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Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of *Enterococcus faecalis*

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Abstract

Enterococcus faecalis is a Gram-positive commensal bacterium of the gastrointestinal tract. *E. faecalis* is also an opportunistic pathogen that frequently exhibits resistance to available antibiotics. Despite the clinical significance of the enterococci, genetic analysis has been restricted by limitations inherent in the available genetic tools. To facilitate genetic manipulation of *E. faecalis*, we developed a conjugative delivery system for high-frequency introduction of cloned DNA into target strains of *E. faecalis* and a host-genotype-independent counterselectable marker for use in markerless genetic exchange. We used these tools to construct a collection of *E. faecalis* mutant strains carrying defined mutations in several genes, including *ccfA*, *eep*, *gelE*, *sprE*, and an alternative sigma factor (*sigH*). Furthermore, we combined these mutations in various permutations to create double mutants, triple mutants, and a quadruple mutant of *E. faecalis* that enabled tests of epistasis to be conducted on the pheromone biosynthesis pathway. Analysis of cCF10 pheromone production by the mutants revealed that both the *ccfA2* and Δ *eep10* mutations are epistatic to mutations in *gelE/sprE*. To our knowledge, this represents the first example of epistasis analysis applied to a chromosomally encoded biosynthetic pathway in enterococci. Thus, the advanced tools for genetic manipulation of *E. faecalis* reported here enable efficient and sophisticated genetic analysis of these important pathogens.

Keywords

counterselection; *pheS*; markerless exchange; epistasis; *Enterococcus faecalis*; *p*-chloro-phenylalanine; pheromone biosynthesis

Introduction

The bacterium *Enterococcus faecalis* is a Gram-positive member of the intestinal microflora (Tannock and Cook, 2002). As a frequent etiological agent of nosocomial infections (Richards *et al.*, 2000), *E. faecalis* is also a leading health concern. *E. faecalis* exhibits relatively high-level intrinsic resistance to some antibiotics and freely shares antibiotic resistance determinants with neighboring bacteria on mobile genetic elements (Kak and Chow, 2002), leading to the emergence of multi-resistant clones in the hospital setting. Thus, treatment of enterococcal infections is an increasingly difficult problem for clinicians armed with currently available

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therapeutic agents. Effective development of new antibiotics to combat multi-resistant strains demands an intimate understanding of enterococcal physiology and genetics. Thus, improved techniques for sophisticated genetic analysis of enterococci are urgently needed.

A procedure known as allelic exchange (Maloy *et al.*, 1996) or markerless exchange (Pritchett *et al.*, 2004)) is often used to replace a wild-type allele of a gene of interest with a cloned allele that has been manipulated in vitro. Markerless exchange is typically performed using a two-step integration-segregation strategy based on homologous recombination. Although an antibiotic-resistance marker enables selection to be used at the first step, inclusion of a counterselectable marker in the exchange plasmid is required to permit selection, rather than laborious screening, at the second step of the process (Maloy *et al.*, 1996).

Counterselection strategies function by inhibiting growth of bacterial clones that carry a counterselectable marker. Common examples include: inhibition of growth in the presence of sucrose, mediated by the *sacB* gene product (Ried and Collmer, 1987); inhibition of growth in the presence of lipophilic chelators such as fusaric acid, mediated by gene products conferring tetracycline-resistance (Maloy and Nunn, 1981); and inhibition of growth by purine or pyrimidine analogs, mediated by phosphoribosyl transferases of the purine and pyrimidine base salvage pathways (Spring *et al.*, 1994; Fukagawa *et al.*, 1999; Peck *et al.*, 2000; Fabret *et al.*, 2002; Bitan-Banin *et al.*, 2003; Pritchett *et al.*, 2004).

Genetic analysis of enterococci has thus far been restricted by limitations of the available genetic tools. Several approaches have been used for targeted mutagenesis of chromosomal genes in *E. faecalis*, each of which suffers from significant drawbacks. For example, Campbell-type homologous recombination of a non-replicating plasmid has been used to disrupt target genes (Qin *et al.*, 1998). However, due to low frequencies of electroporation and recombination in *E. faecalis*, recombinants are difficult to obtain, and any such recombinants suffer from the problems inherent in this type of mutation (for example, mutants that carry antibiotic resistance markers, mutations that are polar, and reversion to the wild-type genotype by plasmid excision). Another approach to perform allelic exchange in *E. faecalis* relies on the use of the temperature-sensitive replicon, pG⁺host, to deliver substrate DNA for recombination (Arbeloa *et al.*, 2004), but the pG⁺host-based approaches have met with limited success in our hands. A variation on this theme, originally developed for use in lactococci (Leenhouts *et al.*, 1996), can be used in *E. faecalis*, but this system lacks the benefits provided by a functional counterselectable marker. Recently, we described a markerless exchange system that employs the first counterselectable marker functional in *E. faecalis* (Kristich *et al.*, 2005); however, that strategy suffers from the drawback that the strain targeted for mutagenesis must also carry an independent mutation (Δupp) for counterselection to be applied. Thus, a Δupp mutant must be constructed in the target strain prior to manipulation of a gene of interest, and the Δupp mutation itself may have undesirable or unknown phenotypic effects.

For each of the approaches mentioned above, a significant difficulty lies in the first step of mutant construction: getting manipulated DNA into *E. faecalis* cells. *E. faecalis* is not known to be naturally competent for transformation, and although electroporation can be used, current electroporation protocols suffer from low efficiency of DNA uptake. As a result, recombinants can be difficult to isolate after electroporation with a non-replicating plasmid (typically, 0–5 recombinants per μ g of plasmid DNA). Consequently, a means to introduce DNA into target cells at high frequency would substantially enhance mutant generation capabilities in *E. faecalis*.

To facilitate efficient genetic manipulation of *E. faecalis*, we developed 2 key improvements of our previous markerless genetic exchange system that overcome the aforementioned problems. First, a conjugative delivery system for high-frequency introduction of cloned DNA

into target strains of *E. faecalis*, based on the enterococcal pheromone-responsive conjugative plasmid pCF10, surmounts the barrier to DNA introduction. Second, a host-genotype-independent counterselectable marker for use in markerless genetic exchange, based on the *E. faecalis* phenylalanyl-tRNA synthetase, eliminates the requirement for a specific pre-existing mutation in the target strain in order for counterselection to be applied. The effectiveness of these new tools was demonstrated by the construction of a panel of *E. faecalis* mutant strains carrying defined, stable mutations in several genes. These included an alternative sigma factor (*sigH*), potentially involved in regulating post-exponential phase gene expression, as well as several genes encoding products known to be involved in the synthesis, processing, or degradation of enterococcal peptide pheromones (*ccfA*, *eep*, *gelE*, and *sprE*). Furthermore, we combined these mutations in various permutations to create double mutants, triple mutants, and a quadruple mutant of *E. faecalis* that enabled tests of epistasis to be conducted on the pheromone biosynthesis pathway. The epistasis analysis revealed that, as expected, both the *ccfA2* and Δ *eep10* mutations are epistatic to mutations in *gelE/sprE*. Thus, this system enables efficient markerless genetic exchange and sophisticated genetic analysis of *E. faecalis*. The approach described in this report should be of general use for enterococcal researchers and may be adapted for use in other Gram-positive organisms.

Materials and Methods

Bacterial strains, growth media, and chemicals

Bacterial strains used in this study are listed in Table 1. Bacteria were stored at -80°C in Todd-Hewitt broth or brain-heart infusion (THB or BHI, respectively; prepared according to the manufacturer's instructions) supplemented with 30% glycerol. Unless otherwise indicated, all culture media were purchased from Difco and all chemicals were purchased from Sigma (St. Louis, MO). *p*-Cl-phenylalanine was obtained from Sigma as a mixture of the L- and D-isomers (Cat. C 6506), added to culture media prior to autoclaving, and mixed thoroughly immediately upon completion of the autoclave cycle. Bacto Agar was used as a solidifying agent for all semi-solid media. Casamino Acids were obtained from Fisher Biotech. MM9YEG medium (a variation of the previously described M9YE medium (Dunny and Clewell, 1975)), is a semi-defined M9-based medium supplemented with 0.25% yeast extract and 0.5% glucose (but prepared without MgSO_4 or CaCl_2). When required for selective growth of *E. faecalis*, erythromycin (Em), tetracycline (Tc), and chloramphenicol (Cm) were used at 10 $\mu\text{g/ml}$; rifampicin (Rf) at 200 $\mu\text{g/ml}$; fusidic acid (Fa) at 25 $\mu\text{g/ml}$; and spectinomycin (Sp) and streptomycin (Sm) at 1000 $\mu\text{g/ml}$. When required for *E. faecalis*, X-Gal was added at 250 $\mu\text{g/ml}$. When required for selective growth of *Escherichia coli*, erythromycin was used in BHI at 100 $\mu\text{g/ml}$. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed at the Biomedical Genomics Center, University of Minnesota.

Plasmid construction

Plasmids used in this study are listed in Table 1. All plasmids derived from pORI280 were propagated in *E. coli* strain EC1000 (Leenhouts *et al.*, 1996), which supplies RepA in trans to allow replication of these plasmids. When isolating plasmids from EC1000, we found that cultivation of recombinant strains at 30°C produced the best results. All other plasmids were propagated in *E. coli* XL1Blue or DH5 α .

The counterselectable P-*pheS** cassette was constructed using an overlap extension PCR strategy (Fig. 1), which introduced the *pheS312AG* mutation and fused a constitutive promoter upstream of the *E. faecalis pheS312AG* allele. First, the synthetic constitutive promoter driving expression of *upp* in pCJK2 (Kristich *et al.*, 2005), which was originally derived from pFW11 (Podbielski *et al.*, 1996), was amplified by PCR. The *pheS* gene from *E. faecalis* OG1RF was

amplified from chromosomal DNA as 2 fragments, using primers designed according to the sequence of the *E. faecalis* V583 *pheS* gene (EF1115), available at The Institute for Genomic Research website (<http://www.tigr.org>). The A312G mutation responsible for relaxed substrate specificity was encoded by the primers annealing internal to the *pheS* gene. These 3 amplicons were used as templates with the outside primers for a second PCR step, fusing the amplicons together and creating the intact P-*pheS** cassette (Fig. 1). This cassette was cloned into the low-copy number shuttle plasmid pWM401 (Wirth *et al.*, 1987) using primer-encoded *SphI*/*XbaI* restriction sites, creating pCJK45 (Fig. 1). The nucleotide sequence of the P-*pheS** cassette in pCJK45 was determined to verify its integrity.

A mobilizable and counterselectable donor plasmid (pCJK47) was constructed in two steps (Fig. 1). First, a 243-bp fragment containing the origin of transfer (*oriT*) from pCF10 (Staddon *et al.*, in press) was amplified by PCR and cloned between the *AcI* sites of the *repA*⁻ plasmid, pORI280 (Leenhouts *et al.*, 1996), using primer-encoded *AcI* restriction sites, thereby creating pCJK34. Second, the P-*pheS** cassette was excised from pCJK45 by digestion with *SphI*/*BglII* and cloned into similarly-digested pCJK34, creating pCJK47.

A donor plasmid used to create an in-frame deletion of *E. faecalis sigH* (pCJK66; Fig. 1) was constructed by first amplifying fragments of OG1RF chromosomal DNA flanking the *sigH* gene to create the upstream and downstream PCR amplicons. The resulting amplicons contained 995 and 949 bp of *E. faecalis* DNA, respectively. These amplicons included flanking sequences as well as sequences encoding the first 5 residues and the last 5 residues of the *sigH* ORF, respectively, which were retained in the deletion construct in an effort to avoid any unanticipated effects on the expression of adjacent genes. The 2 PCR amplicons were sequentially cloned into pSK⁻ to create pCJK52, using the primer-encoded *NotI*/*SpeI* restriction sites for the upstream fragment and the primer-encoded *SpeI*/*XmaI* restriction sites for the downstream fragment. In the final deletion construct, the *SpeI* site forms the fusion point between the two flanking fragments and is in-frame with the few remaining codons of the *sigH* ORF, such that 2 *SpeI*-encoded amino acids are inserted into the 10-amino acid product that is produced from the Δ *sigH2* allele. Over 94% of the *sigH* gene was eliminated in this construct. The Δ *sigH2* allele was excised from pCJK52 by digestion with *NotI*/*XmaI* and cloned into similarly-digested pCJK47, creating pCJK66.

A donor plasmid used to create an in-frame deletion of the adjacent *E. faecalis* genes *gelE* and *sprE* (pJRC105; Table 1) was constructed according to the procedure described above for pCJK66, with modifications. The upstream and downstream PCR amplicons contained 585 and 586 bp of *E. faecalis* DNA, respectively, and included sequences encoding the first 33 residues of *GeLE* and the last 25 residues of *SprE*, which were retained in the deletion construct in an effort to avoid any unanticipated effects on the expression of adjacent genes. The “internal” end of each amplicon (i.e., the ends destined to be fused together to create the deletion) encoded a *BsaI* restriction site, designed such that *BsaI* cleavage and ligation of the amplicons would seamlessly fuse the amplicons together creating an in-frame deletion (i.e., no restriction sites or other extra nucleotides are found between the two amplicons in the fusion construct) according to a previously described procedure (Derre *et al.*, 1999). To create the deletion allele, the upstream amplicon was digested at the primer-encoded *XbaI*/*BsaI* sites, the downstream amplicon was digested at the primer-encoded *NcoI*/*BsaI* sites, and the two amplicons were simultaneously cloned into *XbaI*/*NcoI*-digested pCJK47, creating pJRC105. Over 93% of the *gelE* and *sprE* coding sequences were eliminated in our construct.

A donor plasmid used to create an in-frame deletion of *E. faecalis eep* (pJRC106; Table 1) was constructed according to the procedure described above for pJRC105 with slight modifications. The upstream and downstream PCR amplicons contained 838 and 1036 bp of *E. faecalis* DNA, respectively, and included sequences encoding the first 15 residues and the last 14 residues of

Eep, which were retained in the deletion construct in an effort to avoid any unanticipated effects on the expression of adjacent genes. The amplicons were fused together and cloned into pCJK47 as described for pJRC105, except that the *XbaI/NcoI* restriction sites used for cloning are naturally present in the adjacent DNA sequence and therefore were not primer-encoded. Over 93% of the *eep* gene was eliminated in our construct.

A donor plasmid used to introduce point mutations encoding stop codons in *E. faecalis* *ccfA* (pJRC104; Table 1) was constructed by subcloning the 2.37-kb *NcoI/SpeI* fragment encoding the *ccfA2* allele from pJRC102 (Chandler *et al.*, 2005) into *NcoI/XbaI*-digested pCJK47, creating pJRC104.

Plasmids to transfer *P₂₃-repA* alleles to the *upp* locus of the *E. faecalis* chromosome (pCJK12 and pCJK41) were created as follows. First, the *P₂₃-repA1* allele was amplified by PCR from the chromosome of *E. coli* EC1000. This allele encodes a fusion of the lactococcal *P₂₃* promoter (van der Vossen *et al.*, 1987) with the pWVO1 *repA* gene, preceded by its natural ribosome-binding site (Leenhouts *et al.*, 1991). Primer-encoded *XhoI* restriction sites were used to clone *P₂₃-repA1* between the 2 *XhoI* sites found in the temperature-sensitive plasmid pCJK11 (Kristich *et al.*, 2005), thereby replacing the *erm* determinant (found in the pCJK11-encoded *upp::erm* cassette) with *P₂₃-repA1* (creating pCJK12). In this construct, the *P₂₃-repA1* allele separates two fragments of *E. faecalis* chromosomal DNA that flank the *upp* gene, effectively creating a gene replacement allele in which most of the *upp* ORF is replaced by *P₂₃-repA1*. A plasmid bearing a modified ribosome-binding site upstream of *repA* (pCJK41, encoding RBS of AGGAGC) was created using the QuikChange mutagenesis strategy (Stratagene) with pCJK12 as the template for mutagenesis. The nucleotide sequence of the entire *P₂₃-repA* allele was determined for each plasmid to verify the presence of the desired mutation.

Transformation of *E. faecalis* by plasmid DNA was carried out via electroporation of lysozyme-treated cells as previously described (Bae *et al.*, 2002).

Construction of donor strains for conjugative delivery of markerless exchange plasmids

A spectinomycin-resistant derivative of *E. faecalis* OG1 (OG1Sp) was isolated by selecting for a spontaneous mutant of OG1 after plating on THB agar supplemented with 1000 µg/ml Sp at 37°C.

To serve as *E. faecalis* host strains for *repA*-dependent donor plasmids, we constructed a strain bearing a chromosomally-borne *repA* allele whose expression is driven by the lactococcal *P₂₃* promoter (strain CK111; Table 1). Our strategy was to integrate the *P₂₃-repA* allele into the *E. faecalis* chromosome at the *upp* locus, thereby creating a stable gene replacement mutant with a selectable phenotype (*upp* mutants of *E. faecalis* are resistant to 5-fluorouracil; (Kristich *et al.*, 2005)). This *E. faecalis* mutants was constructed using the temperature-sensitive plasmid, pCJK41. OG1Sp carrying pCJK41 was cultured overnight at 30°C in M9YE supplemented with 0.5% glucose and Cm (for maintenance of the plasmid). The cells were washed twice and diluted ten-fold in M9YE supplemented with 0.5% glucose, followed by incubation at 30°C for 1 h. The culture was shifted to the nonpermissive temperature of 42°C for 4 h, and aliquots were plated on BHI agar supplemented with 1 mM 5-fluorouracil at 42°C to select for *upp* mutants. Colonies that arose were screened using a previously described colony-PCR procedure (Kristich *et al.*, 2005) to identify isolates carrying the *P₂₃-repA* allele in the chromosome.

High-frequency conjugative delivery and markerless exchange to construct a Δ *sigH2* mutant

The donor plasmid, pCJK66, was introduced into the conjugative donor strain, CK111(pCF10-101), by electroporation. Replication of pCJK66 as an episome in the donor strain was verified

by re-isolating the recombinant plasmid using a Qiagen miniprep kit (not shown). The resulting transformant, CK111(pCF10–101, pCJK66) was cultivated overnight in BHI supplemented with Em and Sp at 37°C. The recipient strain (*E. faecalis* OG1RF) was cultivated overnight in BHI at 37°C. Both strains were washed twice in BHI, diluted ten-fold in fresh BHI (no antibiotics), and incubated separately at 37°C for 1 h. Donor and recipient cultures were mixed at a ratio of 1:9, followed by centrifugation of 0.8 ml of the resulting mixture. The cell pellet was resuspended in ~0.1 ml of supernatant and spread on a 100-mm BHI agar plate to permit conjugation. After ~5 h of incubation at 37°C, bacteria were recovered from the surface in 2 ml of PBS supplemented with 2 mM EDTA (to disrupt bacterial aggregates). Serial dilutions were made in PBS and enumerated on BHI agar supplemented with Sp, Tc, and Em (to enumerate donors) and BHI agar supplemented with Rf, Fa, Em, and X-Gal (to enumerate transconjugants harboring integrated copies of pCJK66). Blue transconjugant colonies arose within 24–36 h at 37°C at a frequency of approximately 6×10^{-5} per donor. These recombinants exhibited a severe growth defect relative to OG1RF when patched to MM9YEG agar supplemented with 10 mM *p*-Cl-Phe due to the presence of the *P-pheS** marker in the backbone of the integrated plasmid. Because it is formally possible that pCJK66 could integrate into the recipient chromosome by homologous recombination at the *pheS* locus (via the plasmid-encoded *P-pheS** allele), a PCR assay was performed on chromosomal DNA obtained from colony-purified transconjugants using flanking primers external to the cloned *pheS* gene. Of 6 isolates tested, all yielded an amplicon corresponding in size to the expected wild-type product, indicating that plasmid integration had not occurred at the *pheS* locus. A similar PCR assay performed with primers external to the cloned *sigH* locus failed to produce a product from the 6 mutants, indicating that plasmid insertion had occurred at this locus.

To isolate secondary recombinants in which the integrated plasmid had been excised and lost, several primary recombinants were cultured in BHI lacking Em for approximately 20 generations at 37°C. Serial dilutions of the cell suspension were plated on MM9YEG agar supplemented with 10 mM *p*-Cl-Phe and X-Gal at 37°C. White colonies arose within ~16–20 h of incubation at a frequency of approximately 5×10^{-4} per viable CFU. Patching to Em-containing agar confirmed that the white colonies were also sensitive to Em, as expected if the integrated plasmid had been excised.

As outlined in Fig. 4, the plasmid-free recombinants could carry either the wild-type allele or the mutant allele. To identify recombinants carrying the chromosomal *sigH* deletion, 18 white *p*-Cl-Phe^R colonies were analyzed using a PCR assay to determine if they carried the wild-type or mutant allele at the target locus. Chromosomal DNA prepared from colony-purified isolates (with the DNEasy Tissue Kit, Qiagen) was used as the source of template DNA. Primers for these amplifications annealed to flanking DNA sequences external to the duplicated (cloned) chromosomal regions. Following electrophoresis on agarose gels, the wild-type and deleted alleles could be differentiated on the basis of the size of the amplicon, revealing 14 recombinants that retained the wild-type *sigH* locus and 4 recombinants that carried the $\Delta sigH2$ deletion. One such $\Delta sigH2$ recombinant (strain CK117) was retained for phenotypic analysis.

Markerless exchange to construct other *E. faecalis* mutants

To construct the remaining single, double, triple, and quadruple mutants in *E. faecalis*, a procedure analogous to that described above for deletion of *sigH* was used, with appropriate combinations of donors and recipients. In some cases, the growth conditions prior to conjugation or the screening approach used to differentiate wild-type recombinants from those carrying the desired mutant alleles were slightly modified, based on the anticipated mutant phenotypes. For example, JRC104 candidates were initially screened using a conjugation assay to measure the ability of the candidates to serve as recipients for pCF10 conjugative transfer,

because previous work demonstrated that *ccfA2* mutants exhibited a defect in such an assay (Chandler *et al.*, 2005). Mutants carrying the Δ *eep10* allele were initially screened using a conjugation assay to measure the ability of the candidates to serve as recipients for pAM714 conjugative transfer, because previous work demonstrated that an *eep* mutant exhibited a reduction in cAD1 pheromone production (An *et al.*, 1999). Mutants carrying the Δ (*gelE-sprE*)10 allele were routinely screened on THB agar supplemented with 3% gelatin to identify clones lacking the characteristic halo indicative of GelE activity (Waters *et al.*, 2003). Triple and quadruple mutants were constructed using an iterative approach, beginning with different combinations of conjugative donors and recipients. The presence of all mutant alleles was verified after each iteration during the construction of multiply-mutant strains. When strains exhibiting reduced pheromone production were used as recipients (*ccfA2*, Δ *eep10*), the pCF10–101 conjugative machinery was induced in the donors by the addition of synthetic cCF10 pheromone (BioMedical Genomics Center, University of Minnesota) at 10 ng/ml during the 1-h growth period prior to conjugation.

Determination of cCF10 pheromone activity in culture supernatants

Strains to be tested were cultivated overnight in THB at 37°C (~15 h). The OD₆₀₀ of the overnight cultures was determined for each, and equivalent amounts of biomass were harvested by centrifugation at 8,000 rpm (Beckman J2–21 centrifuge, JA20 rotor) for 10 min. The supernatant from each sample was retained and evaluated with an aggregation assay to measure the biological activity of cCF10 (Buttaro *et al.*, 2000). Briefly, twofold serial dilutions of supernatant samples were made in THB medium in a round-bottom microtiter plate. Ten microliters of a 15-h old OG1RF(pCF10) indicator culture (cultivated in THB at 37°C) was added to each well. Samples were incubated with shaking at 37°C for 2 hours, and wells in which aggregation of the indicator strain occurred were scored. The titer is reported as the reciprocal of the highest supernatant dilution that induced aggregation of the indicator strain. In cases where no aggregation was observed at any dilution, the results are reported as 0.

Analysis of conjugative plasmid transfer

To determine the extent of conjugative transfer of pheromone-responsive plasmids pCF10 and pAM714 into plasmid-free recipients, strains OG1SSp(pCF10) or OG1SSp(pAM714) were used as conjugative donors. Overnight cultures of donors and recipients were cultivated at 37°C in THB for 15 h and diluted ten-fold into THB. These subcultures were incubated at 37°C for 1 h, followed by mixing of donors and recipients at a ratio of 1:9. Conjugation occurred during an additional incubation at 37°C for 2 hours before serial dilutions were prepared for enumeration on selective media. Transconjugants were enumerated on THB agar supplemented with Tc and Rf (for pCF10-containing transconjugants) or Em and Rf (for pAM714-containing transconjugants). Donors were enumerated on THB agar supplemented with Sm. Measurements are reported as the ratio of transconjugants/donor (TC/donor) and represent means from duplicate assays.

Results

A genotype-independent counterselectable marker for *E. faecalis*

To circumvent the problems associated with our previous counterselection system (see Introduction), we sought to develop a counterselection strategy for *E. faecalis* that did not require the presence of a particular mutation in the genome of the target strain in order to be functional.

In *E. coli*, a missense mutation in the phenylalanyl tRNA synthetase α -subunit (*pheS294AG*) confers relaxed substrate specificity, such that the mutant enzyme can aminoacylate tRNA^{Phe} not only with phenylalanine but also with halogenated phenylalanine derivatives,

including *p*-chloro-phenylalanine (*p*-Cl-Phe) (Kast and Hennecke, 1991; Ibba et al., 1994). Cultivation of *E. coli* clones expressing *pheS294AG* on medium amended with *p*-Cl-Phe results in inhibition of growth, presumably as a consequence of the production of non-functional proteins in which Phe residues have been replaced with *p*-Cl-Phe. Clones lacking *pheS294AG* are capable of normal growth in the presence of *p*-Cl-Phe (Kast and Hennecke, 1991; Kast, 1994). Thus, the growth properties of *E. coli* strains expressing the *pheS294AG* allele provide the basis for a counterselection strategy.

To determine if an analogous strategy was applicable to *E. faecalis*, we compared the sequence of *pheS* from *E. faecalis* V583 (GenBank Accession: [AAO80915](#)) with that of *E. coli* (GenBank Accession: [NP_416229](#)) using the BLAST-P algorithm at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). Inspection of the sequence alignment revealed substantial sequence identity in the region surrounding A294 of the *E. coli pheS* with the corresponding region of the *E. faecalis* homolog, suggesting that A312 of the *E. faecalis pheS* was the functionally equivalent residue. We cloned *pheS* from *E. faecalis* OG1RF into a low-copy number plasmid and introduced the analogous A312G mutation, creating pCJK45 (Fig. 1). Because the natural promoter driving expression of *pheS* in *E. faecalis* had not been identified, we included a synthetic constitutive promoter (originally derived from plasmid pFW11 (Podbielski et al., 1996)) upstream of *pheS312AG* in the construct, creating the P-*pheS** cassette (Fig. 1). Analysis of pCJK45-containing cells (Fig. 2) revealed that *E. faecalis* carrying *pheS312AG* grew normally in the absence of *p*-Cl-Phe but was substantially inhibited in the presence of 10 mM *p*-Cl-Phe, whereas the growth of *E. faecalis* carrying the parental plasmid was not inhibited under equivalent conditions. Thus, the P-*pheS** cassette confers a dominant growth defect on otherwise wild-type *E. faecalis* in the presence of *p*-Cl-Phe, indicating that the P-*pheS** cassette can form the basis of a host-genotype-independent counterselectable marker for *E. faecalis*.

The composition of the growth medium was found to influence P-*pheS**-mediated growth inhibition by *p*-Cl-Phe. For example, cultivation of *E. faecalis* carrying P-*pheS** on BHI, THB, or tryptic soy agar amended with *p*-Cl-Phe did not inhibit growth nearly as significantly as did cultivation on MM9YEG (not shown). We hypothesize that the presence of excess Phe in these relatively rich types of media (either free Phe or derived from another source, such as Phe-containing peptides) is able to effectively compete with *p*-Cl-Phe either during transport or as a substrate for the phenylalanyl-tRNA synthetase, thereby preventing *p*-Cl-Phe from being incorporated into protein at levels sufficient to inhibit growth. Consistent with this hypothesis, addition of 1% Casamino Acids to MM9YEG attenuates the growth-inhibitory effect of *p*-Cl-Phe (not shown).

Construction of a mobilizable plasmid and conjugative delivery system for efficient markerless exchange in *E. faecalis*

Enterococci are widely known for their proficiency in the use of conjugation as a means of exchanging mobile genetic elements. For example, enterococcal pheromone-responsive conjugative plasmids such as pCF10 (Dunny et al., 1981) can easily transfer by conjugation to plasmid-free recipient cells at remarkably high frequencies of 10^{-1} transconjugants/donor. Recently, pCF10 was found to mediate the conjugative mobilization, in trans, of heterologous shuttle plasmids carrying a cloned pCF10 origin of transfer (*oriT*) at similarly high frequencies (Staddon et al., in press). Furthermore, a mutant derivative of pCF10 carrying a deletion of its *oriT* (pCF10-101) could not transfer itself to recipient cells at detectable frequencies in liquid matings, but retained the ability to mobilize heterologous *oriT*_{pCF10}-containing shuttle plasmids at high frequency (Staddon et al., in press). Therefore, we developed a system to use pCF10-101 for efficient delivery of conditionally-replicating donor plasmids to target cells of *E. faecalis* for markerless genetic exchange.

The donor plasmid for our system is a derivative of pORI280 (Law *et al.*, 1995; Leenhouts *et al.*, 1996), which requires the replication protein RepA in trans to replicate. We sequentially introduced a cloned copy of the pCF10 *oriT* (conferring pCF10–101-dependent mobilization) and the P-*pheS** cassette (providing the counterselectable marker), thereby creating the donor plasmid pCJK47 (Fig. 1). Most of the unique restriction sites found in pORI280 remain in pCJK47 and can be used to clone target DNA into the donor plasmid. To serve as a conjugative donor strain, we constructed an *E. faecalis* strain carrying pCF10–101 that could support replication of pCJK47 derivatives. Our strategy was to integrate a constitutively expressed allele of *repA* into the *E. faecalis* chromosome at the *upp* locus (see Materials and Methods for details), creating strain CK111. Subsequently, pCF10–101 was introduced to yield the conjugative donor strain.

Construction of *E. faecalis* mutant strains

To evaluate the effectiveness of our improved system for efficient markerless exchange in *E. faecalis*, we constructed a panel of mutant strains according to the procedure described in Materials and Methods (see Fig. 3 for an overview of the process). Because *E. faecalis* is known to grow well under stressful conditions, we initially searched the *E. faecalis* V583 genome for genes encoding alternative sigma factors that might regulate adaptation to stressful conditions, such as *sigH* of *Bacillus subtilis*. Using *B. subtilis sigH* as a query (GenBank Accession: **CAB11874**), we identified TIGR locus EF0049 as a *sigH* homolog and constructed an in-frame deletion mutant of *E. faecalis* lacking this gene. We also constructed a panel of strains carrying mutations in several other genes whose products participate in enterococcal pheromone production or degradation. In particular, we constructed mutations in *ccfA*, which encodes the cCF10 pheromone precursor (Antiporta and Dunny, 2002); *eep*, whose product is required for production of the cCF10 and cAD1 pheromones (An *et al.*, 1999) and whose product is thought to be a membrane-bound protease (Brown *et al.*, 2000) involved in proteolytic processing of pheromone precursors; and *gelE* and *sprE*, which encode extracellular proteases known to degrade secreted pheromone (Waters *et al.*, 2003). Previously, each of these genes had been independently inactivated by other means. However, the corresponding mutants each suffer from significant disadvantages due to the inherent limitations of the approaches used for inactivation of these genes. To circumvent the associated problems, we constructed stable, unmarked mutations that inactivated each of the genes in an isogenic, wild-type background (strains JRC104, JRC105, and JRC106).

One important advantage of markerless exchange is the ability to efficiently construct strains carrying multiple mutations, thereby enabling sophisticated genetic analysis such as tests of epistasis. To demonstrate this, we used an iterative process to combine the *ccfA2*, Δ *eep10* and Δ (*gelE-sprE*)10 mutant alleles with each other in various permutations to form triple and quadruple mutant strains (see Table 2 for a complete list). Previously, strains that simultaneously carried mutations in more than 1 of these 3 loci were not available, precluding epistasis analysis of the current model for pheromone biosynthesis and processing. In summary, our markerless genetic exchange system enabled the efficient construction of a variety of stable, unmarked deletion mutants and point mutants, as well as strains carrying combinations of multiple mutations.

During preliminary experiments analyzing conjugative mobilization of pCJK66 into OG1RF, we found that unselected chromosomal markers from the donor strain could sometimes be transferred into the recipient along with pCJK66. The mechanism of chromosomal co-transfer was not investigated, but this phenomenon requires that any transconjugants constructed by this approach are screened to ensure that donor-specific chromosomal markers (e.g., spectinomycin resistance, *upp4::P₂₃repA4*) have not been co-transferred with the donor plasmid. In the case of pCJK66-containing transconjugant derivatives of OG1RF, we screened

for sensitivity to spectinomycin and 5-FU, each of which are properties of the recipient strain but not the donor strain.

Phenotypic analyses of *E. faecalis* mutant strains

The properties of all mutants constructed in this study by markerless exchange technology were compared with wild-type *E. faecalis* OG1RF using several phenotypic assays. Growth rates of all mutants, as measured by changes in optical density at 600 nm, were indistinguishable from that of OG1RF in THB at 37°C (not shown). Cultivation of the mutants on THB agar supplemented with 3% gelatin (a substrate for the secreted GelE protease) overnight at 37°C revealed the characteristic halo indicative of GelE activity (Waters *et al.*, 2003) for all strains that are *gelE*⁺ (OG1RF, CK117, JRC104, JRC106) but no halo for strains carrying the $\Delta(\textit{gelE-sprE})10$ allele (JRC105, JRC107, JRC109, JRC110), as expected (not shown).

Given the known role for *B. subtilis* *sigH* in mediating the transition to stationary phase (Britton *et al.*, 2002), we evaluated growth kinetics of our *sigH2* mutant in several rich and semi-defined media, but were unable to identify a phenotypic defect. We also failed to identify a defect for the *sigH2* mutant in a number of other assays, including sensitivity to a panel of antibiotics, biofilm formation in a microtiter-plate assay, adherence to collagen-coated surfaces, and ability to serve as a donor or recipient for conjugative plasmid transfer (Fig. 4).

Production of cCF10 pheromone by the panel of mutant strains was evaluated with a biological activity assay (Buttaro *et al.*, 2000), using stationary-phase culture supernatants as the source of pheromone (Table 2). Because *E. faecalis* OG1RF produces pheromone-degrading extracellular proteases, cCF10 pheromone produced by OG1RF is not detectable under the conditions used for this assay. Previously, analysis of pheromone production by a mutant carrying an insertion in *gelE* demonstrated that cCF10 accumulation was substantially increased, revealing a key role for GelE in cCF10 degradation (Waters *et al.*, 2003). Consistent with those results, our markerless $\Delta(\textit{gelE-sprE})10$ mutant also exhibited enhanced cCF10 accumulation (Table 2, JRC105). Tests of epistasis conducted by introducing additional mutations in genes known to encode the pheromone or to be required for its processing (the *ccfA2* or $\Delta\textit{eep10}$ mutations introduced in the triple mutants JRC107 and JRC109) show that the phenotypic effects of the $\Delta(\textit{gelE-sprE})10$ mutation is either completely or partially abrogated if pheromone biosynthesis is impaired (Table 2). That the *ccfA2* and $\Delta\textit{eep10}$ alleles are epistatic to the $\Delta(\textit{gelE-sprE})10$ allele suggests that both *ccfA* and *eep* act upstream of *gelE/sprE* in the pathway determining the level of cCF10 activity in culture supernatants, consistent with current understanding (Chandler and Dunny, 2004). Thus, our improved genetic system allowed us to perform genetic tests of the current model of cCF10 biogenesis.

Pheromone production can also be assessed by monitoring the ability of a strain to serve as a recipient for pheromone-responsive conjugative plasmid transfer. Therefore, the mutants were evaluated as recipients for 2 distinct conjugative plasmids: the cCF10-specific plasmid pCF10 and the cAD1-specific plasmid pAM714 (Fig. 4). As expected, the *ccfA2* mutant (JRC104) is defective as a recipient for pCF10 but not for pAM714, whereas the $\Delta\textit{eep10}$ mutant is defective as a recipient for both plasmids. Although plasmid transfer frequencies have not previously been reported for the original *eep* insertion mutant, the latter phenotype is consistent with its deficiency in both cCF10 and cAD1 pheromone production (An *et al.*, 1999). We also note that the 2 triple mutants (JRC107 and JRC109), each of which exhibited a reduction in cCF10 accumulation in culture supernatants (Table 2), also exhibit a reduction in pCF10 recipient ability relative to the otherwise isogenic double mutant (JRC105), confirming the biological relevance of the pheromone accumulation results.

Discussion

Genetic analysis of enterococci has thus far been limited by inefficient or non-existent tools for genetic manipulation. We now report the development of a substantially improved system for efficient markerless genetic exchange in *E. faecalis* that surmounts some of the previous limitations. By exploiting the enterococcal pheromone-responsive conjugative plasmid pCF10 for use as an efficient DNA delivery machine, we have overcome a significant barrier to genetic manipulation of *E. faecalis* – transferring DNA that has been manipulated in vitro back into target cells to serve as a substrate for recombination. Enterococci are not known to be naturally competent for transformation, and current electroporation protocols are inefficient, especially when introducing non-replicating plasmids that must undergo homologous recombination to be retained. However, the enterococcal pheromone-responsive plasmids have evolved a remarkably efficient ability to deliver DNA into neighboring cells, rendering these plasmids ideal tools for genetic manipulation. Furthermore, pCF10 has recently been shown to mobilize heterologous *oriT*_{pCF10}-containing plasmids across species boundaries into *Lactococcus lactis* and *Streptococcus agalactiae* (Staddon *et al.*, in press), suggesting that our conjugative delivery system may facilitate genetic manipulation of other Gram-positive bacteria for which no efficient means of DNA introduction currently exists. In such applications, we expect that the conjugative *E. faecalis* donor strains will require induction by exogenous cCF10 pheromone to enable efficient delivery.

During the construction of the mutants reported in this paper, we observed that chromosomal markers present in the conjugative donor can sometimes be co-transferred to recipients along with the donor plasmid. The mechanism of this co-transfer has not been investigated. However, the frequency of co-transfer of particular chromosomal alleles varies depending on the identity of the chromosomal segment cloned into pCJK47; thus we speculate that chromosomal co-transfer may result from an Hfr-type mechanism following transient integration (by homologous recombination) of the pCJK47-derivative into the chromosome of the conjugative donor strain. In any case, the extent of co-transfer has never been so great as to preclude the isolation of the desired mutant. The issue of co-transfer likely will be irrelevant if this system is used to mobilize DNA into heterologous species, where homologous recombination cannot occur.

We recently described the first counterselectable marker functional in *E. faecalis* (Kristich *et al.*, 2005) and have used it to construct a number of interesting mutants. However, one drawback of the previous counterselection strategy is that the host strain must carry a mutation in the chromosomal *upp* gene for counterselection to be applied. The “second generation” P-*pheS** counterselectable marker described in this report eliminates any such genotypic requirement, as the P-*pheS** allele is dominant over the wild-type *pheS* allele. Therefore, counterselection can be applied in any strain, regardless of its genotype. Preliminary evidence indicates that this P-*pheS** cassette can also inhibit growth of *Staphylococcus aureus* and *Lactococcus lactis* cultivated in the presence of p-Cl-Phe, suggesting that our P-*pheS** counterselection strategy can be applied to facilitate markerless exchange in a variety of low-GC Gram positive bacteria for which no counterselectable marker is currently available.

In summary, we have developed a substantially improved system for efficient markerless genetic exchange in *E. faecalis* and used this system to rapidly construct a panel of single, double, triple, and quadruple mutant strains carrying unmarked in-frame deletions and point mutations. Of note, our system enabled tests of epistasis to be applied, for the first time, to a chromosomally encoded biosynthetic pathway - the enterococcal pheromone biosynthesis pathway. Thus, this technology enables the kind of sophisticated genetic analysis that is required to develop a thorough understanding of the biology of *E. faecalis*. It seems likely that

these tools can also be successfully applied for genetic manipulation of other Gram-positive bacteria for which efficient genetic tools are currently lacking.

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References

- An FY, Sulavik MC, Clewell DB. Identification and characterization of a determinant (*eep*) on the *Enterococcus faecalis* chromosome that is involved in production of the peptide sex pheromone cAD1. *J Bacteriol* 1999;181:5915–21. [PubMed: 10498702]
- Antipporta MH, Dunny GM. *ccfA*, the genetic determinant for the cCF10 peptide pheromone in *Enterococcus faecalis* OG1RF. *J Bacteriol* 2002;184:1155–62. [PubMed: 11807076]
- Arbeloa A, Segal H, Hugonnet JE, Josseume N, Dubost L, Brouard JP, Gutmann L, Mengin-Lecreulx D, Arthur M. Role of class A penicillin-binding proteins in PBP5-mediated beta-lactam resistance in *Enterococcus faecalis*. *J Bacteriol* 2004;186:1221–8. [PubMed: 14973044]
- Bae T, Kozlowicz B, Dunny GM. Two targets in pCF10 DNA for PrgX binding: their role in production of Qa and *prgX* mRNA and in regulation of pheromone-inducible conjugation. *J Mol Biol* 2002;315:995–1007. [PubMed: 11827471]
- Bitan-Banin G, Ortenberg R, Mevarech M. Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyrE* gene. *J Bacteriol* 2003;185:772–8. [PubMed: 12533452]
- Britton RA, Eichenberger P, Gonzalez-Pastor JE, Fawcett P, Monson R, Losick R, Grossman AD. Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. *J Bacteriol* 2002;184:4881–90. [PubMed: 12169614]
- Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 2000;100:391–8. [PubMed: 10693756]
- Buttaro BA, Antipporta MH, Dunny GM. Cell-associated pheromone peptide (cCF10) production and pheromone inhibition in *Enterococcus faecalis*. *J Bacteriol* 2000;182:4926–33. [PubMed: 10940037]
- Chandler JR, Dunny GM. Enterococcal peptide sex pheromones: synthesis and control of biological activity. *Peptides* 2004;25:1377–88. [PubMed: 15374642]
- Chandler JR, Hirt H, Dunny GM. A paracrine peptide sex pheromone also acts as an autocrine signal to induce plasmid transfer and virulence factor expression in vivo. *Proc Natl Acad Sci USA* 2005;102:15617–22. [PubMed: 16223881]
- Derre I, Rapoport G, Msadek T. *CtsR*, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in gram-positive bacteria. *Mol Microbiol* 1999;31:117–31. [PubMed: 9987115]
- Dunny G, Funk C, Adsit J. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis*. *Plasmid* 1981;6:270–8. [PubMed: 6796985]
- Dunny GM, Brown BL, Clewell DB. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. *Proc Natl Acad Sci USA* 1978;75:3479–83. [PubMed: 98769]
- Dunny GM, Clewell DB. Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. *J Bacteriol* 1975;124:784–90. [PubMed: 810482]
- Fabret C, Ehrlich SD, Noirot P. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. *Mol Microbiol* 2002;46:25–36. [PubMed: 12366828]
- Fukagawa T, Hayward N, Yang J, Azzalin C, Griffin D, Stewart AF, Brown W. The chicken HPRT gene: a counterselectable marker for the DT40 cell line. *Nucleic Acids Res* 1999;27:1966–9. [PubMed: 10198428]

- Gold OG, Jordan HV, van Houte J. The prevalence of enterococci in the human mouth and their pathogenicity in animal models. *Arch Oral Biol* 1975;20:473–7. [PubMed: 807189]
- Ibba M, Kast P, Hennecke H. Substrate specificity is determined by amino acid binding pocket size in *Escherichia coli* phenylalanyl-tRNA synthetase. *Biochemistry* 1994;33:7107–12. [PubMed: 8003476]
- Ike Y, Clewell DB. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn917 as an insertional mutagen. *J Bacteriol* 1984;158:777–83. [PubMed: 6327637]
- Kak, V.; Chow, JW. Acquired antibiotic resistances in enterococci. In: Gilmore, MS.; Clewell, DB.; Courvalin, P.; Dunny, GM.; Murray, BE.; Rice, LB., editors. *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. American Society for Microbiology Press; Washington, D.C: 2002. p. 355-383.
- Kast P. pKSS--a second-generation general purpose cloning vector for efficient positive selection of recombinant clones. *Gene* 1994;138:109–14. [PubMed: 8125286]
- Kast P, Hennecke H. Amino acid substrate specificity of *Escherichia coli* phenylalanyl-tRNA synthetase altered by distinct mutations. *J Mol Biol* 1991;222:99–124. [PubMed: 1942071]
- Kristich CJ, Manias DA, Dunny GM. Development of a method for markerless genetic exchange in *Enterococcus faecalis* and its use in construction of a *srtA* mutant. *Appl Environ Microbiol* 2005;71:5837–5849. [PubMed: 16204495]
- Law J, Buist G, Haandrikman A, Kok J, Venema G, Leenhouts K. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J Bacteriol* 1995;177:7011–8. [PubMed: 8522504]
- Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* 1996;253:217–24. [PubMed: 9003306]
- Leenhouts KJ, Tolner B, Bron S, Kok J, Venema G, Seegers JF. Nucleotide sequence and characterization of the broad-host-range lactococcal plasmid pWVO1. *Plasmid* 1991;26:55–66. [PubMed: 1840693]
- Maloy SR, Nunn WD. Selection for loss of tetracycline resistance by *Escherichia coli*. *J Bacteriol* 1981;145:1110–1. [PubMed: 7007341]
- Maloy, SR.; Stewart, VJ.; Taylor, RK. *Genetic Analysis of Pathogenic Bacteria. A Laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1996.
- Peck RF, Dassarma S, Krebs MP. Homologous gene knockout in the archaeon *Halobacterium salinarum* with *ura3* as a counterselectable marker. *Mol Microbiol* 2000;35:667–76. [PubMed: 10672188]
- Podbielski A, Spellerberg B, Woischnik M, Pohl B, Lutticken R. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* 1996;177:137–47. [PubMed: 8921859]
- Pritchett MA, Zhang JK, Metcalf WW. Development of a markerless genetic exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new genetic tools for methanogenic archaea. *Appl Environ Microbiol* 2004;70:1425–33. [PubMed: 15006762]
- Qin X, Singh KV, Xu Y, Weinstock GM, Murray BE. Effect of disruption of a gene encoding an autolysin of *Enterococcus faecalis* OG1RF. *Antimicrob Agents Chemother* 1998;42:2883–8. [PubMed: 9797220]
- Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in combined medical-surgical intensive care units in the United States. *Infect Control Hosp Epidemiol* 2000;21:510–5. [PubMed: 10968716]
- Ried JL, Collmer A. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. *Gene* 1987;57:239–46. [PubMed: 3319780]
- Spring KJ, Mattick JS, Don RH. *Escherichia coli gpt* as a positive and negative selectable marker in embryonal stem cells. *Biochim Biophys Acta* 1994;1218:158–62. [PubMed: 8018715]
- Tannock, GW.; Cook, G. Enterococci as Members of the Intestinal Microflora of Humans. In: Gilmore, MS.; Clewell, DB.; Courvalin, P.; Dunny, GM.; Murray, BE.; Rice, LB., editors. *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. American Society for Microbiology Press; Washington, D.C: 2002. p. 101-132.

- van der Vossen JM, van der Lelie D, Venema G. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. *Appl Environ Microbiol* 1987;53:2452–7. [PubMed: 2447829]
- Waters CM, Antiporta MH, Murray BE, Dunny GM. Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J Bacteriol* 2003;185:3613–23. [PubMed: 12775699]
- Wirth, R.; An, F.; Clewell, DB. Highly efficient cloning system for *Streptococcus faecalis* protoplast transformation, shuttle vectors, and applications. In: Ferretti, JJ.; Curtiss, RI., editors. *Streptococcal Genetics*. American Society for Microbiology Press; Washington, D. C: 1987. p. 25-27.

Fig. 1. Strategy for construction of plasmids used in this study

See Materials and Methods for details. The asterisks indicate the *pheSA312G* mutation. P, synthetic constitutive promoter. “*sigH* up” and “*sigH* down” represent the chromosomal segments upstream and downstream of *sigH*, respectively. Open arrows indicate PCR amplifications, solid arrows indicate cloning steps. Plasmids for markerless exchange of other genes were similarly constructed by cloning into pCJ2K47, as described in Materials and Methods. Unique restriction sites are shown. Note that the plasmids are not shown to scale.

Fig. 2. Conditional growth inhibition mediated by *P-pheS in *E. faecalis***

Wild-type *E. faecalis* OG1RF carrying either an empty shuttle vector (pWM401) or a derivative carrying the *P-pheS** cassette (pCJK45) were cultivated overnight in BHI supplemented with Cm at 37°C. Bacteria were washed twice with PBS and resuspended in PBS to the original volume. After preparing ten-fold serial dilutions in PBS, aliquots (5 µl) of successive dilutions were spotted from left to right on MM9YEG agar supplemented with Cm and the indicated concentration of *p*-Cl-Phe. The plates were incubated at 37°C for 24 h and photographed using dark-field illumination.

Fig. 3. Efficient markerless genetic exchange strategy used to create a deletion of *sigH* in *E. faecalis*

See Materials and Methods for a detailed description of the procedure. The conjugative donor strain (left) contains a mutant pCF10 (pCF10-101) lacking its *oriT*. The donor supports replication of mobilizable *repA*⁻ plasmids due to expression of a chromosomal copy of *repA*. Conjugative transfer functions supplied by pCF10-101 mediated the high-frequency mobilization of pCJK66 into the *repA*⁻ recipient, OG1RF (right). Homologous recombination of pCJK66 occurred at a region of cloned chromosomal DNA (Step 1), generating an erythromycin-resistant recombinant that is sensitive to growth inhibition mediated by *p-Cl-Phe* due to constitutive expression of *pheS**. The *sigH* deletion is encoded by the pCJK66-borne allele. In Step 2, plasmid excision by recombination occurred during nonselective growth, leaving either the wild-type allele (A) or the mutant allele (B) on the chromosome. The excised plasmid cannot replicate and was lost by segregation. Recombinants from Step 2 were selected by plating cells on MM9YEG agar supplemented with 10 mM *p-Cl-Phe* and X-Gal and screened by PCR to differentiate those carrying the wild-type *sigH* allele from those carrying the deletion. Cloned chromosomal DNA fragments flanking *sigH* on the upstream (up) or downstream (down) sides are indicated as white rectangles juxtaposed to the $\Delta sigH$ allele. An equivalent sequence of events can be drawn for plasmid integration at either the upstream or downstream fragment.

Fig. 4. Analysis of conjugative plasmid transfer into *E. faecalis* mutant strains

The ability of a panel of *E. faecalis* strains to serve as recipients for pheromone-responsive conjugative plasmid transfer was determined in a 2-h liquid conjugation assay for (A) the cCF10-specific plasmid pCF10, or (B) the cAD1-specific plasmid pAM714. The data are reported as the ratio of transconjugant CFU/donor CFU (TC/donor) for 2 independent experiments performed on separate days. Strains tested as recipients are: 1, OG1RF (wild-type); 2, CK117 ($\Delta sigH2$); 3, JRC104 (*ccfA2*); 4, JRC105 ($\Delta(gelE-sprE)10$); 5, JRC106 ($\Delta deep10$); 6, JRC107 (*ccfA2* $\Delta(gelE-sprE)10$); 7, JRC109 ($\Delta deep10$ $\Delta(gelE-sprE)10$); 8, JRC110 (*ccfA2* $\Delta(gelE-sprE)10$ $\Delta deep10$).

Table 1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic or description	Reference or Source
Strains		
<i>E. coli</i>		
XL1Blue	<i>E. coli</i> cloning host	Lab stock
DH5 α	<i>E. coli</i> cloning host	Lab stock
EC1000	<i>E. coli</i> cloning host, provides RepA in trans	(Leenhouts <i>et al.</i> , 1996)
<i>E. faecalis</i>		
OG1	Reference strain	(Gold <i>et al.</i> , 1975)
OG1SSp	Spontaneous mutant of OG1; Sm ^R , Sp ^R	(Dunny <i>et al.</i> , 1978)
OG1RF	Spontaneous mutant of OG1; Rf ^R , Fa ^R	(Dunny <i>et al.</i> , 1978)
OG1Sp	Spontaneous mutant of OG1; Sp ^R	This work
CK111	OG1Sp <i>upp4::P₂₃repA4</i>	This work
CK117	OG1RF Δ <i>sigH2</i>	This work
JRC104	OG1RF <i>ccfA2</i>	This work
JRC105	OG1RF Δ (<i>gelE-sprE</i>)10	This work
JRC106	OG1RF Δ <i>deep10</i>	This work
JRC107	JRC104 Δ (<i>gelE-sprE</i>)10	This work
JRC109	JRC105 Δ <i>deep10</i>	This work
JRC110	JRC107 Δ <i>deep10</i>	This work
Plasmids		
pSK	Cloning vector	Stratagene
pAM714	cAD1-inducible conjugative plasmid (pAD1-derivative); Em ^R	(Ike and Clewell, 1984)
pCF10	cCF10-inducible conjugative plasmid	(Dunny <i>et al.</i> , 1981)
pCF10-101	pCF10 Δ <i>oriT2</i>	Staddon <i>et al.</i> , in press
pORI280	Requires RepA in trans for replication; Em ^R	(Leenhouts <i>et al.</i> , 1996)
pWM401	Low-copy shuttle vector; Cm ^R	(Wirth <i>et al.</i> , 1987)
pCJK2	Source of constitutive promoter	(Kristich <i>et al.</i> , 2005)
pCJK11	<i>upp::erm</i> cassette in temperature-sensitive shuttle vector; Cm ^R	(Kristich <i>et al.</i> , 2005)
pCJK12	pCJK11 derivative carrying P ₂₃ <i>repA1</i>	This work
pCJK41	pCJK11 derivative carrying P ₂₃ <i>repA4</i>	This work
pCJK34	pORI280 derivative carrying <i>oriT</i> _{pCF10}	This work
pCJK45	pWM401 derivative carrying P- <i>pheS</i> *	This work
pCJK47	Conjugative donor plasmid, carries <i>oriT</i> _{pCF10} and P- <i>pheS</i> *; pORI280 derivative; Em ^R	This work
pCJK52	carries Δ <i>sigH2</i>	This work
pCJK66	Δ <i>sigH2</i> allele cloned into pCJK47	This work
pJRC104	<i>ccfA2</i> allele cloned into pCJK47	This work
pJRC105	Δ (<i>gelE-sprE</i>)10 allele cloned into pCJK47	This work
pJRC106	Δ <i>deep10</i> allele cloned into pCJK47	This work

Table 2

Determination of cCF10 pheromone activity in culture supernatants.

Strain	Relevant genotype	cCF10 activity* Expt 1	cCF10 activity* Expt 2
OG1RF	Wild-type	0	0
CK117	$\Delta sigH2$	0	0
JRC104	$ccfA2$	0	0
JRC105	$\Delta(gelE-sprE)10$	16	8
JRC106	$\Delta eep10$	0	0
JRC107	$ccfA2 \Delta(gelE-sprE)10$	0	0
JRC109	$\Delta eep10 \Delta(gelE-sprE)10$	2	1
JRC110	$ccfA2 \Delta(gelE-sprE)10 \Delta eep10$	0	0

* cCF10 pheromone activity in stationary-phase culture supernatants was determined using a biological indicator assay, as described in Methods, for 2 independent experiments performed on separate days. cCF10 activity is reported as the reciprocal of the highest supernatant dilution that induced aggregation of the indicator strain. Supernatant samples that did not induce aggregation at any dilution are reported as 0.