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Development of a genomic site for gene integration and expression in *Enterococcus faecalis*

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Abstract

Enterococcus faecalis, a gram-positive opportunistic pathogen, has become one of the leading causes of nosocomial infections. Normally a resident of the gastrointestinal tract, extensive use of antibiotics has resulted in the rise of *E. faecalis* strains that are resistant to multiple antibiotics. This, compounded with the ability to easily exchange antibiotic determinants with other bacteria, has made certain *E. faecalis* infections difficult to treat medically. The genetic toolbox for the study of *E. faecalis* has expanded greatly in recent years, but has lacked methodology to stably introduce a gene in single copy in a non-disruptive manner for complementation or expression of non-native genes. In this study, we identified a specific site in the genome of *E. faecalis* OG1RF that can serve as an expression site for a gene of interest. This site is well conserved in most of the sequenced *E. faecalis* genomes. A vector has also been developed to integrate genes into this site by allelic exchange. Using this system, we complemented an in-frame deletion in *eutV*, demonstrating that the mutation does not cause polar effects. We also generated an *E. faecalis* OG1RF strain that stably expresses the green fluorescent protein and is comparable to the parent strain in terms of *in vitro* growth and pathogenicity in *C. elegans* and mice. Another major advantage of this new methodology is the ability to express integrated genes without the need for maintaining antibiotic selection, making this an ideal tool for functional studies of genes in infection models and co-culture systems.

Keywords

Enterococcus faecalis; genomic integration; complementation; green fluorescent protein

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1. Introduction

Enterococcus faecalis is a gram-positive commensal inhabitant of the intestinal tract of humans, animals and insects and has been used as an ingredient in probiotics (Aarestrup et al., 2002; Tannock and Cook, 2002). However, it is also an opportunistic pathogen and has emerged as a leading cause of hospital-acquired extraintestinal infections, including urinary tract infections, bacteremia, wound infections and endocarditis (Malani et al., 2002). *E. faecalis* exhibits high levels of resistance to several antibiotics and is talented in horizontal exchange of antibiotic resistance determinants (Kak and Chow, 2002). Such features, compounded with the intrinsic ability of enterococci to thrive in various environmental conditions, have made it increasingly difficult for clinicians to combat this pathogen. Thus the need to understand this infectious agent at the genetic and physiological level is clearly important. An integral requirement of such inquiry and analysis is the availability of molecular and genetic tools. One important category of tools in functional studies is the means to complement genes-of-interest (GOI).

Several plasmids have been developed that allow complementation in *E. faecalis*. One is the pDL267 vector that contains a kanamycin cassette and a pUC origin (Dunny et al., 1991). Another set of vectors has been developed from the streptococcal plasmid pMV158, a natural replicon of *Streptococcus agalactiae* (Burdett, 1980). One example is pLS1, a broad-host-range vector that has tetracycline resistance (Lacks et al., 1986). A set of vectors, pMSP3535 and pMSP3535VA, were developed to allow nisin-controlled expression of selected genes in enterococci (Bryan et al., 2000). Kristich *et. al.* did further engineering and developed plasmid pCJK96 for rhamnose-inducible expression in *E. faecalis* by replacing the nisin system found in pMSP3535 with a rhamnose-inducible system (Kristich et al., 2007b). Both these plasmids use an erythromycin resistance determinant for selection in both *E. coli* and *E. faecalis*. However we have found erythromycin resistance difficult to select for in *E. coli*. To address this, we previously modified pCJK96 by adding an ampicillin resistance cassette for better selection in *E. coli*. The vector, pSD2, retained the *erm* cassette for selection in *E. faecalis* (Ramesh, 2012).

These vectors can be used for complementation of mutant strains to directly attribute a functional property to the absence of a specific gene. However, these plasmids are not stably transmitted, and maintenance requires that they carry a resistant determinant so that constant antibiotic selection can be applied. Because many strains of *E. faecalis* are resistant to many antibiotics that are routinely used in the laboratory, there are a limited number of markers that can be used. As a result, introducing certain plasmids into certain strains of this bacterium becomes problematic. Also, the need to maintain antibiotic selective pressure to retain a complementing gene is undesirable if the phenotype of the mutant being studied requires assessment in an infection model, where it can become difficult or impossible to maintain antibiotic exposure.

In the recent past, Kristich *et. al.* developed a markerless allelic exchange system that can efficiently insert fragments into a targeted site in the *E. faecalis* genome (Kristich et al., 2007a). In this study, we identified a site within the genome of *E. faecalis* OG1RF, flanked by two convergent reading frames that is a desirable site for insertion of a GOI. To take advantage of this site, we developed a vector system that allows the insertion of any GOI into the specific genomic location by allelic exchange. The system provides a useful new addition to the *E. faecalis* molecular toolbox. First, it allows complementation in scenarios that would otherwise require the maintenance of two different plasmids in the same bacterium. One such case is elaborated in this study. Second, it circumvents the need for antibiotic markers and selection to maintain a complemented phenotype, making it particularly suitable for demonstrating the contribution of a specific gene in an infection

model or other host-related experiment. Third, it complements by introducing a gene in single rather than multiple copy. Finally, it allows the stable addition of genes that are not part of the native genome.

Using our new system, we successfully complimented a mutant phenotype to demonstrate the role of a response regulator, EutV, in the regulation of the ethanolamine utilization genes. We also generated another useful tool for the *E. faecalis* community, a strain of OG1RF that stably and evenly produces green fluorescent protein (GFP) in a population of cells, as determined by flow cytometry. Additionally, the strain's properties are identical to wild type OG1RF, in terms of growth in culture and infectivity of the model nematode host *C. elegans* and mice.

2. Material and methods

2.1 Bacterial strains and media

All bacterial strains used in this study are listed in Table 1. Media was purchased from DIFCO and chemicals from Sigma, unless otherwise mentioned. *E. coli* strains were routinely cultured in Luria Bertani broth at 37°C. Antibiotics were added at the following concentrations: spectinomycin, 100µg/ml; erythromycin, 300µg/ml; and streptomycin, 25µg/ml. *E. faecalis* strains were cultured in Brain Heart Infusion (BHI) at 37°C. Antibiotics were added at the following concentrations : erythromycin, 50µg/ml; and rifampicin, 100µg/ml.

2.2 DNA manipulations, primers and sequencing

All primers used in this study are listed in Table 1. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Sequencing was done by GeneWiz (South Plainfield, NJ). High Fidelity Taq polymerase (Invitrogen, Carlsbad, CA) was used for all PCR reactions. Restriction enzymes (NEB) and T4 DNA ligase (Promega, WI) were used as per manufacturer's instructions. Plasmid extraction (Qiagen) and transformation were performed as per standard protocols.

2.3 Plasmid construction and allelic exchange

All plasmids used in this study are listed in Table 1. The primers ef2238f/r and ef2239f/r were used to amplify the ORFs of *ef2238* and *ef2239* respectively from *E. faecalis* OG1RF genomic DNA. The *ef2238* reverse primer was designed to incorporate a single SmaI site in the intergenic sequence between the two genes. These two amplicons were then used as templates with outer primers to generate a second PCR product that consisted of the amplicons fused together with a unique SmaI site between them. This cassette was digested with PstI and NcoI and ligated into a similarly digested pCJK47 vector to yield pRV1. The same undigested cassette was also inserted into pCR8/GW/TOPO to generate pTOPOIC. The multiple cloning site of the vector pSTBlue was synthesized as oligomers with terminal SmaI sites. These oligomers were annealed and ligated into the unique SmaI site between *ef2238* and *ef2239* in pTOPOIC to generate pSD15. To generate pSD16, the constitutive promoter was amplified using SD42 and SD106 from pHFZ2. The *eutV* gene was amplified with SD107 and SD110. The promoter was then fused to the *eutV* gene using crossover PCR and digested with SalI and NotI. Both fragments were ligated into similarly digested pSD15 to generate pSD16. The inserted fragments along with the remaining MCS was excised by SmaI digestion and ligated into SmaI-digested pRV1, resulting in pSD17. These constructs were introduced into *E. faecalis* CK111 by electroporation and introduced into either the AR2 or the Δ *eutV* strain by conjugation. Allelic exchange was performed as described in Kristich et. al (Kristich et al., 2007a). The reporter plasmid pSD3 was introduced into these strains by electroporation.

The *gfp* used in this study was amplified from pMV158*gfp* with primers SD143 and SD144 and digested with PstI and NotI. Plasmids pSD18 and pSD19 was generated using similar cloning procedures as described above and SD234 was obtained by standard allelic exchange methods as described (Kristich et al., 2007a).

2.4 β -galactosidase assays

Beta-galactosidase assays were done as described previously (Ramesh, 2012). Briefly, overnight cultures grown in BHI broth with 50 μ g/mL erythromycin were diluted 1:25 into MM9HY medium or MM9HY supplemented with 33mM ethanolamine and 40 μ g/mL AdoCbl. Strains were then grown anaerobically, without agitation, for 3.5hrs in the dark. Cells were resuspended in 1:10 Z buffer and lysed using 0.1mm glass disruption beads (Research Products International) for 2 minutes in a mini bead beater. Samples were cleared and incubated with *o*-nitrophenyl β -D-galactoside (ONPG). Color development was measured at 414 nm using a plate reader. Total protein in samples was estimated using a Pierce BCA kit (Thermo Scientific, Rockwood, IL) as per manufacturer's instructions. Reporter activity was reported in arbitrary units that indicate absorbance per μ g/ μ l total protein. Samples were assayed in triplicate. Mean and standard deviations were calculated from experimental replicates. The experiment was repeated on three independent occasions.

2.5 *C. elegans*

C. elegans strains were grown and maintained as previously described (Hope, 1999). The following bacterial strains were used in this study: *E. coli* OP50, *E. faecalis* OG1RF and *E. faecalis* SD234. *C. elegans* strains used in this study are indicated in Table 1. The killing assays and statistical analysis, by the Kaplan-Meier method, were done as described previously (Garsin et al., 2001). The experiment was replicated on three independent occasions.

2.6 Fluorescence microscopy

A suspension of *E. faecalis* SD234 in PBS was mounted on glass slides for microscopy. L4 *C. elegans* worms were exposed to *E. faecalis* strains for 24 hours at 25°C. Worms were collected, paralyzed with 1mM levamisole and mounted on 2% agarose pads. Bacterial and worm samples were imaged at 100X and 10X magnification respectively, using an Olympus IX81 automated inverted microscope. Data was processed using Slidebook (version 5.0) software.

2.7 Flow cytometry analysis

Overnight cultured cells were inoculated in BHI medium to a starting OD₆₀₀ of 0.1 and incubated at 37°C without shaking. Aliquots of one OD₆₀₀ equivalents of *E. faecalis* cells in BHI medium were harvested at exponential and post-exponential phase and washed twice with PBS, fixed with 1% paraformaldehyde and analyzed with a BD FACS calibur flow cytometer (BD Bioscience, CA). Mean fluorescent intensity was measured using the FL1 (fluorescence at 530 nm) from 10,000 events with triplicates.

2.8 Murine peritonitis infections

For monoinfections, *E. faecalis* strains OG1RF and SD234 were tested following our previously published method (Singh et al., 1998). In brief, mice were injected intraperitoneally with bacteria (grown in BHI medium), premixed with sterile rat fecal extract (SRFE) and were observed for 4 days for survival. Inocula of (1.2×10^7 , 1.2×10^8 , 1.2×10^9 CFU/ml) for OG1RF and (8×10^6 , 8×10^7 , 8×10^8 CFU/ml) for SD234 were used to compare animal survival/mortality using five mice per inoculum. For mixed infection competition assays, OG1RF and SD234 at approximately the same OD₆₀₀, i.e. 2.190 and

2.165 respectively, were mixed in equal amounts (~1:1) and inoculated using the same method as described above for mono-infection. Bacterial CFUs determined for OG1RF and SD234 from the inoculum were 5×10^8 /mouse and 4×10^8 /mouse, respectively. After 24 hrs of infection, kidney pairs and spleen were aseptically removed from dead animals, homogenized in 5 ml of saline and dilutions were plated on to Enterococcosel agar (EA) supplemented with 100 µg/ml rifampicin. Bacteria were examined and enumerated by fluorescence microscopy using a Leica MZ16F stereoscope. Comparison of the survival curves at similar inocula was performed using a log-rank test and the percentages of SD234 or OG1RF strains in the inoculum versus in kidney pair and spleen for individual mice were analyzed for significance by the paired *t* test. Graph Pad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) was used for graphs and statistical analysis.

2.9 Ethics statement

The mouse peritonitis model and procedures were performed in accordance with the institutional policies and the guidelines stipulated by the animal welfare committee, University of Texas Health Science Center at Houston (AWC, UTHSC), protocol # HSC-AWC-10-135.

3. Results and discussion

3.1. The *E. faecalis* genomic insertion site for expression (GISE)

To identify a site appropriate for inserting genes, we looked for an intergenic region flanking two convergently expressed genes. Our rationale was that such a site would be unlikely to cause polar effects, i.e. changes in gene expression in the surrounding region, due to insertion of a gene that is expressed. We also tried to choose a region shared by most strains of *E. faecalis*. The region between *ef2238* and *ef2239* fit these criteria and became our genomic insertion site for expression (GISE). The region containing the genes *ef2238* and *ef2239* and the intergenic sequence will henceforth be referred to as the insertion cassette (IC).

The GISE is present in both *E. faecalis* OG1RF and V583 strains (Bourgogne et al., 2008). BLAST searches also revealed that the GISE is nearly 100% conserved in all 16 of the *E. faecalis* strains sequenced by the Broad Institute (Palmer et al., 2010), with the exception of *E. faecalis* X98. In addition, the GISE is also conserved in the *Enterococcus* sp. 7L76 and *E. faecalis* strain ATCC29200 and TX1322. No regions of similarity were found in a number of clinical isolates. Hence, this site can be utilized for expression of GOI, not only in OG1RF, but in many other *E. faecalis* strains. This region had no homology to any of the sequenced *E. faecium*, *E. casseliflavus* and *E. gallinarum* strains, making it an *E. faecalis* – specific site. The utility of the GISE is at least two-fold. It can be utilized for complementation studies and for the expression of non-native genes, such as those that encode a fluorescent protein. In this study, we demonstrated both uses of the GISE.

3.2 Construction of pRV1

We developed the vector, pRV1, for insertion of GOIs into the GISE using the allelic exchange method (Kristich et al., 2007a). We engineered a single *Sma*I site between *ef2238* and *ef2239* by crossover PCR. Ligation of this IC into pCJK47 eliminated the existing *Sma*I site in the multiple cloning site (MCS), resulting in a unique site for insertion of the GOI. This vector was named pRV1 (Fig. 1). Since pRV1 was limited to a single blunt site for ligation of the GOI, we decided to introduce a more elaborate MCS at this *Sma*I site for greater ease of GOI introduction. We chose the MCS of the pSTBlue vector because it has a large number of usable restriction sites. Because we found the pRV1 plasmid difficult to manipulate in *E. coli* compared to other plasmids (see below), we decided to perform further

manipulations in the pCR8/GW/TOPO vector. Therefore, the IC was introduced into pCR8/GW/TOPO and the pSTBlue MCS cloned into the SmaI site to generate pSD15 (Fig 1). GOIs were first cloned into the IC in pSD15, and then excised and ligated into pRV1, as shown in Fig. 1. Though cloning directly into pRV1 is theoretically possible, we found this two-step cloning process preferable for the following reasons. In comparison to pRV1, the pCR8/GW/TOPO vector is much smaller. Unlike pRV1, it is a high copy number vector and can be easily retrieved in large quantities from small culture volumes by mini prep. Using of pCR8/GW/TOPO also facilitates transformation into common *E. coli* host strains such as DH5 α , compared to EC1000, which is required for working with pCJK47 (Kristich et al., 2007a) and is less efficiently transformed by electroporation. Thus, subcloning into the pSD15 intermediate alleviates several inherent technical difficulties.

3.3. Complementation of the *E. faecalis* EutVW two-component system

E. faecalis has a very flexible metabolism that allows it to utilize a wide range of nutritional sources. Ethanolamine, which is present in abundance in the intestine, can be used by *E. faecalis* as a source of both carbon and nitrogen. Utilization of this compound requires the upregulation of the ethanolamine utilization (*eut*) genes (reviewed by (Garsin, 2010)). The *E. faecalis eut* genes are positively regulated by an adenosylcobalamin-binding riboswitch and a two-component regulatory system (TCS). The presence of both ethanolamine and adenosylcobalamin is required for the induction of the *eut* genes (Baker and Perego, 2011; Del Papa and Perego, 2008; Fox et al., 2009; Ramesh, 2012). The *eut* TCS consists of a sensor histidine kinase, EutW, which autophosphorylates in the presence of ethanolamine and then transfers the phosphate to its cognate response regulator, EutV (Del Papa and Perego, 2008; Fox et al., 2009). EutV is a member of the ANTAR (AmiR and NasR transcriptional anti-terminator regulators) family of proteins that regulates its target genes by interacting with their nascent transcripts and preventing the formation of transcriptional terminators (Shu and Zhulin, 2002). Antitermination by EutV occurs at each of four terminators that are present in the *eut* locus (Baker and Perego, 2011; Fox et al., 2009; Ramesh, 2012). Deletion of either *eutW*, *eutV*, or both, rendered the *eut* genes uninducible under conditions that normally favor expression. The *eutV* and *eutW* genes are under the control of a promoter that precedes *eutS*. A transcriptional terminator follows the *eutS* promoter, which is the target of antitermination by EutV when the system is induced. Deletion of the terminator renders the promoter constitutively active (Ramesh, 2012).

To demonstrate that we could complement a *eutV* mutation in the *eut* locus by replacing the gene at the GISE, we generated constructs where the *eutV* gene was under the control of a constitutive *eutS* promoter (P_C *eutV*). This P_C *eutV* was inserted into the GISE in an OG1RF host background that had a deletion in *eutV*, or in both *eutV* and *eutW*. In all instances we used a PCR verification step to ensure that the insertion event had occurred at the correct site in the genome. In our previous studies, we have used the reporter pSD3 to measure the activity of the *eut* TCS (Ramesh, 2012). This reporter contains a translational fusion of *lacZ* to the 5' UTR of *eutP*, the first gene of the *eut* locus. The 5' UTR of *eutP* is also a target for antitermination by EutV upon activation and hence functions as a reporter of positive regulation by the EutVW TCS (Fox et al., 2009). Since EutV is activated by phosphorylation by EutW upon sensing ethanolamine (Del Papa and Perego, 2008; Fox et al., 2009), expression of EutV from the remote site was expected to activate the reporter only in the *eutV* background. Our results indicate that the pSD3 reporter is activated only under inducing conditions in the *eutV* host background but not in the *eutVW* double mutant (Fig. 2). This provides evidence that our previously generated *eutV* in-frame deletion does not disrupt the downstream expression of *eutW*. We can express *eutV* in a remote location and recover the ability to induce gene expression, which has been shown to require *eutW*. We were unable to complement this mutation previously by expressing *eutV* on a plasmid

because that would have required the strain to carry two plasmids – the complementing plasmid, and the *lacZ* reporter. This was not possible due to the lack of a plasmid with different origin of replication that could co-exist with pSD3 in the same bacterial cell. Thus, expression of *eutV* from this remote site makes complementation of our deletion in the *eut* locus possible.

3.4. Stable expression of *gfp*

To test the utility of the GISE for expression of a foreign gene, we chose the gene that encodes green fluorescent protein (*gfp*). The *gfp* gene was amplified from a Streptococcal plasmid vector pMV158*gfp* where the *gfp* gene is under the control of the inducible promoter of the pneumococcal *malM* gene, P_{mal} (Nieto and Espinosa, 2003). This promoter is negatively regulated by the MalR transcriptional repressor in *Streptococcus pneumoniae*, and repression is released in the presence of maltose (Nieto et al., 1997). However, the interaction between the MalR/P_{mal} repressor/promoter pair is not functional in *E. faecalis*. The expression of *gfp* from the pMV158*gfp* plasmid has been shown to be constitutive in both *E. faecalis* OG1RF and V583 strains (Lorenzo-Diaz and Espinosa, 2009; Thomas et al., 2008). The *gfp* reporter under the control of the *malM* promoter was integrated into the GISE, resulting in the SD234 strain.

Observation of dilute suspensions of the *E. faecalis* SD234 strain showed strong production of GFP based on color (Fig. 3A). We employed FACS methodology to analyze what percent of the cell population at exponential and post-exponential stages were producing GFP. In previous attempts to generate *gfp*-expressing strains of *E. faecalis*, we had observed that GFP was not present uniformly throughout the population (Garsin lab, data not shown). However, as shown in Fig. 3B, the SD234 cells exhibited a mean fluorescence intensity (MFI) of 13.19, 7-fold higher than that of for the wild type OG1RF cells (MFI = 1.5). No minor peak was observed in the histogram of the SD234 cells, indicating the ability of SD234 to stably produce GFP. No major change in morphology of SD234 was observed based on the forward and side scatter profile of the population. Analysis of post-exponential phase cells showed similar results. The method of growth, static or shaking, also did not impact the production of GFP in these cells (data not shown). Analysis of the growth curve of the *gfp*-expressing SD234 strain showed no difference from that of wild type OG1RF (Fig 3C), suggesting that expression of *gfp* from the GISE did not have any gross deleterious effect on the laboratory fitness of the strain.

3.5. Stable in vivo expression of *gfp* in strain SD234 results in no loss of virulence in animal infection models

We next tested the ability to track the *gfp*-expressing *E. faecalis* strain in an infection model. We used the model nematode host *Caenorhabditis elegans*, which is transparent and has successfully been used as a model host for many human bacterial pathogens, including *E. faecalis* (Garsin et al., 2001). *C. elegans* worms were fed either wild type OG1RF or the *gfp*-expressing SD234 strain for a duration of 24 hours at which point they were collected, washed and anesthetized before being analyzed by fluorescence microscopy. As shown in Fig. 4, the SD234 strain stably and strongly produced the fluorophore in the host. In comparison, the pictures of the worms infected with the wild type strain, OG1RF, manifested only weak, background autofluorescence from the worm, which is a normal occurrence when they are infected (Chavez et al., 2007). In the images captured at higher magnification, single bacterial colonies of SD234 are discreetly visible and expression of *gfp* appeared uniform. We also compared the wild type OG1RF and the *gfp*-expressing SD234 strain for their ability to kill the *C. elegans* host. As shown in Fig. 5Aa, SD234 mirrors OG1RF in the kinetics of killing *C. elegans*.

We next analyzed the expression and infectivity of SD234 in an intraperitoneal mouse infection model. Groups of mice were injected with either OG1RF or SD234 and survival was monitored. Both groups of mice showed similar mortality rates (Fig 5B). SD234 colonies recovered from these mice were fluorescent when plated on solid media. As in the *C. elegans* model, no significant difference was observed between the virulence of wild type OG1RF and SD234 in mice. We also performed competition assays in which mice were inoculated with an equal mix of OG1RF and SD234. After 24 hours of infection, the numbers of OG1RF and SD234 in the kidneys and spleen of the mice were estimated. Dilutions of organ homogenates were plated, and the two bacterial species were differentiated visually on the basis of fluorescence. As shown in Figure 5C, both strains were recovered in similar numbers suggesting that SD234 was as fit as the wild type strain in causing infection and dissemination in the mouse model.

Thus, production of GFP from gene insertion into the GISE does not cause any gross physiological shortcomings and is comparable to the wild type strain in terms of *in vivo* virulence in the animals tested. This makes SD234 a potentially useful tool for tracking *E. faecalis* cells in infection models, co-cultures and biofilm formation. Generation of mutants in the SD234 background will allow easy and efficient enumeration of the wild type and mutant populations visually. Although *gfp*-expressing *E. faecalis* strains have been generated in the past, these strains have suffered from lack of robust and uniform expression. The added advantage of the SD234 strain is its independence from selection by antibiotic pressure. Expressing *gfp* from the chromosome rather than an extrachromosomal plasmid circumvents the need to maintain its presence by antibiotic selection.

4. Conclusions

In this study we have identified a site in the *E. faecalis* OG1RF genome that allows the introduction of a gene of interest by markerless allelic exchange. We have designed a new vector for the insertion of a GOI and demonstrated two different utilities of this GISE. Complementation of a mutation in the *E. faecalis* EutVW TCS was demonstrated successfully, by inserting the gene encoding the response regulator into the GISE. Since, integration of genes into the GISE is not accompanied by addition of an antibiotic marker, one of the advantages of this method is its independence from maintaining selective pressure to ensure stable expression. This is particularly useful, since *E. faecalis* is resistant to several routinely used antibiotics. Strains created using this method are ideal for assessment in an infection model where maintenance of antibiotic selection is challenging. It also is a viable alternative for studies that would otherwise require the presence of two plasmids in *E. faecalis*. Due to the presence of multiple copies of plasmids, expression of genes from plasmids is normally higher and can have deleterious effects on the normal physiology of the bacterium. Such a situation is avoided by insertion into the GISE, as expression occurs from a single copy of the gene. We also showed that the GISE can be used to express a foreign gene such as *gfp*. The GFP-labeled *E. faecalis* strain showed no defects in growth in media, and production of the fluorophore was stable and even throughout the population of cells, including within the *C. elegans* host. The GFP-labeled strain also retained its fluorescence when recovered from infected mice. Additionally, the GFP-labeled strain was as efficient as wild type in killing both *C. elegans* and mice, suggesting that expression from the GISE does not have any gross physiological effects on bacterial virulence. It was also shown to survive in numbers similar to that of the wild type when inoculated in mice as an equal mixture. Therefore, the GFP producing strain, SD234, could provide an ideal background for the development of mutants that can be followed in infection models or co-cultures in an antibiotic-independent manner. The GISE is well conserved in most *E. faecalis* strains and this technique could potentially be used in strains other than OG1RF. Finally, as the GISE allows expression of foreign genes, this technology could lead to the

development of OG1RF as a heterologous host for expression and analysis of genes present in other less genetically amenable enterococcal strains and species.

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Highlights

1. A vector was designed to allow integration of DNA into a specific site in the *E. faecalis* genome.
2. The method bypasses the need to maintain antibiotic pressure for expression of genes of interest.
3. Using this system, we complimented a mutant in the EutVW two component system.
4. An *E. faecalis* strain that stably and evenly produces green fluorescent protein (GFP) was created.
5. The *gfp*-expressing strain was identical to wild type for *in vitro* growth and killing of *C. elegans* and mice.

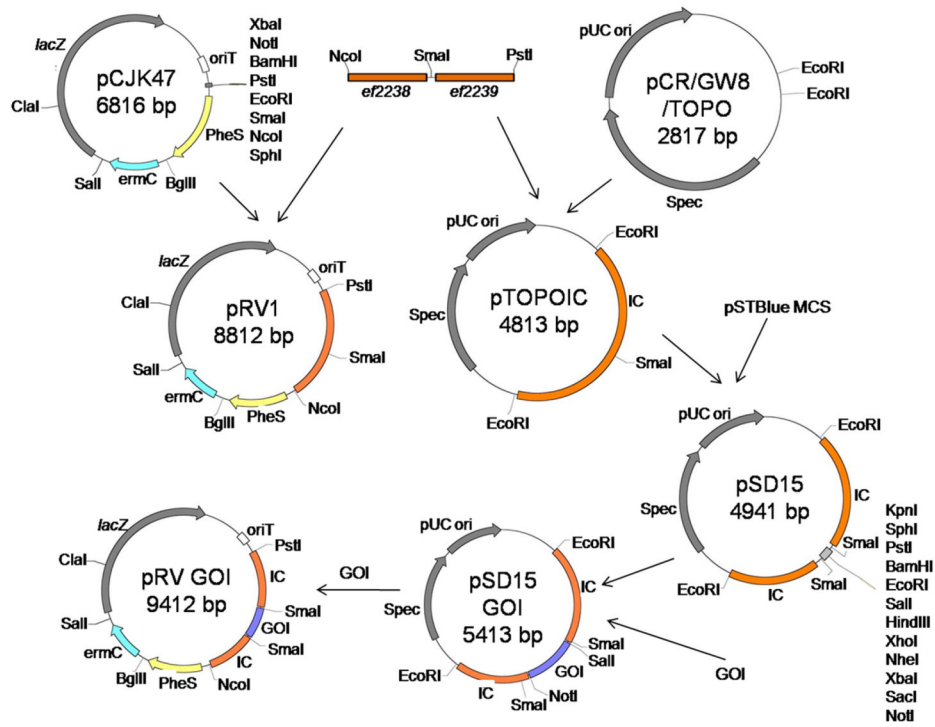


Figure 1. Schematic representation of the construction of clones in pRV1. See Methods section for details. The gene-of-interest represented is the *P_{cut}V* gene described in the text. IC-1 and IC-2 represent *ef2238* and *ef2239* respectively. Plasmids are not drawn to scale.

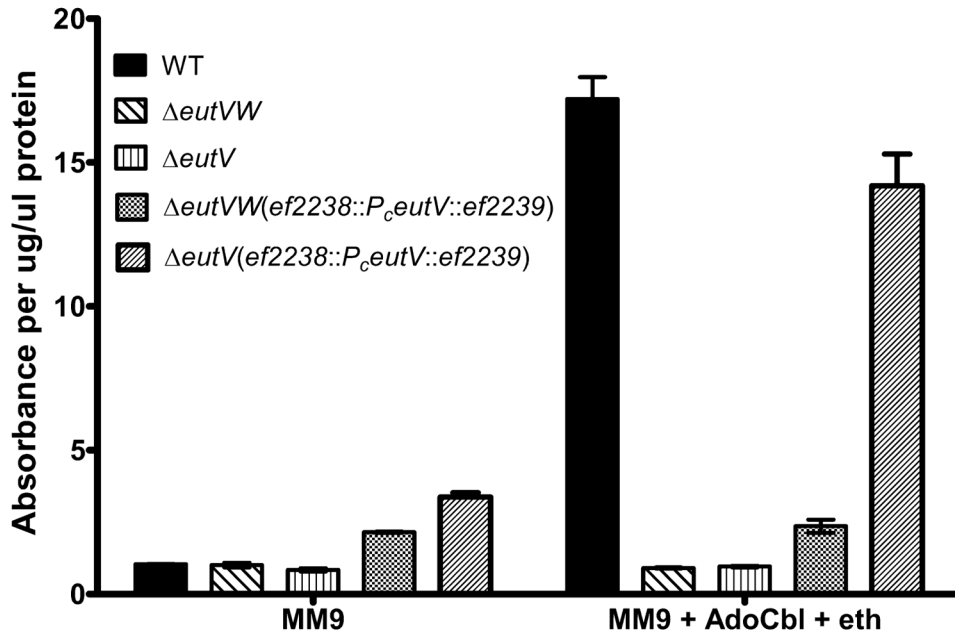


Figure 2. Complementation of the EutVW two-component system in *E. faecalis*. Expression of the *eutP::lacZ* reporter, as measured by β -galactosidase assays, is shown in different host backgrounds. Expression from the reporter is observed in wild type OG1RF in the presence of adenosylcobalamin and ethanolamine, but not in mutants lacking either the entire EutVW two-component system or just the EutV regulator. Expression of the integrated $P_{c}eutV$ is unable to induce reporter activity in a *eutVW* deletion mutant but shows near wild type levels of reporter activity in a *eutV* deletion background. Data for each culture condition was collected in triplicates. Error bars indicate standard deviation.

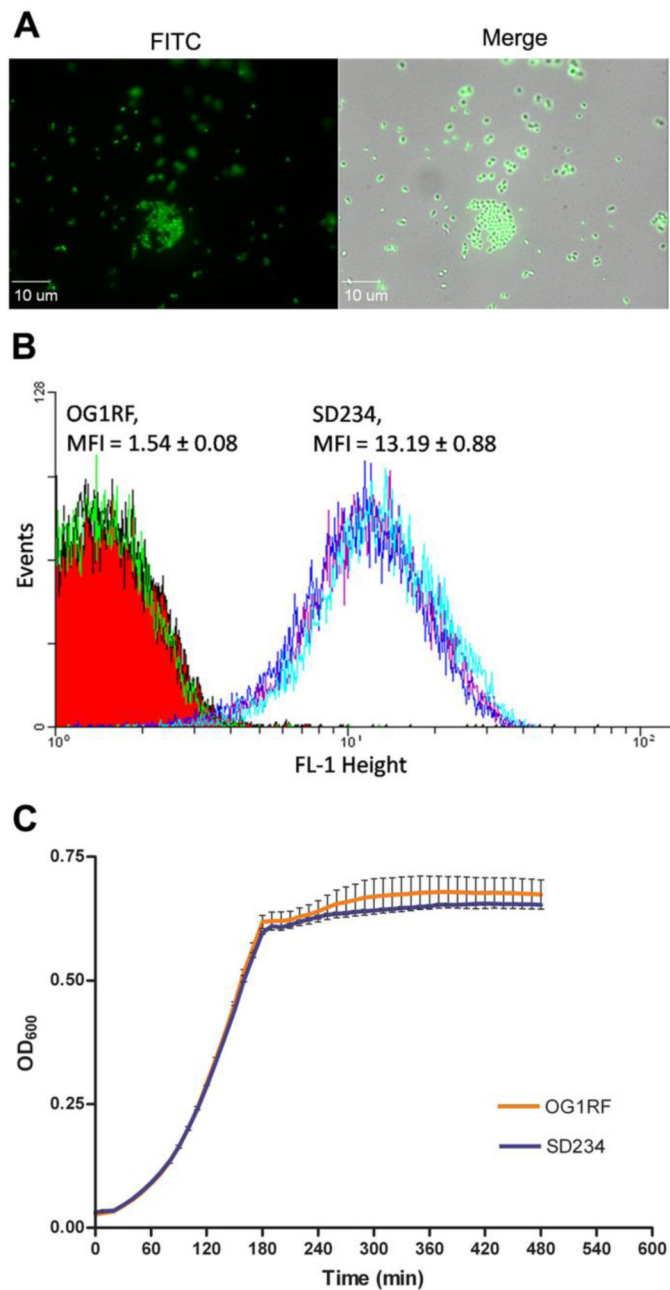


Figure 3. Expression of *gfp* in *E. faecalis*. A) Fluorescent micrographs of SD234 expressing *gfp*. B) FACS analysis of exponential cultures of OG1RF wild type and SD234 strains. SD234 forms a tight peak representing a single population of fluorescent cells suggesting ubiquitous expression of *gfp*. C) *In vitro* growth of OG1RF and SD234 in BHI medium at 37°C. Each data point represents triplicate samples and error bars indicate standard error.

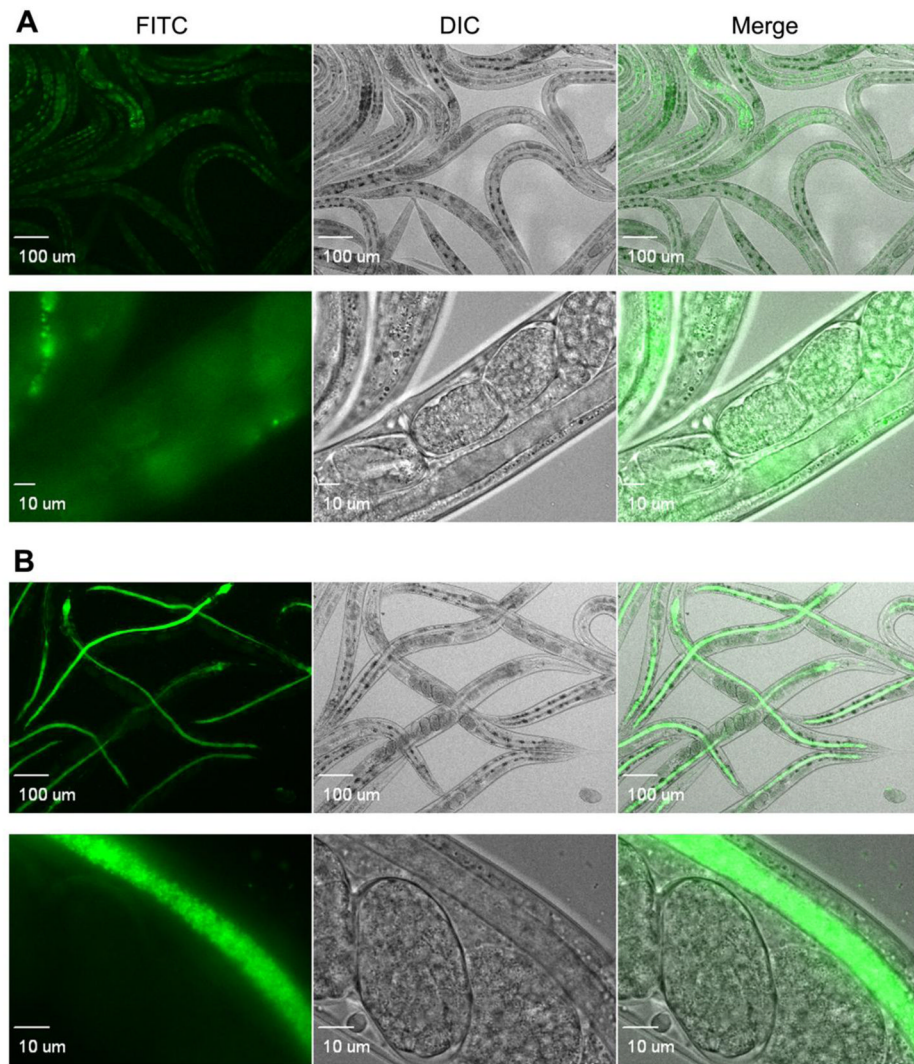


Figure 4. Production of GFP by SD234 inside *C. elegans*. Fluorescent micrographs of *C. elegans* nematodes infected with *E. faecalis* A) OG1RF and B) SD234 after 24 hours of feeding. Diffuse fluorescence observed in OG1RF-fed *C. elegans* is a result of autofluorescence that accumulates in the intestine of the worm. Fluorescent SD234 is distinctly seen within the nematode gut.

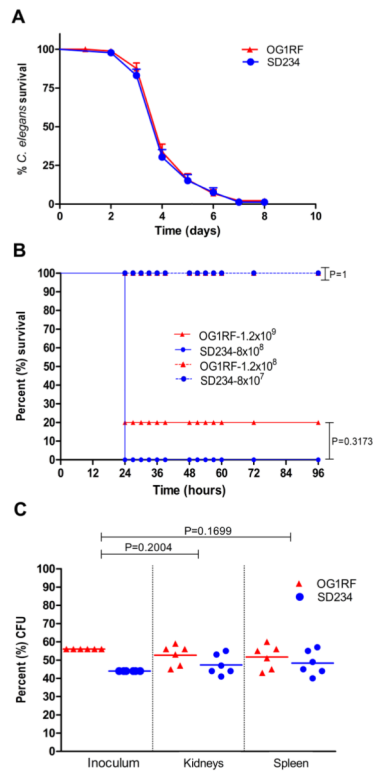


Figure 5. Infection of *C. elegans* and mice with *E. faecalis* OG1RF and SD234. A) Killing assay of *C. elegans* worms infected with *E. faecalis* OG1RF and SD234. Each data point reflects the average of three groups of thirty worms each ($P = 0.5949$). Intraperitoneal infection of mice with B) monocultures or C) mixed cultures of OG1RF and SD234. B) Kaplan-Meier survival plots of wild type OG1RF (red) and SD234 (blue) are shown. Inoculum is indicated. C) Mice were infected with a 1:1 mix of OG1RF (red) and SD234 (blue). Percentage CFU of OG1RF and SD234 present in the inoculum, and recovered from the kidneys and spleens of mice are shown.

Table 1

Strains, plasmids and primers used in this study

	Description	Source
Strains		
<i>E. faecalis</i>		
OG1RF	Wild type strain, Fa ^R , Rf ^R	Lab stock
AR2	Δ cutVW, Fa ^R , Rf ^R	Fox et al.
Δ cutV	Markerless deletion of cutV in OG1RF, Fa ^R , Rf ^R	This work
CK111	Conjugative donor strain	Kristich et al. (2007)
SD48	OG1RF pSD3, Fa ^R , Rf ^R , Em ^R	Ramesh et al. (2012)
SD49	Δ cutVW pSD3, Fa ^R , Rf ^R , Em ^R	Ramesh et al. (2012)
SD218	Δ cutVW [e2238::PCcutV::e2239] pSD3, Fa ^R , Rf ^R , Em ^R	This work
SD219	Δ cutV [e2238::PCcutV::e2239] pSD3, Fa ^R , Rf ^R , Em ^R	This work
SD234	OG1RF [e2238::Pmalgfp::e2239]	This work
<i>E. coli</i>		
TOP10	Strain used for construction of pSD2-based plasmids	Lab stock
EC1000	Host for pCJk47-based plasmids; provides RepA in trans	Lab stock
OP50	Strain used as food source for <i>C. elegans</i>	Lab stock
<i>C. elegans</i>		
N2	Wild type Bristol strain	Lab stock
Plasmids		
pCJk47	Plasmid for markerless exchange	Kristich et al. (2007)
pCR8/GW/TOPO	TA cloning vector from Invitrogen	Lab stock
pMV158 gfp	Source of gfp	Nieto & Espinosa (2003)
pRV1	pCJk47 (e2238 - e2239)	This work
pTOPOIC	pTOPO (e2238 - e2239)	This work
pSD15	pTOPOIC MCS	This work
pSD16	pSD15 PCcutV	This work
pSD17	pRV1 PCcutV	This work
pSD3	pSD2 cutP5' UTR	This work
pSD18	pSD15 gfp	This work
pSD19	pRV1 gfp	This work
pHFZ2	pSD2 cutSAT	Ramesh et al. (2012)

Primers	Description	Source
Ef2238 F	5' CCCATGGCTATAATAGTACTTGAGAAGGAGGC3'	This work
Ef2238R	5' AAAAAATAAAGGACGGTTCCTTTATAGGAGCCCGGGGGT CCTTATTTTTTATTCTGGCGTGG3'	This work
Ef2239F	5' GCTCCTATAAAGGAACCGTCTTATTTTT3'	This work
Ef2239R	5' AACTGCAAGGTAAAACTAGGAGGGAAGCATATG3'	This work
SD100	5' ^{Pho88} GGGTACCAACCGCATGCTCGAGACCGGTTACGFA TCGGATCCAGAA TTCGTGATATCTGAATTCGTGCACAAGCTTCTCGAGCCTAGGCTAGCTTAGACACACAGCTGTGGGGGCC CGAGCTCCGGCCGCCcc3'	This work
SD101	5' ^{Pho88} GGGGCCGGAGCTCGGGCCCCACACGCTGTGCTAGAGCTAGCCTAGCCTAGGCTCGAGAAAGCTTGTGAGAAATTCACGAAATTCGGATCCGATACCGTAACCGGCTGTG CAGCATGCGGTGTACcc3'	This work
SD143	5' GCCTGCAAGGAGCTCGGTACCCAGCTTGTGATTTGATAGC3'	This work
SD144	5' GGCGGGCCGCTATTGTATAGTTCATCCATGCCATGTG3'	This work
SD42	5' GCGTCGACCGACAAT CAAGCAACTCCGTCAA3'	This work
SD106	5' CGTAATAGGTTCAATCAGGACTATTACAAATTCGTCATCC ATCCAGACTCGCTCCTTTAATTTAGC3'	This work
SD107	5' ATGGATGGACGAAATGTAAATAGTCG3'	This work
SD110	5' GCAGCGCCGCTTATTCATATCCAATTACAATCAATTCGTG3'	This work