No. of RIVET clones	Strain	OG1RF locus	V583 (EF) locus	Symbol	Annotated function in the OG1RF genome <sup>b</sup>	Persistence in <i>M. sexta</i>	Pathogenesis in <i>M. sexta</i>	Biofilm on microtiter plates
—	OG1RF	—	—	—	—	WT	WT	WT
34	OG1RFΔ <i>pglA</i>	11582	1918	<i>pglA</i>	6-Phosphogluconolactonase	Defective	Defective	Increased
—	OG1RFΔ <i>pglA</i> _TCDR_97	—	—	—	—	WT	WT	WT
6	OG1RFΔ <i>radA</i>	10039	0040	<i>radA</i>	DNA repair protein RadA	WT	WT	WT
3	OG1RFΔEF1144	10921	1144	—	Lipoate-protein ligase A family protein, putative	WT	Inconsistent <sup>c</sup>	WT
3	OG1RFΔ <i>rpoN</i>	10514	0782	<i>rpoN</i>	DNA-directed RNA polymerase sigma subunit RpoN	WT	Defective	Increased
3	OG1RFΔ <i>rnc2</i>	12365	3097	<i>rnc2</i>	RNase III	WT	Inconsistent	WT
2	OG1RFΔ <i>menB</i>	10330	0445	<i>menB</i>	Naphthoate synthase	WT	Inconsistent	WT

<sup>a</sup> WT, wild type. Phenotypes are described relative to the WT. Dashes indicate “not applicable” or “no value assigned.”

<sup>b</sup> GenBank accession number CP002621.

<sup>c</sup> “Inconsistent” pathogenesis indicates clones for which reduced killing was observed in at least one experiment and wild-type killing was observed in at least one experiment.

OG1RFΔ*pglA* in *M. sexta* differed, and the ratio of recovered bacterial populations of strain OG1RF to strain OG1RFΔ*pglA* varied over time (see Fig. S2 in the supplemental material).

Each mutant was also tested for competition and invasion, defined as persistence in *M. sexta* when coted with a spectinomycin-resistant strain of *E. faecalis*, strain OG1Sp, and when fed to larvae precolonized with normal microbiota, respectively. All mutants tested had wild-type OG1RF phenotypes except for strain OG1RFΔ*pglA*, which did not persist, regardless of the presence of other bacteria in the gut (data not shown).

**Strain OG1RFΔ*pglA* forms enhanced biofilms in polystyrene microtiter plates.** The six deletion mutants were tested for the ability

to form biofilms on polystyrene microtiter dishes (Table 2 and Fig. 3B). Most mutants formed wild-type biofilms, as measured by crystal violet staining. However, both strain OG1RFΔ*pglA* (Fig. 3B) and strain OG1RFΔ*rpoN* (Table 2) generated up to twice as much biofilm biomass as strain OG1RF or the other mutants. Wild-type levels were restored in strain OG1RFΔ*pglA*\_TCDR\_97 (Fig. 3B). The enhanced biofilm formation phenotype of a Δ*rpoN* strain was previously reported (32), and our biofilm studies reinforce this finding (Table 2). ANOVA found significant differences in mean biomass among the strains ( $P < 0.001$ ). Strain OG1RFΔ*pglA* differed significantly from wild-type OG1RF (Dunnett's posttest,  $P < 0.0001$ ), but the complemented strain OG1RFΔ*pglA*\_TCDR\_97 did not.

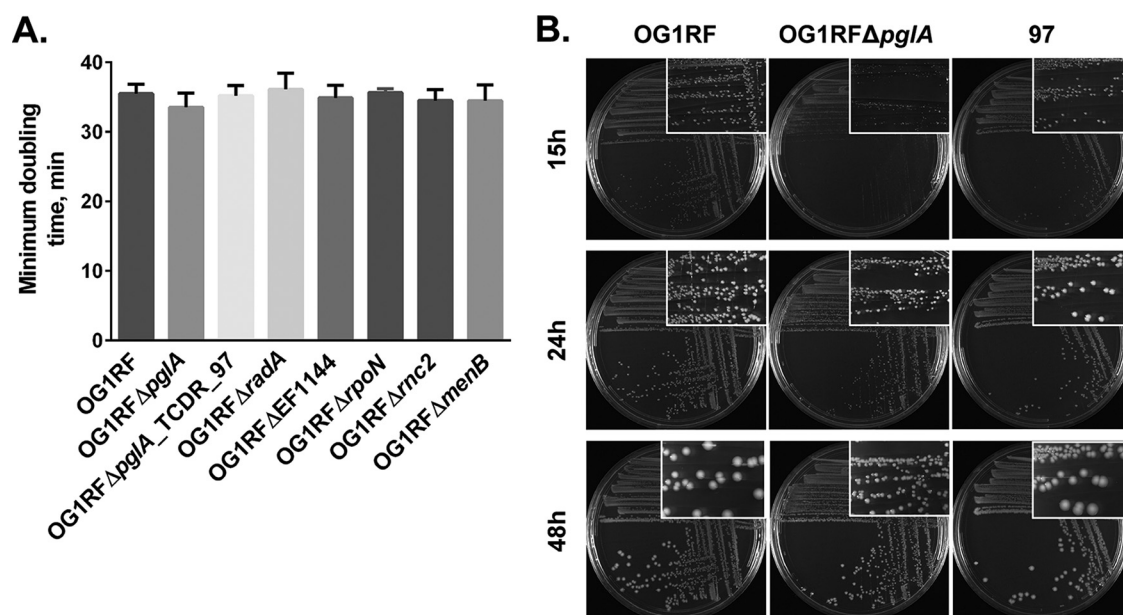
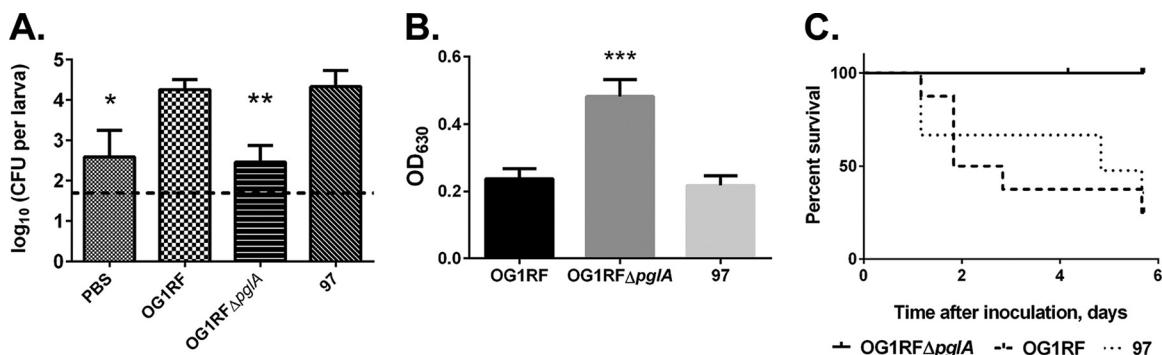


FIG 2 Strain OG1RFΔ*pglA* has a small-colony growth phenotype on agar plates. (A) Minimum doubling times of mutants grown in rich medium. Log-phase cells were inoculated into BHI broth and incubated at 37°C without shaking for 16 h. Doubling times were calculated from at least eight sequential points as described in Materials and Methods. Data shown are the means of the results from three independent experiments performed using at least four replicate wells each. Error bars represent the standard errors from three experimental means. Analysis of variance found no difference between the doubling time of any strain and the overall mean doubling time. (B) Growth of OG1RFΔ*pglA* on BHI agar. Photographs show colony formation of wild-type *E. faecalis* OG1RF (left), deletion strain OG1RFΔ*pglA* (middle), and complemented strain OG1RFΔ*pglA*\_TCDR\_97 (right) on BHI agar plates. Bacteria were streaked from overnight BHI broth cultures onto plates that were incubated at 37°C and photographed at 15, 24, and 48 h postinoculation. Inset photos show  $\times 7.5$  magnification of streaks. The same plates are shown for each time point.

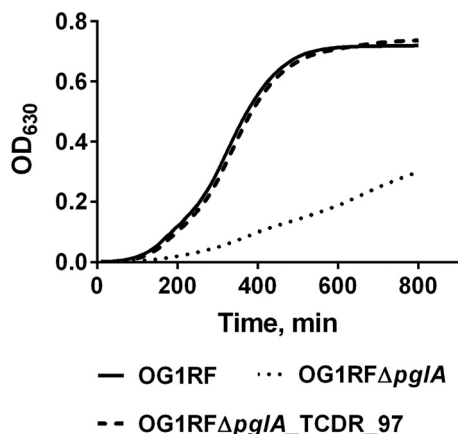




**FIG 3** Persistence, biofilm formation, and pathogenesis of strain OG1RF $\Delta$ pglA. (A) Persistence assays indicating the ability of strains OG1RF, OG1RF $\Delta$ pglA, and OG1RF $\Delta$ pglA\_TCDR\_97 to survive in the *M. sexta* gut 48 h after feeding. Bars represent the log-transformed viable counts obtained on BHI agar with 200  $\mu$ g ml<sup>-1</sup> rifampin from one representative experiment of two performed with these strains. Error bars represent standard errors of the means (SEM) ( $n \geq 10$ ). The dotted black line represents the lower detection limit. Counts shown in PBS control groups indicate background non-*Enterococcus* rifampin-resistant fungal isolates (confirmed by cellular morphology and lack of positive PCR amplification with bacterial 16S rRNA-specific primers). 97, OG1RF $\Delta$ pglA\_TCDR\_97. Asterisks indicate significant differences from the wild type identified by Dunnett's posttest. \*, adjusted  $P < 0.05$ . \*\*, adjusted  $P < 0.01$ . (B) *in vitro* biofilm formation. Experiments were repeated in triplicate with six replicate wells per strain evaluated per experiment. Bars represent means of the results determined from all wells from three experiments, and error bars represent standard errors from all wells. \*\*\*,  $P < 0.0001$ . (C) Pathogenesis of *E. faecalis* strains toward *M. sexta*. The graph data represent survival of *M. sexta* after injection of *E. faecalis* strains behind the right first proleg. In all treatments,  $n = 8$  or higher. A strain was considered defective for pathogenesis in an experiment if it differed significantly from the wild type in the Mantel-Cox log rank test with  $P < 0.05$ .

**Several mutants that persist normally are deficient in insect pathogenesis.** Wild-type OG1RF causes rapid, dose-dependent killing when injected into larval hemocoel (12). Strain OG1RF $\Delta$ pglA consistently exhibited slower killing (Fig. 3C), as did the OG1RF $\Delta$ rpoN deletion mutant (Table 2). The complemented OG1RF $\Delta$ pglA\_TCDR\_97 strain displayed increased pathogenicity compared to the  $\Delta$ pglA strain (Fig. 3C). Three additional mutants had moderate or inconsistent pathogenesis defects (Table 2).

**Strain OG1RF $\Delta$ pglA growth is impaired in hemolymph.** To determine whether altered growth in *M. sexta* was responsible for the pathogenesis defect in strain OG1RF $\Delta$ pglA, growth was measured *ex vivo* in *M. sexta* hemolymph. In hemolymph, strain OG1RF $\Delta$ pglA grew slowly relative to the wild-type and complemented strains (Fig. 4). Since exponential growth of the mutant was not observed, doubling times were not calculated.



**FIG 4** Growth of *E. faecalis* strains in *M. sexta* hemolymph. Log-phase cultures of *E. faecalis* strains were inoculated into pooled, homogenized *ex vivo* hemolymph from *M. sexta* larvae. The  $OD_{630}$  was measured every 10 min to determine growth at 30°C. Representative data shown are from one of two experiments. Solid line, OG1RF; dotted line, OG1RF $\Delta$ pglA; dashed line, OG1RF $\Delta$ pglA\_TCDR\_97.

## DISCUSSION

In this work, we used a RIVET screen to identify genes from *E. faecalis* that contribute to its commensalism with *M. sexta*. The abundance of RIVET clones recovered from growth of a library under our selective condition of interest, the gut of *M. sexta* larvae, served as a measure of gene expression in the gut environment (see Dataset S1 in the supplemental material). Several clones were overrepresented in the results of our RIVET screen (Table 2) that were not overrepresented in previous screens with this library in other systems (18, 28). In this paper, we establish that the most frequently recovered gene, locus OG1RF\_11582, is a 6-phosphogluconolactonase based on functional complementation and *in vivo* enzymatic activity in *E. coli* and enzymatic activity of the purified gene product. In light of these results, we have designated the OG1RF\_11582 locus *pglA*. Based on weaker homology, there remains a formal possibility that PglA also has *cis,cis*-muconate lactonizing activity, although this has yet to be investigated.

*pglA* appears important for both commensal and pathogenic interactions with larvae of *M. sexta*. A mutant lacking *pglA* was affected in persistence in the gut (Fig. 3A) and larval killing when injected directly into the hemocoel (Fig. 3C). The *pglA* mutant also produces enhanced biofilms (Fig. 3B) and adheres better than the wild type to several surfaces, including polypropylene, charged glass, and untreated glass (data not shown). The role of *pglA* in interactions with surfaces might indicate that surface interactions are important in the larval gut. Perhaps elevated nonspecific adhesion enables the  $\Delta$ pglA strain to bind to either the food bolus or the peritrophic matrix lining the insect gut and to be transported with these materials out of the gut, reducing the mutant's persistence.

Several other hypotheses explain the defective persistence of the  $\Delta$ pglA mutant in the *M. sexta* gut. The phenotype could be related to the reduced growth on solid media, although it is not known if growth is required for persistence. Although its role is not known, 6-PGL is also essential for *Lactococcus lactis* colonization of the mouse intestine (26). The role of *pglA* in gut persistence by two different genera within the lactic acid bacteria in diverse

hosts suggests an ecological role for 6-PGL. The parallels among model systems indicate that the *M. sexta*-*E. faecalis* system provides a valid alternative to vertebrate hosts for the study of commensal behaviors of mammalian pathogens.

The mutant's altered surface interactions are also reflected in its growth defect on solid, but not liquid, media (Fig. 2). The growth defect was not rescued by addition of dihydroorotate or inosine to media (data not shown), indicating that this phenotype is not attributable to depletion of metabolites produced by the pentose phosphate pathway, of which *pglA* is likely a constituent. Furthermore, the reaction catalyzed by 6-PGL enzymes (conversion of  $\delta$ -6-phosphogluconolactone to 6-phosphogluconate) occurs spontaneously, if slowly, under physiological conditions (33), and therefore a mutant strain lacking 6-PGL should not have a dramatic phenotype. However, the absence of a 6-PGL enzyme would allow  $\delta$ -6-phosphogluconolactone to accumulate, and this could have multiple effects on the cell.  $\delta$ -6-Phosphogluconolactone can spontaneously isomerize to form  $\gamma$ -6-phosphogluconolactone, which is considered a metabolic "dead end" (33). Previous work has shown that  $\delta$ -6-phosphogluconolactone is highly reactive with cellular nucleophiles, suggesting that it could cause nonspecific disruption of proteins if it accumulated in a cell, which could reduce growth. In addition, Tu et al. showed that  $\delta$ -6-phosphogluconolactone inhibits maltodextrin phosphorylase, potentially leading to alterations in carbohydrate polymerization in *E. coli* (34). Modification of carbohydrates could result in altered surface characteristics, which may explain the differences observed for OG1RF $\Delta$ *pglA* in attachment to surfaces and biofilm formation and warrants further study.

The insect gut screen identified a set of genes different from those that have been identified in other *in vivo* screens. For example, over one-third of the active clones recovered from the same RIVET library in a rabbit abscess model contained putative promoters oriented in the direction opposite that of nearby ORFs (28), whereas only 1 of our 113 clones contains an antisense locus (see Dataset S1 in the supplemental material), suggesting that there are significant differences in global transcription regulation between a mammalian abscess and an insect gut. Comprehensive transcriptomic analysis would be required to test this hypothesis. Likewise, the loci identified in a screen for gene activation in the hemocoel of *Galleria mellonella* did not overlap those reported here, likely reflecting the differences between the gut and the hemocoel (35).

RIVET methodology has identified factors in *E. faecalis* that contribute to *in vitro* biofilm formation (18), rabbit subdermal abscess infection (28), rabbit endocarditis (K. L. Frank and G. M. Dunny, unpublished data), and pathogenesis in another lepidopteran insect, *G. mellonella* (36). The commensal-to-pathogen switch in *M. sexta* contributes a low-cost, nonmammalian model system with the potential for high-throughput applications to study the cosmopolitan bacterium *E. faecalis*.

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