The Enterococcus Cassette Chromosome, a Genomic Variation Enabler in Enterococci

A. Sivertsen, J. Janice, T. Pedersen, T. M. Wagner, J. Hegstad, K. Hegstad

ABSTRACT

Enterococcus faecium has a highly variable genome prone to recombination and horizontal gene transfer. Here, we have identified a novel genetic island with an insertion locus and mobilization genes similar to those of staphylococcus cassette chromosome elements SCCmec. This novel element termed the enterococcus cassette chromosome (ECC) element was located in the 3’ region of rlmH and encoded large serine recombinases ccrAB similar to SCCmec. Horizontal transfer of an ECC element termed ECC::cat containing a knock-in cat chloramphenicol resistance determinant occurred in the presence of a conjugative rep_{pLG1} plasmid. We determined the ECC::cat insertion site in the 3’ region of rlmH in the E. faecium recipient by long-read sequencing. ECC::cat also mobilized by homologous recombination through sequence identity between flanking insertion sequence (IS) elements in ECC::cat and the conjugative plasmid. The ccrAB_{Ent} genes were found in 69 of 516 E. faecium genomes in GenBank. Full-length ECC elements were retrieved from 32 of these genomes. ECCs were flanked by attR and attL sites of approximately 50 bp. The attECC sequences were found by PCR and sequencing of circularized ECCs in three strains. The genes in ECCs contained an amalgam of common and rare E. faecium genes. Taken together, our data imply that ECC elements act as hot spots for genetic exchange and contribute to the large variation of accessory genes found in E. faecium.

IMPORTANCE

Enterococcus faecium is a bacterium found in a great variety of environments, ranging from the clinic as a nosocomial pathogen to natural habitats such as mammalian intestines, water, and soil. They are known to exchange genetic material through horizontal gene transfer and recombination, leading to great variability of accessory genes and aiding environmental adaptation. Identifying mobile genetic elements causing sequence variation is important to understand how genetic content variation occurs. Here, a novel genetic island, the enterococcus cassette chromosome, is shown to contain a wealth of genes, which may aid E. faecium in adapting to new environments. The transmission mechanism involves the only two conserved genes within ECC, ccrAB_{Ent}, large serine recombinases that insert ECC into the host genome similarly to SCC elements found in staphylococci.

KEYWORDS

Enterococcus faecium, enterococci, mobile genetic element, serine recombinase, ccrAB_{Ent}, SCCmec

Enterococci are a public health concern as a common cause of hospital-associated infections and a burden to patient morbidity and mortality. They have acquired antimicrobial resistance mechanisms toward many currently available antibiotics through horizontal gene transfer (HGT) of mobile genetic elements (MGEs) (1–4). They
are also able to survive a broad range of environments and environmental stressors to which other bacteria succumb (5, 6). Enterococci contain a broad diversity of large integrative conjugative elements (ICEs) and nonconjugative genomic islands (GIs) believed to contribute to their genomic diversity (7).

The staphylococcus cassette chromosome element SCCmec is a GI in Staphylococcus aureus harboring the mecA gene providing resistance toward beta-lactams (8). Movement of SCCmec occurs by the serine recombinases CcrA and CcrB that recognize a specific attachment site (attB) in the 3' region of rlmH, a conserved tRNA methyltransferase gene in S. aureus (9, 10), and a corresponding attachment site (attSCC) on the circularized SCCmec intermediate. CcrAB use these sites to integrate SCCmec, after which attL (5') and attR (3') sites are generated as excision sites on either end of the element (9, 11, 12). SCCmec elements show a large degree of diversity in the gene content in both S. aureus (13) and in other species within the Staphylococcus genus (14–16). HGT of SCCmec between staphylococci has been observed during antimicrobial therapy (17), in the lab using bacteriophages as transfer vehicles (18, 19), and by conjugation after SCCmec integration by homologous recombination of IS elements into a staphylococcal conjugative plasmid in vitro (20).

Orthologues to the S. aureus ccrAB genes have been found by screening a collection of several species of the Enterococcus genus (21). These ccrABEnt genes were expressed as a bicistronic mRNA (21) in reference strain Enterococcus faecium DO (22).

Here, we demonstrate the mobility of the novel genetic island enterococcus cassette chromosome (ECC) in E. faecium. ECC shares insertion site and movement by large serine recombinases with SCCmec. ECCs are present in 9% of available E. faecium genomes in the NCBI database, and their gene content is highly variable. We postulate that ECCs act as gene traffickers between the enterococcal chromosomes.

RESULTS AND DISCUSSION

ECC::cat was successfully transferred between strains by the help of a conjugative rep<sub>pLG1</sub> megaplasmid. UWECC::cat is a clinical plasmid-cured E. faecium strain with a knocked-in selectable marker, the chloramphenicol resistance-encoding gene cat immediately downstream of ccrAB<sub>Ent</sub>. Filter mating experiments using strain UWECC::cat without a helper plasmid failed to produce transconjugants with ECC::cat within the detection limits (10<sup>-10</sup> to 10<sup>-9</sup> transconjugants/donor cell). To mobilize ECC::cat into recipient strain BM4105-RF, a conjugation apparatus was provided by filter mating a 298-kb rep<sub>pLG1</sub> megaplasmid into UWECC::cat via BM4105-RF from clinical isolate K60-19 (Table 1; see also Fig. S1 in the supplemental material) (23). The rep<sub>pLG1</sub> megaplasmid contains a type IV secretion system (T4SS), an aac(6')Ie-aph(2")Ia gentamicin resistance selection determinant, and belongs to the RepA_N family which previously has been shown to mobilize large chromosomal stretches of DNA in E. faecium (24, 25). Transconjugants occurred at frequencies of 3 × 10<sup>-7</sup> per donor in UWECC::cat rep<sub>pLG1</sub> × BM4105-RF filter mating experiments.

We also obtained horizontal transfer of ECC::cat by the aid of five other rep<sub>pLG1</sub> megaplasmids from clinical E. faecium strains (results not shown), confirming that they are vehicles for mobilization of genetic elements in E. faecium.

ECC::cat was inserted into the recipient chromosome in an SCCmec-like fashion. E. faecium UWECC::cat, recipient BM4105-RF, and two transconjugants were long read sequenced to resolve genomic structures and identify insertion sites of ECC::cat. A 32-kb ECC element was inserted chromosomally downstream of rlmH in the transconjugants BMECC::cat, flanked by direct repeat regions representing att sites (Fig. 1 and Table S1). BM4105-RF contains an ECC remnant, as an attL site is contained within rlmH and an attR site could be identified downstream. Three genes near the attR site had been lost in the transconjugant compared to recipient BM4105-RF (Fig. 1). The likely explanation for the organization of the ECC::cat chromosomal region in transconjugant BMECC::cat is excision and loss of the recipient’s ECC remnant and subsequent replacement with ECC::cat.
| Species and strain | Plasmid | Relevant resistance characteristic(s) [gene(s)] | Relevant description | Type of sequence data | Reference | GenBank accession no. |
|--------------------|---------|-----------------------------------------------|----------------------|-----------------------|-----------|----------------------|
| E. faecium strains |         |                                               |                      |                       |           |                     |
| UW1551             |         |                                               | ECC-containing clinical isolate |                       | 52        |                     |
| UWΔp               |         |                                               | UW1551 cured of most plasmids |                       | This study |                     |
| UWEC::cat          | rep$_{plG1}$ | Chl$^r$ [cat]                                      | Plasmid-cured UW1551 with cat resistance marker inserted in ORF1 next to ccrAB$_{rep}$ |                       | This study |                     |
| K60-19             | rep$_{plG1}$ | Genr [aac(6')le-aph(2'')]la]                               | Clinical rep$_{plG1}$ plasmid donor with many other plasmids |                       | 23        |                     |
| BM4105-RF rep$_{plG1}$ | rep$_{plG1}$ | Rif', Fus', Genr [aac(6')le-aph(2'')la]                              | rep$_{plG1}$ Plasmid donor containing only this plasmid |                       | This study |                     |
| UWEC::cat rep$_{plG1}$ | rep$_{plG1}$ | Chl$^r$ [cat], Genr [aac(6')le-aph(2'')la]                          | Donor UWEC::cat with rep$_{plG1}$ |                       | PacBio    | This study NMZL01000001.1, NMZL01000002.1, NMZL01000003.1 |
| BM4105-RF          |         |                                               | Recipient            |                       |           |                     |
|                     |         |                                               | Nanopore and Illumina combined |                       | 54        | CP03010.1           |
| BMECC::cat         | rep$_{plG1}$ | Chl$^r$ [cat], Rif, Fus, Gen$^r$ [aac(6')le-aph(2'')la] | Transconjugant containing ECC::cat on BM4105-RF chromosome |                       | PacBio    | This study NMZK01000001.1, NMZK01000002.1 |
| BM pECC::cat       | rep$_{plG1}$, ECC::cat | Chl$^r$ [cat], Rif, Fus, Gen$^r$ [aac(6')le-aph(2'')la] | Transconjugant containing ECC::cat on plasmid |                       | PacBio    | This study NMZJ01000001.1, NMZJ01000002.1 |
| E. coli strains    |         |                                               |                      |                       |           |                     |
| pTEX500ts          |         | Chl$^r$ [cat], Gen$^r$ [aph(2'')-Id]                     | Shuttle plasmid, temperature sensitive in Gram-positive host |                       | 53        |                     |
| pORF1a             |         | Chl$^r$ [cat], Gen$^r$ [aph(2'')-Id]                     | pTEX500ts with cloned ORF1 fragment upstream of the cat gene |                       | This study |                     |
| pORF1b             |         | Chl$^r$ [cat], Gen$^r$ [aph(2'')-Id]                     | pTEX500ts with cloned ORF1 fragments flanking the cat gene |                       | This study |                     |

aThe r superscript indicates resistance. Drugs are abbreviated as follows: Chl, chloramphenicol; Gen, gentamicin; Rif, rifampin; Fus, fusidic acid.
The identified direct repeats of approximately 50 nucleotides flanking ECC::cat showed similarity to direct repeats found in SCCmec-containing S. aureus N315 (Fig. 2), and thus represent att sites compatible with ccrABEnt-mediated specific excision and insertion. The repeats contained a conserved central motif (5'-TATCATAA-3') identical to SCCmec att sites.

Excision of ECC. ECC is expected to circularize during excision, as is observed in SCCmec. Circularization PCRs of ECC::cat and ECC elements from E. faecium DO and K59-68 (Fig. 2, primers in green arrows) were Sanger sequenced, showing circularization of ECC in these strains (attECC sequences in Table S1). The consensus sequences in Fig. 2 show how the attECC and attR sites contain inverted repeats (red arrows), creating a dyad symmetry characteristic of serine recombinase att sites (26).

ECC elements in enterococcal genomes. In order to evaluate the presence of ECCs in enterococci, 1,478 enterococcal genomes downloaded from NCBI, including three PacBio-sequenced strains in our own collection (UWECC::cat, K59-68, and 9-F-6) were analyzed by BLASTn searches for ccrABEnt. The ccrABEnt genes spread sporadically throughout the Enterococcus genus, as BLAST hits were found in E. faecium, E. faecalis, E. durans, E. hirae, and E. mundtii in addition to five enterococci without species designations (Table 2).

We decided to analyze elements in E. faecium, as they contained the most ccrABEnt-positive strains (Fig. 3A, Table 2, and Table S1). It was of interest to see whether ECC was enriched in specific lineages or environments. As determined by the E. faecium whole-genome sequence (WGS) phylogeny and metadata (shown for complete ECCs in Fig. S2 and Table S1), the ccrABEnt-containing isolates are found in both commensal and nosocomial lineages and originate from both clinical, farm animal, and commensal sampling sites without apparent preference.

Complete ECC elements were identified by two criteria: ccrABEnt located downstream of rlmH and the presence of identifiable attL and attR sites. The ccrABEnt genes
were often located on small contigs and/or near contig ends in many short-read-based assemblies thus impairing analysis of the up- and downstream regions. Thirty-two complete or scaffolded ECC elements with ccrAB\textsubscript{Ent} and both att\textsubscript{L} and att\textsubscript{R} could be identified in \textit{E. faecium}.

**ECC putative att\textsubscript{L} and att\textsubscript{R} sites are conserved 50-bp sequences.** To evaluate \textit{att} site conservation among ECC elements, we searched for \textit{att} sites in ECC-containing strains with BLASTn-short (Table S1) and concatenated all identified \textit{att} site regions into MEME motifs (Fig. 2B). The putative att\textsubscript{L} and att\textsubscript{R} sites consist of 50-bp direct repeats, containing inverted repeats in att\textsubscript{R} and in attECC after ECC excision/circularization (Fig. 2B, red arrows). The \textit{att} sites from \textit{S. aureus} strain N315 (12) were included for

### TABLE 2 Number of enterococcal genomes analyzed and positive for ccrAB\textsubscript{Ent}

| Species             | No. of genomes analyzed | No. of genomes positive for ccrAB\textsubscript{Ent} |
|---------------------|-------------------------|------------------------------------------------------|
| \textit{E. faecium} | 516                     | 69                                                   |
| \textit{E. faecalis} | 677                     | 4                                                    |
| \textit{E. durans}  | 10                      | 4                                                    |
| \textit{E. hirae}   | 34                      | 8                                                    |
| \textit{E. mundtii} | 20                      | 1                                                    |
| Enterococcus sp.    | 221                     | 5                                                    |
| **Total**           | **1,478**               | **91**                                               |
A conserved (5′-TATCATAA-3′) motif in SCCmec is conserved in ECC att sites and is also partly present on the complementary strand (5′-ATGATA-3′) within the inverted repeat in attR and attECC (Fig. 2). According to Wang et al. (12), the only essential nucleotide capable of completely abrogating SCCmec CcrAB function if substituted is the C surrounded by the TAT/ATA palindrome (5′-TATCATAA-3′). This nucleotide was conserved in all ECC attR sites.

To investigate the number of att sites present in each genome, att sites were queries in BLASTn-short analyses. This consistently resulted in less than five hits per genome and att sites most often located near ccrABEnt in circularized genomes. Multiple attR sites could be found in 16 of 32 ECCs (Table S1), as has also been observed in S. aureus strains containing complex SCC elements, see Wang et al. (12) and references therein. One isolate (GCA_000321805/EnGen0001) had ECC on two contigs, of which one spanned both att sites. However, tandem ECCs with multiple ccrABEnt genes were not observed directly.

ECCs are highly variable in gene content. After identifying 32 ECC elements in enterococci, the basal features of size and content were analyzed. The sizes of the ECCs varied from 21 kb to 78 kb (Table S1), with an average of 42 kb. There were on average 42 ORFs in each ECC, and the largest contained 92 ORFs.
A Roary pan-genome analysis was done to evaluate the gene content of ECCs and identified 283 gene clusters. Most genes were present in one ECC or in a few ECCs (Fig. 3A and Table S2). This is also reflected in the core/pan-genome plot (Fig. 3B), which shows a limited number of shared genes (ccrA, ccrB, and insertion gene rlmH).

We hypothesized that the most abundant genes in ECCs were specific to this element and would not be present in strains without ECC. A BLAST database of representative genes clustered in ECC as determined by Roary was created and used as the basis to search for ECC genes among the 516 E. faecium genomes investigated. Interestingly, many of the ECC genes are common in E. faecium genomes, but not necessarily as part of ECCs (Fig. 3C).

The ECC insertion locus rlmH resides in >99% of the enterococcal strains and therefore could serve as an entry point for ECCs in most enterococci. The analyses showed two alleles of this gene with less than 75% DNA identity. Both rlmH1 and rlmH2 contained the attL site. The locations of the 283 ECC genes within circularized genomes (n = 26) were plotted to investigate the locations of these genes in E. faecium genomes relative to rlmH. These genes were found located throughout the whole genome in strains both with and without ECC elements (Fig. S3). This finding either supports that gene synteny conservation in E. faecium is limited or that ECCs may acquire gene cargo with limited conservation with regard to the position in the chromosome. Often, ECC-associated genes are enriched in the vicinity of rlmH, possibly representing ECC remnants or showing that ECCs are prone to engulf neighboring DNA. IS elements and transposases are found in abundance within ECCs and are likely carriers of genetic cargo entering ECCs by composite transposition or by representing homologous recombination sites between IS elements in ECCs and other genomic regions.

**ECC gene content may vary by ecological background.** The gene synteny of the 32 ECCs was assessed via a Mauve alignment. Fifty-four percent of the ECC genes were unique to only one ECC (Fig. 3C and Table S2) and tended to be connected within particular local colinear blocks (LCBs) (Fig. S2), thus representing independent genetic acquisitions. Strain habitat and phylogenetic proximity influence LCB content, as there is more variability between phylogenetic clades than within the clades, and ECCs in isolates from similar origins share more LCBs (Fig. S2). These observations indicate that ECC elements have a role in enterococci similar to that of SCCmec in staphylococci where the surrounding regions have been described as sequence variation “hot spots” (13, 27).

**Notable functions of genes enriched in ECC elements.** Mir-Sanchis et al. (28) characterized conserved hypothetical genes in SCCmec, containing domains with unknown functions DUF 927, DUF 950, DUF 960, and DUF 1643. Among these, DUF 960 (n = 30) and DUF 927 (n = 20) were found in ECCs (Table S2) including ECC::cat, which supports the idea that these ORFs may encode central unknown functions in both SCCmec and ECC. DUF 927 is predicted to encode a helicase, which implies autonomous replication of SCCmec in its circular state.

Genes associated with carbohydrate transport and metabolism were enriched among ECC genes and largely consisted of phosphotransferase systems (PTS) (Table S2). PTS genes associated with increased virulence such as ptsO encoding the PTS IID subunit which has been implicated in improved intestinal colonization during antimicrobial treatment (29) or the bepA gene encoding PTS permease implicated in endocarditis and biofilm formation (30) were not found.

Of interest, many ECC elements contained defense system-related genes (Table S2). Mostly, they were identified as hsdR, hsdS, and hsdM genes, which when all are present, encode a functional EcoKl type I restriction/modification (R/M) system. EcoKl has been observed in staphylococcal SCC elements and is thought to contribute to SCC persistence in its host (16, 31). Another R/M system (SfaNI) has previously been associated with ccrABEnt-positive E. faecalis strain (32), which further suggests that R/M systems are associated with cassette chromosome elements. Incomplete type I R/M systems (n = 13) occur more often than complete ones (n = 8) in ECC elements,
which is surprising given that orphan methylases from type I R/M systems are rarely found (33) and should be inactive without hsdS (34).

One ECC harbors the tetracycline resistance gene tetM (Table S2) as part of a Tn916-like ICE. The reason why we do not see more of this resistance gene in other ECC elements may be that tetM is already present on a Tn916-like element which successfully transfers itself and inserts into random genomic sites with little sequence homology (35).

Alternative mobilization by integration of ECC into the conjugative plasmid. Species in which HGT frequently occur demonstrate “Russian doll”-like dissemination patterns of MGEs, permitting multiple pathways of movement within the cell as well as by HGT (36). Horizontal movement of large segments of chromosomal DNA has previously been shown in enterococci through conjugative plasmid cointegration of chromosomal DNA and subsequent integration into the recipient chromosome by recombination along homologous regions (24, 37).

Strain UWECC::cat contained a novel IS982-family ISEfm1-like element (purple in Fig. 4) within two 10-kb repeats (yellow) up- and downstream of the central region containing ccrABent. This ISEfm1-like element has a size of 2427 bp. As seen in Fig. 4, the region transferred from UWECC::cat into the plasmid was bounded exactly by the two ISEfm1-like copies. Likely, plasmid integration was enabled by homologous recombination between ISEfm1-like elements. This hybrid plasmid was then transferred from strain UWECC::cat to BM4105-RF, resulting in BMpECC::cat, which contains an altered ECC::cat lacking attL.

In one closed chromosome (6E6/GCA_001518735), ccrABent was present but was not located downstream of rlmH. Two attR sites were found downstream of the ccrABent genes, but no attL site was found upstream. There are several IS elements up- and downstream of this ccrABent, which could have contributed to alternative mobility.

The ECC of strain 9-F-6 harbors parts of Tn6085 (38), a Tn916-like ICE, which may allow cotransfer of ECC with Tn916. Cotransfer of GIs by Tn916 has previously been seen for the small GI MTnSag1 in Streptococcus agalactiae (39).

Mobilization of GIs by plasmids and ICEs has previously been shown (40, 41) and is dependent on compatibility between the hitchhiking GI and the conjugative element. Mobilizable GIs either encode a relaxase that is compatible with a type 4 coupling protein (T4CP) of a T4SS expressed by another conjugative MGE, or the T4SS may have
a relaxase recognizing an oriT within the mobilizable GI to enable hitchhiking. Some relaxases show a less strict requirement for the base sequences within oriT sequences and can initiate transfer from a variety of sites (42, 43). The most likely ECC transfer mechanism is recognition of an oriT within the circular ECC by the rep_pLG1 replication and conjugation apparatus.

Alternatively, ECC::cat encodes its own relaxase able to interact with the T4CP of the rep_pLG1 T4SS apparatus. A gene determinant thought to engage in rolling-circle replication (rep) was detected in six of 32 investigated ECC elements, including isolate DO and isolate UW ECC::cat used in the mobilization experiments. This is the same putative replication gene others have associated with ccrABEnt (21, 28).

**Conclusions.** For the first time, SCCmec-like elements have been identified in *Enterococcus*. The novel element was named enterococcus cassette chromosome (ECC) and shared characteristics like the insertion site downstream of rlmH, att site sequences, and variable gene content with SCCmec. We also show mobilization with the help of a conjugative rep_pLG1 megaplasmid.

Cassette chromosome elements had previously been found only in the *Staphylococcus* genus. The existence of a similar element in *Enterococcus* suggests that cassette chromosome elements are more abundant than previously thought. Several resistance genes (toward methicillin, kanamycin, tobramycin, bleomycin, penicillins, heavy metals, tetracycline, macrolide, lincosamide, and streptogramin) have been found in SCCmec (44, 45), but only one ECC harbored a tetracycline resistance gene. Introduction of other clinically important resistance genes in the ECC element such as mecA in SCCmec may result in more spread and stability of this type of element due to antimicrobial selection.

The ECC gene content variability parallel results of Farrugia et al. (46) who found a family of GIs in *Proteobacteria* which were characterized by site-specific insertion in tRNA-dihydrouridine synthase A (*dusA*) by *dusA*-associated integrases (DAIs). The only universal features of these GIs were presence of DAIs and a consensus insertion sequence within the *dusA* gene, while the accessory genes within the GI varied extensively.

On the basis of the genetic contents in the studied ECC elements, we propose that they act as vehicles for exchange of genes in *E. faecium*. In SCCmec typing systems, accessory genes are located in the originally termed “junkyard” or “joining” “regions” (47). Little is known about the accessory genes in SCCmec and what effect they confer to their hosts in various environments. Accessory genes in general often seem to encode functions associated with peripheral functions thought to aid survival of bacterial populations in changing environments (48, 49).

Several others have indicated that genomic islands perform an adaptive evolutionary role for their hosts (50–52). Introduction into new ecological niches may be aided by gene acquisition and loss within these genomic sites. Understanding the underlying dynamics of such events is crucial to understand the evolution of their respective hosts, as well as the stability and dissemination of the individual GI itself.

The enterococci have already been shown to contain a vast array of mobile genetic elements. Here we add another layer of complexity to the *E. faecium* pan-genome through the discovery of an element with a variable gene content. Future endeavors connecting the genes of the mobilome by how they travel between MGEs such as ECC could shine light on the genetic connectivity of a highly recombinogenic species such as *E. faecium*.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth or agar and *E. faecium* in brain heart infusion (BHI) broth or agar at 37°C unless specified otherwise.

The German clinical *E. faecium* ST17 UW1551 (53) was first partially plasmid cured by growth in novobiocin at 45°C overnight. After curing, the strain designated UWΔp showed a different plasmid profile (results not shown) visualized by gel electrophoresis of plasmid DNA isolated by alkaline lysis (54) and had lost resistance to vancomycin, gentamicin, and tetracycline. A chloramphenicol resistance-
encoding gene (cat) was then inserted by a double crossover into an ORF encoding a hypothetical protein immediately downstream of ccrB\textsubscript{ent} using pTEX5501ts (S5), resulting in strain UW\textsubscript{ECC::cat}. The rep\textsubscript{Aec} helper plasmid originating from the clinical E. faecium isolate K60-19 was first mated into isolate BM4105-RF (S6) and from there into UW\textsubscript{ECC::cat}.

**Introduction of chloramphenicol resistance gene downstream of ccrB\textsubscript{ent}** The gene replacement protocol described by Nallapareddy et al. (S5) with minor modifications was used to insert a chloramphenicol acetyltransferase (cat) gene into the open reading frame (ORF1) downstream of ccrB\textsubscript{ent}. In brief, an 822-bp-long upstream region of ORF1 designated ORF1\textsubscript{UpDel} was amplified from genomic DNA from strain UW\textsubscript{Dap} by using the ORF1\textsubscript{UpDel} primers with restriction sites Nhel and HindIII, respectively (Table S3). The PCR product was digested with Nhel and HindIII and ligated to similarly digested pTEX5500\textsubscript{ts}, resulting in pORF1a. Subsequently, an 842-bp-long downstream region of ORF1 designated ORF1\textsubscript{DownDel} was amplified from genomic DNA from strain UW\textsubscript{Dap} by using the ORF1\textsubscript{DownDel} primers with restriction sites NheI and HindIII, respectively. This PCR product was digested with NheI and HindIII and ligated to similarly digested pORF1b, resulting in pORF1, which is pTEX5500\textsubscript{ts} with cloned ORF1 fragments flanking the cat gene. pORF1a and pORF1b were transferred into E. coli TOP10 cells (Invitrogen) for propagation and plasmid purification. pORF1b was introduced into strain UW\textsubscript{Dap} by electroporation to generate an insertion of cat into ORF1. Correct insertion of cat in ORF1 was checked by PCRs using primers for amplification of single and double crossovers (Table S3), by Smal PFGE, Southern hybridization with ccrB\textsubscript{ent} and Cm (cat) probes using protocols described by Sivertsen et al. (S7), and DNA sequencing.

Genomic DNA from E. faecium was purified using the Qiagen genomic DNA kit (Qiagen). PCRs were performed with a Gene Amp PCR system 9700 thermal cycler (Applied Biosystems) using Pfu turbo polymerase (Promega). PCR products were purified using a ZR PCR Clean-up Kit (Zymo Research). Plasmid DNA from E. coli was purified using the ZR Plasmid Prep Miniprep Kit (Zymo Research). Primers are listed in Table S3. Transconjugants were further verified and characterized by the use of Smal and S1 nuclease PFGEs, Southern hybridizations with Dig-labeled probes, and by sequencing.

**Filter mating and verification of transconjugants** The filter mating protocol from Sivertsen et al. (S7) was used with the following antibiotics and concentrations: chloramphenicol (Chl), 30 mg/liter; gentamicin (Gen), 30 mg/liter; rifampin (Rif), 20 mg/liter; fusidic acid (Fus), 10 mg/liter. For schematic presentation of experiments and which elements were transferred, see Fig. S1 in the supplemental material. All experiments were done using BHI agar. The presence of ccrB\textsubscript{ent} in strains was determined by primers FB and RB from Bjerken et al. detecting ccrB (21). The presence of the rep\textsubscript{Aec} plasmid was determined by primers aac(6')-Ie-aph(2')-la F and R detecting the LGLR determinant (S4). PCRs specific to strains UW\textsubscript{ECC::cat} and BM4105-RF were designed by identifying genes unique to each genome through Roary (S8) comparisons. Primers are listed in Table S3. Transconjugants were further verified and characterized by the use of Smal and S1 nuclease PFGEs, Southern hybridizations with Dig-labeled ccrB\textsubscript{ent} and aac(6')-Ie-aph(2')-la probes.

**Genome sequencing** Experimental isolates were cultured on blood agar overnight and a single colony was transferred to BHI broth and grown overnight. Genomic DNA (gDNA) was extracted using the Promega Wizard genomic DNA purification kit with the addition of 30 U mutanolysin in the lysis step. gDNA was sent to the Norwegian Sequencing Centre (NSC) (University of Oslo) where the 20-kb library preparation protocol and 6-kb cutoff BluePippin (Sage Sciences) size selection were done and sequenced with the Pacific Biosciences HiPi sequencer using P6-C4 chemistry, 360-min movie time, and one SNP per sample. Illumina sequencing was performed at the Genomics Support Center Tromsø, with Nextera 500 Illumina technology. For Oxford Nanopore sequencing, gDNA was purified using the QIAquick DNA purification kit (Qiagen) using standard settings. Phylogeny of E. faecium was produced by constructing a whole-genome alignment using Parsnp v.2.1.8 (70). Consensus motifs for att sites were produced at the

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**Bioinformatic analyses** Reads from Pac-Bio sequencing were assembled and polished at NSC using the HGAP v3 (Pacific Biosciences, SMRT Analysis Software v2.3.0) software (S9). Unitigs were circularized by Minimus2 from the AMOS package (60), and dnaA (chromosome) or repA (plasmid) genes were set at the first nucleotide positions of unitigs using the circulator software (61), as well as closed with PCRs (data not shown). BM4105-RF Illumina and Nanopore data were combined in Unicycler v0.4.4 (62) and polished with Pilon v1.22 (63) after initial nanopore base calling with albacore v2.1.7, standard trimming by porechop v0.2.3 (https://github.com/nvick/Porechop), removal of reads of <2 kb, downsampling to 1 gb, and Illumina data adaptor trimming and quality trimming (Q > 28) with Trim Galore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Nanopore reads were then mapped to the circularized genome using minimap2 (64) and processed by samtools (65) to confirm uniform coverage. All E. faecium and E. faecalis genome assemblies available as of June 2016 and other enterococcal genomes available as of May 2018 (n = 1,478) were downloaded from NCBI. Searches for ccrB\textsubscript{ent} (UniProt accession nos. Q3Y3B0 and Q3Y3B1) were done with BLASTn and BLASTp. Perfect and imperfect repeats were identified using the NCUMer (v3.1) software (66) with a window size of 20 nt. Searches for att sites in enterococcal sequences were done with BLASTn-short. Pairwise alignment figures were created with EasyFig v2.2.2. All ECC elements and novel genome sequences were annotated using prokka v1.11 (67) and further manual curation was done with BLASTp results. Transfer of ccrB\textsubscript{ent}-containing elements and surrounding regions were manually inspected in Artemis and Artemis Comparison Tool (68). ProgressiveMauve (69) from Mauve v.2.3.1 with standard settings was used to find common local colinear blocks and produce an alignment figure of ccrB\textsubscript{ent} elements. Gene clustering of ccrB\textsubscript{ent} elements were done with Roary (S8) using standard settings. Phylogenetic trees were constructed using the whole-genome alignment using Parsnp v.2.1.8 (70). Consensus motifs for att sites were produced at the
MEME webpage (71). Functional annotation of ECC genes was done using the eggNOG mapper (72) and eggNOG database (73). To find ECC genes in E. faecium genomes, a representative gene of each gene cluster as determined by Roary v.3.6.8 was included in a custom nucleotide BLAST database in ABRicate. ECC genes were found in E. faecium genomes with inclusion criteria defined as >75% DNA identity to include functionally equivalent genes and >95% coverage to exclude smaller genes.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00402-18.

TABLE S1. XLSX file, 0.02 MB.
TABLE S2. XLSX file, 0.1 MB.
FIG S1. PDF file, 0.7 MB.
FIG S2. PDF file, 2.3 MB.
FIG S3. PDF file, 0.3 MB.
TABLE S3. PDF file, 0.05 MB.

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