**ccrAB_{Ent}** serine recombinase genes are widely distributed in the *Enterococcus faecium* and *Enterococcus casseliflavus* species groups and are expressed in *E. faecium*

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The presence, distribution and expression of cassette chromosome recombinase (*ccr*) genes, which are homologous to the staphylococcal *ccrAB* genes and are designated *ccrAB_{Ent}* genes, were examined in enterococcal isolates (*n*=421) representing 13 different species. A total of 118 (28 %) isolates were positive for *ccrAB_{Ent}* genes by PCR, and a number of these were confirmed by Southern hybridization with a *ccrA_{Ent}* probe (*n*=76) and partial DNA sequencing of *ccrA_{Ent}* and *ccrB_{Ent}* genes (*n*=38). *ccrB_{Ent}* genes were present in *Enterococcus faecium* (58/216, 27 %), *Enterococcus durans* (31/38, 82 %), *Enterococcus hirae* (27/52, 50 %), *Enterococcus casseliflavus* (1/4, 25 %) and *Enterococcus gallinarum* (1/2, 50 %). In the eight other species tested, including *Enterococcus faecalis* (*n*=94), *ccrAB_{Ent}* genes were not found. Thirty-eight sequenced *ccrAB_{Ent}* genes from five different enterococcal species showed 94–100 % nucleotide sequence identity and linkage PCRs showed heterogeneity in the *ccrAB_{Ent}* flanking chromosomal genes. Expression analysis of *ccrAB_{Ent}* genes from the *E. faecium* DO strain showed constitutive expression as a bicistronic mRNA. The *ccrAB_{Ent}* mRNA levels were lower during log phase than stationary phase in relation to total mRNA. Multilocus sequence typing was performed on 39 isolates. *ccrAB_{Ent}* genes were detected in both hospital-related (10/29, 34 %) and non-hospital (4/10, 40 %) strains of *E. faecium*. Various sequence types were represented by both *ccrAB_{Ent}* positive and negative isolates, suggesting acquisition or loss of *ccrAB_{Ent}* in *E. faecium*. In summary, *ccrAB_{Ent}* genes, potentially involved in genome plasticity, are expressed in *E. faecium* and are widely distributed in the *E. faecium* and *E. casseliflavus* species groups.

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Received 12 May 2010
Revised 27 August 2010
Accepted 1 September 2010

Abbreviations: ATCC, American Type Culture Collection; CDSs, coding sequences; JGI, Joint Genome Institute; MLST, multilocus sequence type; ST, sequence type; SCC, staphylococcal cassette chromosome.

The GenBank/EMBL/DBJ accession numbers for the *ccrAB_{Ent}* gene sequences from *E. faecium* (*n*=14), *E. hirae* (*n*=10 for *ccrA_{Ent}* and 11 for *ccrB_{Ent}*), *E. durans* (*n*=10), *E. gallinarum* (*n*=1) and *E. casseliflavus* (*n*=1) isolates are FJ572967–FJ573039.

A supplementary figure, showing representative real-time PCR results for the expression of five genes, and a supplementary table, showing the domains for Ccr_{Ent} proteins predicted in the Pfam database, are available with the online version of this paper.
INTRODUCTION

The emergence of multidrug-resistant hospital-acquired Enterococcus faecium as one of the most important pathogens in the developed world has been a remarkable development in the last two decades (Leavis et al., 2006; Werner et al., 2003). Molecular epidemiological studies and comparative genomic hybridization analyses of E. faecium (Leavis et al., 2007; Werner et al., 2003) have revealed genotypic differences between hospital and community isolates (Leavis et al., 2006). Mixed whole genome arrays demonstrated a distinct genetic make-up of hospital-associated E. faecium with more than 100 extra genes, possibly acquired by horizontal gene transfer (Leavis et al., 2007). The esp virulence gene, located on a putative pathogenicity island, is one of the determinants acquired by hospital-associated E. faecium. These observations, as well as current multilocus sequence typing (MLST) data, strongly indicate that gene flux and recombination contribute significantly to diversification and adaptation of E. faecium (Leavis et al., 2006; van Schaik et al., 2010).

Recombinases facilitate the exchange of DNA fragments within and between bacteria and are thus pivotal in genome plasticity. Staphylococcal cassette chromosome (SCC) elements are vehicles for exchange of genetic information in staphylococci. These elements are characterized by the presence of terminal inverted repeats and unique recombinase genes, and are flanked by direct repeats (Ito et al., 2001, 2004; Katayama et al., 2003). So far, the major group of elements described are SCCmec I–VIII (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009) responsible for the spread of methicillin resistance between staphylococci. The movement of SCC elements is dependent on the gene products of the cassette chromosome recombinase genes (ccr), either the ccrA–ccrB complex or the single product of ccrC (Katayama et al., 2000; Noto & Archer, 2006). These proteins are serine recombinases of the resolvase/invertase family which integrate the SCC element in a site-specific manner (Ito et al., 1999). To our knowledge, ccr genes have only been reported in staphylococcal species.

Here, we report for the first time to our knowledge, the presence of ccrAB genes in enterococci, hereby designated ccrABEnt and show that they are expressed under standard in vitro growth conditions. Our analyses show that the ccrABEnt genes are widely distributed in Enterococcus species belonging to the E. faecium and Enterococcus casseliflavus species groups.

METHODS

Bacterial isolates. A total of 421 Enterococcus isolates of 13 species from three continents (Europe, USA and Australia) were included in the study; E. faecium (n=216), E. faecalis (n=94), E. durans (n=38), E. hirae (n=52), E. casseliflavus (n=4), E. avium (n=4), E. raffinosus (n=3), E. canadensis (n=2), E. casseliflavus (n=2), E. gallinarum (n=2), E. eceorum (n=2), E. asini (n=1) and E. dispar (n=1). Among the 216 E. faecium isolates, 72 were of human origin of which 58 were clinical isolates. Among the 94 E. faecalis isolates, 13 were of human origin of which eight were clinical isolates. Other enterococcal species included were exclusively of animal origin (poultry, dog, bovine and pig). Six ATCC strains were also included. Isolates used for phylogenetic analyses, MLST and/or PCRs to link ccrABEnt with surrounding genes are displayed in Table 1.

The E. faecium ATCC 19434, E. faecalis ATCC 29212, E. gallinarum ATCC 49608, E. faecalis ATCC 19433 and E. faecalis ATCC 51575 were used as controls in species identification. All species were identified by ddl PCR (Dutka-Malen et al., 1995) or trnA intergenic spacer PCR (Baele et al., 2000).

Detection of ccrABEnt genes in the E. faecium DO genome by in silico analyses. Preliminary sequence data of the E. faecium DO strain were obtained from The Joint Genome Institute (JGI) website at http://genome.jgi-psf.org/mic_home.html (version 08.06.04). Searches for homologous proteins were performed using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/, on 8 February 2010) and FASTA 33 (http://www.ncbi.nlm.nih.gov/fasta33/, on 8 February 2010). Translation of coding sequences (CDSs) into amino acid sequences was done using Expasy proteomic tools (http://au.expasy.org/tools/, on 8 February 2010).

For prediction of CDSs we used ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html, on 8 February 2010), Gene Mark (v2.4) (Besemer & Borodovsky, 1999), FgenesB (http://softberry.com, on 2 August 2010) and ARTEMIS (WELCOME Trust Genome Campus, Hinxton, Cambridge, UK). Pairwise comparison and multiple sequence alignments were performed between the E. faecium CcrABEnt proteins and the previously identified four pairs of Staphylococcus aureus CcrABs (CcrAB1, CcrAB2, CcrAB3, CcrAB4; GenBank accession nos AB033763, D86934, AB037671 and AF411935) and CcrC (GenBank accession no. AB121219). Since ccrB1 and ccrB4 were truncated due to frame shift mutation, 1626 bp (ccrB1) and 1629 bp (ccrB4) ORFs were reconstituted by adding adenine to deleted positions in order to make the alignment better with the Staphylococcus hominis ccr sequence (GenBank accession no. AB063171) which has been fully sequenced (Ito et al., 2001). The comparison of DNA sequences was performed in BioEdit v7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), while multiple alignments were done using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) or T-Coffee (http://www.ebi.ac.uk/Tools/t-coffee/index.html).

The evolutionary relationships of CcrABEnt, Ccr of staphylococci (deduced from ccrA1, ccrA2, ccrA3, ccrA4, ccrB1, ccrB3, ccrB4 and ccrC) and three other site-specific recombinases (site-specific integrase of bacteriophage ϕ-FC1 found in E. faecalis and two site-specific recombinases from Clostridium acetobutylicum ATCC8242) were further investigated. These were included because they have been part of previous similar analyses (Ito et al., 2004) and because the ccrA and ccrB, as well as one of the recombinases from C. acetobutylicum (AE001437; locus tag no. CAC.2247), have been annotated as if they were DNA inverting Pro homologue proteins. The full-length ccrB1 of NCTC10442 and ccrB4 of HDE288 were reconstituted as described earlier (Ito et al., 2004). A neighbour-joining tree was constructed using MEGA3 (Kumar et al., 2004) by creating 2000 bootstrap replicates. Sites with gaps missing data were excluded during analyses. Recombination within the sequenced regions of ccrAEnt and ccrBEnt was determined by phi test (Bruen et al., 2006).

Protein structures were predicted using PSTPRED v2.4 (http://bioinf.cs.ucl.ac.uk/pstpred/, on 17 December 2008) and the determinations of protein superfamilies were done using the HMM library, Genome assignment v1.65 (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/, on 8 February 2010), InterProscan (http://www.ebi.ac.uk/InterProScan/, on 8 February 2010) and Pfam (http://pfam.sanger.ac.uk/, on 8 February 2010). The programs EditSeq and SeqMan (DNASTAR) were used for sequence analysis. To detect repeat sequences, Nucleic Acid Dot Plot
Table 1. *Enterococcus* isolates selected for MLST typing, phylogenetic analyses and/or PCRs to link ccrAB<sub>Ent</sub> with surrounding genes

Type of vancomycin resistance, ST or hospital-related ST (CC17 genogroup), and the presence of ccrAB<sub>Ent</sub> genes is shown. ND, not determined; −, negative.

| Isolate name | Origin country/region | Sample source | Epidemiology* | van type | ST | ccrAB<sub>Ent</sub> | Reference/source |
|--------------|------------------------|---------------|---------------|----------|----|----------------|------------------|
| E. faecium   |                        |               |               |          |    |                |                  |
| C68          | USA/Ohio               | Human faeces  | CI            | vanB     | 16 (CC17) | AB  | Carias et al. (1998) |
| E0470        | Netherlands/Amsterdam  | Human blood   | HO            | vanA     | 16 (CC17) | AB  | Willems et al. (2005) |
| E0734        | Netherlands/Amersfoort | Hospital faeces | HO          | vanA     | 16 (CC17) | AB  | Willems et al. (2005) |
| E0745        | Netherlands/Utrecht    | Human faeces  | HO            | vanA     | 16 (CC17) | AB  | Willems et al. (2005) |
| TUH7-15      | USA                    | Human blood   | HO            | vanB     | 16 (CC17) | AB  | Dahl et al. (1999) |
| E0510        | Australia/Melbourne   | Human blood   | HO            | vanB     | 17 (CC17) | –   | Willems et al. (2005) |
| TUH2-18      | Norway/Bergen          | Human urine   | HO            | vanB     | 17 (CC17) | –   | Dahl et al. (1999) |
| TUH2-19      | Norway/Bergen          | Human wound   | HO            | vanB     | 17 (CC17) | –   | Dahl et al. (1999) |
| TUH7-55      | Germany                | Human urine   | CI            | vanB     | 17 (CC17) | AB  | Dahl et al. (1999) |
| DO (TX0016)  | USA/Houston            | Human blood   | CI            | –        | 18 (CC17) | AB  | Arduino et al. (1994) |
| E1652        | Netherlands/Amersfoort | Human faeces  | HO            | vanA     | 18 (CC17) | –   | Willems et al. (2005) |
| E1406        | Spain/Madrid           | Human blood   | HP            | ND       | 63 (CC17) | –   | T M. Coque/R. Willems |
| E1392        | Great Britain/Centre H | Human         | HP            | ND       | 64 (CC17) | –   | N. Woodford/R. Willems |
| E1181        | Austria/Linz           | Human blood   | HP            | ND       | 78 (CC17) | –   | ENARE/R. Willems |
| E1186        | Germany                | Human blood   | HP            | ND       | 78 (CC17) | –   | ENARE/R. Willems |
| E1321        | Italy/Rome             | Human catheter | HP          | ND       | 78 (CC17) | –   | L. Baldassarri/R. Willems |
| E1644        | Germany/Freiburg       | Human catheter urine | HP | ND      | 78 (CC17) | – | D. Jonas/R. Willems |
| E0333        | Israel/Centre I        | Human blood   | HP            | ND       | 80 (CC17) | –   | R. Schouten/R. Willems |
| E1775        | Belgium                | Pig faeces    | ND            | 121 (CC17) | AB  | E. de Leener/R. Willems |
| E1173        | Portugal/Coimbra       | Human wound   | CI            | vanA     | 125 (CC17) | –   | Willems et al. (2005) |
| E1304        | Portugal/Coimbra       | Human blood   | CI            | vanA     | 132 (CC17) | AB  | Willems et al. (2005) |
| E1762        | Australia/Perth        | Human Hospital survey | ND | 174 (CC17) | – | W. Grubb/R. Willems |
| U0105        | Netherlands            | Human blood   | HP            | ND       | 267 (CC17) | –   | A. Troelstra/R. Willems |
| 3332         | USA/Ohio               | Human         | HO            | vanB     | 308 (CC17) | AB  | Carias et al. (1998) |
| TUH4-65      | USA                    | Human         | CI            | vanB     | 313 (CC17) | –   | Dahl et al. (1999) |
| E0125        | Netherlands/Rotterdam  | Human bile    | CI            | vanA     | 5    | –   | Willems et al. (2005) |
| 399/F98/H2   | Norway/Ostfold         | Human faeces  | CS            | vanA     | 8    | –   | Johnsen et al. (2005) |
| 64/3         | Germany                | Human faeces  | HP            | –        | 21   | –   | Werner et al. (2003) |
| E0073        | Netherlands/Amersfoort | Human faeces  | CS            | vanA     | 22   | –   | Willems et al. (2005) |
| S399/S99/H5  | Norway/Ostfold         | Human faeces  | CS            | –        | 48   | –   | Johnsen et al. (2005) |
| 64/F98/H1    | Norway/Ostfold         | Human faeces  | CS            | vanA     | 48   | –   | Johnsen et al. (2005) |
| E1293        | Italy/Genova           | Human blood   | CI            | –        | 50   | AB  | Willems et al. (2005) |
| E1626        | Netherlands            | Human peritoneal fluid | CI | –      | 92   | –   | Willems et al. (2005) |
| BM4105-RF    | France                 | Human faeces  | Wild strain   | –        | 172  | –   | Poyart & Trieu-Cuot (1994) |
| 399/F99/H8   | Norway/Ostfold         | Human faeces  | CS            | vanA     | 195  | AB  | Johnsen et al. (2005) |
| 64/F99/H6    | Norway/Ostfold         | Human faeces  | CS            | vanA     | 246  | AB  | Johnsen et al. (2005) |
| 399/F99/A10  | Norway/Ostfold         | Animal faeces | CS            | vanA     | 310  | AB  | Johnsen et al. (2005) |
| 399/F98/A1   | Norway/Ostfold         | Animal faeces | CS            | vanA     | 311  | –   | Johnsen et al. (2005) |
| S399/F98/H3  | Norway/Ostfold         | Human faeces  | CS            | –        | 312  | –   | Johnsen et al. (2005) |
| K17a         | Belgium                | Chicken       | ND            | ND       | AB   | P. Butaye |
| K40b         | Belgium                | Chicken       | ND            | ND       | AB   | P. Butaye |
| S399/S99/A4  | Norway/Ostfold         | Animal faeces | CS            | –        | ND   | AB  | Johnsen et al. (2005) |
| S399/F99/A14 | Norway/Ostfold         | Animal faeces | CS            | –        | ND   | AB  | Johnsen et al. (2005) |
| V63b         | Belgium                | Pig           | ND            | ND       | AB   | P. Butaye |
| V128         | Belgium                | Pig           | ND            | ND       | AB   | P. Butaye |
| E. durans    |                        |               |               |          |    |                |                  |
| K101b        | Belgium                | Chicken       | ND            | ND       | AB   | P. Butaye |
| K4a          | Belgium                | Chicken       | ND            | ND       | AB   | P. Butaye |
| K21b         | Belgium                | Chicken       | ND            | ND       | AB   | P. Butaye |
Table 1. cont.

| Isolate name | Origin country/region | Sample source | Epidemiology* type | van | ST | ccrAB\text{Ent} | Reference/source |
|--------------|------------------------|---------------|-------------------|-----|----|----------------|-----------------|
| K70          | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K89          | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K116a        | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K118c        | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K120a        | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K121         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| 96b          | Belgium                | Dog           | ND                | ND  | ND | AB             | P. Butaye        |
| **E. hirae** |                        |               |                   |     |    |                |                 |
| K51b         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K56b         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K66a         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K73a         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K74b         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K79b         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K115b        | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| K141         | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| V70b         | Belgium                | Pig           | ND                | ND  | ND | B              | P. Butaye        |
| V106c        | Belgium                | Pig           | ND                | ND  | ND | B              | P. Butaye        |
| 81a          | Belgium                | Dog           | ND                | ND  | ND | AB             | P. Butaye        |
| **E. casseliflavus** |                |               |                   |     |    |                |                 |
| 86           | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |
| 327          | Belgium                | Chicken       | ND                | ND  | ND | AB             | P. Butaye        |

*CI, Clinical isolate; HO, hospital outbreak; HP, hospitalized patient; CS, community survey. Details are provided for human isolates only.
†Positive for ccrA\text{Ent} only by Southern hybridization.

(http://arbl.cvmbs.colostate.edu/molkit/dnadot/index.html, on 17 June 2010) and the Dotlet database (http://myhits.isb-sib.ch/cgi-bin/dotlet, on 8 February 2010) were used.

DNA extraction, PCR amplification and DNA sequencing. Bacterial DNA extraction for PCR analyses was performed manually by using the InstaGene matrix kit (Bio-Rad) or the GenoM-48 robotic workstation using GenoPrep DNA from blood, standard kit (Genovision). DNA for hybridization purposes was isolated using guanidium isothiocyanate (Dahl & Sundsfjord, 2003).

For long range PCR, 2 U DNA polymerase enzyme rTth XL (Perkin Elmer) was used per reaction and 1.4 mM Mg(OAc)$_2$ in a standard XL PCR mix, or a 0.7 × Pfu Ultra mix (Stratagene) with 2.5 U Pfu Ultra polymerase per reaction. DNA sequencing was performed using BigDye 3.1 technology (Applied Biosystems). Real-time PCR was performed using ABI Prism 7300 real-time PCR system (PE Biosystems) and TaqMan universal mastermix (Applied Biosystems).

Detection of ccrAB\text{Ent} genes and PCR linkage to surrounding genes. ccrAB\text{Ent} genes were detected by PCR, using the primer pairs FA–RA and FB–RB, respectively (Table 2), and genes in selected isolates were detected by Southern hybridization and DNA sequencing. PCRs were also performed on 13 of 14 ccrAB\text{Ent}-positive E. faecium isolates selected for MLST as well as two ccrAB\text{Ent}-positive E. faecium animal isolates from Norway, to search for the presence and conservation of gene synteny in the surrounding genes (Table 2 and Fig. 1a). Primers and probes were designed using E. faecium DO sequences as template.

Expression analysis of ccrAB\text{Ent} genes by real-time quantitative PCR. To analyse if ccrAB\text{Ent} genes are expressed, E. faecium DO was grown aerobically in BHI broth at 37 °C for 18–24 h. Subsequently the culture was diluted 1 : 50 in BHI broth and grown with agitation to OD$_{600}$ 0.7 or to stationary phase (grown overnight). The cell suspension was centrifuged and the cells were immediately frozen on dry ice or liquid nitrogen before adding an RNA stabilizing solution, RNA later (Ambion). Alternatively, RNA later or RNA protect (Qiagen) was added directly to the inoculum, according to the manufacturer’s instructions. RNA extraction was performed by using the RNeasy mini kit (Qiagen) using a prolonged lysis step of 45 min with 10 mg lysozyme and 10 U mutanolysin in a total volume of 100 μl. On-column DNase treatment was performed according to the manufacturer’s instructions. A successive removal of DNA was performed using Turbo DNase (Ambion) according to the manufacturer’s instructions. RNA integrity was determined by agarose gel electrophoresis. Reverse transcription of the total RNA was performed using the ABRT1 primer and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) or Superscript III Rnase H-reverse transcriptase (Invitrogen). Real-time PCR was performed on the cDNA using primers ccrAFre, ccrARre, ccrBFre, ccrBRre, recAFre, recARre, pbp3Fre, pbp3Rre, adkFre and adkRre, and probes ccrAEnt, ccrBEnt, recA, pbp5 and adk (Table 2). Expression of ccrAB\text{Ent} genes was compared with the expression of recA, pbp5 and adk. Ten-fold serial dilutions of E. faecium DO genomic DNA were used to make standard curves to determine PCR efficiency, using the equation: 

$$E = 10^{-\frac{1}{10 \text{(slope)}}} - 1.$$ 

The PCR efficiencies ranged from 88 to 104 % in one assay and 99 to 100 % in a second assay and were considered similar enough to be able to compare only Ct (threshold cycle) values for a semiquantitative relative measurement of expression. The expression experiments were
performed in three triplicates; a no template control (NTC) and a minus reverse transcriptase control (−RT) was included in each assay. The −RT controls were in the range of an acceptable difference from the cDNA expression analysis (ΔCt difference).

### Analysis of ccrAB<sub>Ent</sub> mRNA linkage by RT-PCR.
RNA isolation was performed as described above. RNA was treated with the DNA-free kit (Ambion). Reverse transcription of total RNA was performed with SuperScript III reverse transcriptase (Invitrogen) using primers CcrBRTR1 or CcrBxR. RT-PCR without reverse transcriptase was performed on total RNA to check for DNA contamination. Linkage of ccrA<sub>Ent</sub> and ccrB<sub>Ent</sub> mRNAs as a bicistronic mRNA was analysed by PCRs on cDNAs using primers located in ccrA<sub>Ent</sub> (CcrARTR1 and CcrARre) and ccrB<sub>Ent</sub> (ccrBRTR1 and CcrBxR) (Fig. 1b and Table 2).

### Southern blot hybridization analyses.
RFLP with XbaI (Promega) was performed on total genomic DNA for selected E. faecium isolates (DO, TUH7-55, E0470, E0745, E1304 and E1293). PFGE of SmaI-digested DNA from 76 E. faecium isolates was performed as described by Dahl et al. (1999). DNA fragments separated by gel electrophoresis were transferred to a positively charged nylon membrane (Boehringer) by vacuum blotting using a Vacugene XL system (Amersham Biosciences). Southern blot hybridization was performed with a DIG-labelled (Boehringer) ccrA<sub>Ent</sub> probe based on E. faecium DO.
RESULTS AND DISCUSSION

**ccrAB<sub>Ent</sub> sequences in the E. faecium DO genome**

Genes similar to the **ccrA** and **ccrB** genes of *S. aureus* (GenBank accession no. D86934) were identified in the draft sequence of *E. faecium* DO (Katayama et al., 2000). BLAST searches indicated two CDSs (locus tag nos 2319 and 2398) in *E. faecium* DO contig 655 (version 08.06.04) similar and in an identical order to the staphylococcal **ccrA** and **ccrB**. They were named **ccrA<sub>Ent</sub>** and **ccrB<sub>Ent</sub>**. No available reports have previously shown **ccrA<sub>Ent</sub>** and **ccrB<sub>Ent</sub>** genes in enterococci. The **ccrA<sub>Ent</sub>** and **ccrB<sub>Ent</sub>** CDSs are 1374 bp and 1638 bp in size, respectively. The two **ccrB<sub>Ent</sub>** genes in *E. faecium* DO were similar in length to the staphylococcal **ccrB<sub>2</sub>** (Katayama et al., 2000).

The **ccrA<sub>Ent</sub>** gene synteny was confirmed to be the same as in staphylococci (Katayama et al., 2000) for 14 of 15 *E. faecium* isolates by linkage PCR (Table 3). No available results have previously shown whether staphylococcal **ccrA** and **ccrB** genes are transcribed as separate units or as a bicistronic mRNA. RT-PCR analysis of total RNA from *E. faecium* DO revealed that the **ccrA<sub>Ent</sub>** genes were transcribed as a bicistronic mRNA, confirming the bioinformatics results. Knowing the function of **ccrAB** in staphylococci, we hypothesize that **ccrA<sub>Ent</sub>** genes in enterococci might be part of a larger integrative genetic element in *E. faecium*. The GC content of *E. faecium* DO contig 655 (35%), the **ccrA<sub>Ent</sub>** CDSs (35%) and the whole genome (38%) is not substantially different. No putative termini (repeats) were identified in contig 655 by nucleic acid dot plot or DotLet analyses. Thus it was not possible to identify a putative integrative element. The genome sequence of contig 655 is limited to the **tnp** transposase determinant (Fig. 1a) at the left side and it has not been possible to identify the continuation of this sequence in another DO contig. The sequence at the other side of **ccrA<sub>Ent</sub>** also contains a lot of putative transposases (belonging to several insertion sequence families) in addition to hypothetical proteins (http://maple.lsd.ornl.gov/cgi-bin/JGI_microbial/contig_viewer.cgi?org=efae&chr =08jun04&contig=Contig655&sort=left_bp, on 21 June 2010) which may well be part of an integrative element.

Pairwise comparison and multiple sequence alignments were performed between the *E. faecium* **ccrA<sub>Ent</sub>** proteins and the **Ccr** proteins of *S. aureus*. The similarities of **CcrA** and **CcrB** between *E. faecium* and *S. aureus* N315 were 55 and 69%, respectively. The N-terminal resolvase and recombinase domains, as well as the predicted catalytic serine residue of the recombinase active site were highly conserved between the *Staphylococcus* and *Enterococcus* CcrAB proteins. Moreover, the *Enterococcus* **CcrB<sub>Ent</sub>** was predicted to include an Ogr/delta-like domain (a phage transcription activator). Two algorithms, Pfam and ProScan, predicted both the resolvase and recombinase domains in the examined Ccr protein sequences (Supplementary Table S1, available with the online version of this paper).

The evolutionary relationships of **CcrA<sub>Ent</sub>**, **Ccr** of staphylococci and three other site-specific recombinases were further investigated. The phylogenetic analyses revealed an evolutionary relationship between **CcrA<sub>Ent</sub>** and **CcrB<sub>Ent</sub>** from enterococci and the staphylococcal CcrAB cluster (Fig. 2). However, the low identity score between the enterococcal and staphylococcal proteins does not support a recent horizontal transfer of the **ccr** genes between these species.

**ccrA<sub>Ent</sub>** genes are expressed in *E. faecium*

Analyses of **ccrA<sub>Ent</sub>** gene expression were performed during both the exponential and stationary phase of *E. faecium* DO grown in rich medium. Both genes were expressed in approximately the same amounts in exponential phase. **ccrA<sub>Ent</sub>** genes were expressed >70-fold lower than the **rpa5**, **reoA** and **adk** genes (Supplementary Table S1).
Table 3. Long-range linkage PCR results for the \( \text{ccrA}_{\text{Ent}} \) and \( \text{ccrB}_{\text{Ent}} \) chromosomal region among 15 \( \text{ccrAB}_{\text{Ent}} \)-positive \( E. \text{faecium} \) isolates

+ , Positive; − , negative; NA, not applicable (one of the genes/CDSs not present); ND, not determined.

| Isolate | ST* | \( \text{ccrA}_{\text{Ent}}-\text{REP factor} \) | \( \text{ccrB}_{\text{Ent}}-\text{ccrA}_{\text{Ent}} \) | orf1–\( \text{ccrB}_{\text{Ent}} \) | \( \text{tnp–orf1} \) |
|---------|-----|------------------------------------------------|---------------------------------|----------------|----------------|
| DO      | 18  | ND                                            | +                               | +              | +              |
| E1304   | 132 | +                                             | +                               | +              | +              |
| TUH7-55 | 17  | +                                             | +                               | +              | +              |
| 3332    | 308 | +                                             | +                               | ND             | ND             |
| C68     | 16  | +                                             | +                               | +              | +              |
| E0470   | 16  | +                                             | +                               | +              | +              |
| E0734   | 16  | +                                             | +                               | +              | +              |
| E0745   | 16  | +                                             | +                               | +              | +              |
| TUH7-15 | 16  | +                                             | +                               | +              | +              |
| 64/F99/H6 | 48  | −                                             | −                               | −              | −              |
| 399/F99/A10 | 310 | −                                             | −                               | −              | −              |
| 399/F99/H8 | 195 | −                                             | −                               | −              | −              |
| E1293   | 50  | NA                                            | ND                              | ND             | ND             |
| S399/F99/A14 | ND  | −                                             | −                               | −              | −              |
| S399/S99/A4 | ND  | −                                             | −                               | −              | −              |

* STs in bold belong to the CC17 genogroup.

Fig. S1). The mRNA abundance of \( \text{ccrAB}_{\text{Ent}} \) was lower in stationary phase than in exponential phase.

**\( \text{ccrAB}_{\text{Ent}} \) genes are dispersed among Enterococcus species belonging to the \( E. \text{faecium} \) and \( E. \text{casseliflavus} \) species groups**

Of a total of 421 enterococcal isolates, 118 (28%) were positive for \( \text{ccrAB}_{\text{Ent}} \) genes in five species by PCR: \( E. \text{faecium} \) (58/216, 27%), \( E. \text{durans} \) (31/38, 82%), \( E. \text{hirae} \) (27/52, 50%), \( E. \text{casseliflavus} \) (1/4, 25%) and \( E. \text{gallinarum} \) (1/2, 50%) (Table 1). One \( E. \text{hirae} \) isolate was positive by PCR for \( \text{ccrB}_{\text{Ent}} \) only. Eight other species including \( E. \text{faecalis} \) were negative for \( \text{ccrAB}_{\text{Ent}} \) (data not shown).

A BLAST search for the \( \text{ccrAB}_{\text{Ent}} \) genes and the surrounding regions against Enterococcus strains revealed the presence of \( \text{ccrAB}_{\text{Ent}} \) in \( E. \text{faecium} \) E1071, 1,231,408 and C68 (http://www.ncbi.nlm.nih.gov/genomes/geblast.cgi?gi=6512#SearchSet, on 21 June 2010) and no \( \text{ccrAB}_{\text{Ent}} \) sequence or protein matches with high identity scores in other available Enterococcus genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi, on 21 June 2010). \( E. \text{faecium} \) E1071 and \( E. \text{faecium} \) 1,231,408 showed sequence similarity with the DO sequence in parts of the hypothetical protein, \( \text{ccrB}_{\text{Ent}} \) and parts of \( \text{ccrA}_{\text{Ent}} \). \( E. \text{faecium} \) C68 showed similarity with DO in parts of the hypothetical protein, both \( \text{ccrA}_{\text{Ent}} \) and \( \text{ccrB}_{\text{Ent}} \) and parts of the replication initiation factor (REP factor).

\( \text{ccrAB}_{\text{Ent}} \) gene sequences (GenBank accession nos FJ572967–FJ573039) from \( E. \text{faecium} \) (n=14), \( E. \text{hirae} \) (n=10 for \( \text{ccrA}_{\text{Ent}} \) and 11 for \( \text{ccrB}_{\text{Ent}} \)), \( E. \text{durans} \) (n=10), \( E. \text{gallinarum} \) (n=1) and \( E. \text{casseliflavus} \) (n=1) isolates were aligned and a neighbour-joining phylogenetic tree was made with 2000 bootstrap replicates using the P-distance model (Fig. 3). The \( \text{ccrAB}_{\text{Ent}} \) genes both clustered into two major clades represented by the majority of \( E. \text{faecium} \) (clade I) and \( E. \text{hirae} \) (clade II) isolates, respectively. With 7 of 10 isolates clustering in clade II, \( E. \text{hirae} \) appears to be slightly more dispersed between the two \( \text{ccrA}_{\text{Ent}} \) clades. \( \text{ccrAB}_{\text{Ent}} \) from the \( E. \text{gallinarum} \) and \( E. \text{casseliflavus} \) isolates clustered in clade II with the majority of \( \text{ccrAB}_{\text{Ent}} \) from the \( E. \text{hirae} \) isolates. In \( E. \text{durans} \), 6 of 10 \( \text{ccrA}_{\text{Ent}} \) genes clustered in clade I, while 7 of 10 \( \text{ccrB}_{\text{Ent}} \) clusters were in clade II. Except for \( \text{ccrA}_{\text{Ent}} \) from \( E. \text{faecium} \) E1304, the \( \text{ccrAB}_{\text{Ent}} \) genes of the human isolates clustered in clade I whereas the animal isolates were found in both clades. Incongruence between \( \text{ccrA}_{\text{Ent}} \) and \( \text{ccrB}_{\text{Ent}} \) phylogenies within an isolate was noted for 11 isolates, all of animal origin (Fig. 3, isolates marked with asterisks). Phi tests revealed no statistically significant evidence for recombination within the sequenced regions of the \( \text{ccrA}_{\text{Ent}} \) and \( \text{ccrB}_{\text{Ent}} \) genes. However, the incongruence suggests recombination of the \( \text{ccrA}_{\text{Ent}} \) genes outside the sequenced regions of the two genes. Incongruence between these genes has also been seen for \( S. \text{aureus} \) (Ito et al., 2004).

\( \text{ccrAB}_{\text{Ent}} \) genes were only found in isolates belonging to the \( E. \text{faecium} \) and \( E. \text{casseliflavus} \) species groups that belong to the same tree branch in phylogenetic trees based on enterococcal 16S and \( \text{sodA} \) gene diversity (Devriese et al., 1993; Poyart et al., 2000). The absence of \( \text{ccrAB}_{\text{Ent}} \) in the other species could be explained by the low number of isolates tested, except for \( E. \text{faecalis} \), or by a lack of integration sites recognized by \( \text{ccrAB}_{\text{Ent}} \) in the
strains not belonging to the *E. faecium* or *E. casseliflavus* groups. Alternatively, their *ccrAB* genes may exhibit such a low sequence identity to the *ccrAB* genes identified in this study that they are missed using the PCR and hybridization conditions used in the present study.

**Variations of the *ccrAB* genes and the surrounding region between selected *E. faecium* isolates**

PFGE analysis and Southern hybridization of 76 *E. faecium* isolates with the *ccrA* probe confirmed the PCR results. One *ccrA* PCR-negative strain (399/F98/A1) was *ccrA*-positive by Southern blot hybridization (data not shown) indicating that sequence diversity affects PCR amplification. Also, *Xba*I analyses of *ccrA* and *ccrB* genomic regions revealed heterogeneity and only one copy of *ccrA*. The *ccrA* probe hybridized to an approximately 10 kb fragment in DO, TUH7-55, E1304 and E1293 isolates; however, the *ccrA*-positive fragment of E0470 and E0745 was approximately 24 kb (data not shown). To investigate this in more detail, the presence of *ccrAB*-flanking genomic genes identified in the DO genome was determined by multiple PCRs in 15 *ccrAB*-positive and 16 *ccrAB*-negative isolates (Fig. 1a). Examinations of the *ccrAB* surrounding region in several isolates showed a variable pattern of the *ccrAB* flanking sequences with hospital-associated isolates showing most sequence similarity with the DO sequence (Table 3). All 31 isolates were positive for the *tnp* gene-specific PCR (*tnp* belongs to the IS30 family) as well as for orf1 PCR and three *ccrAB*-positive isolates of different sequence types (STs) were also positive for the REP factor gene PCR. This REP factor gene harbours a REP_trans domain belonging to superfamily pfam02486. This family represents probable topoisomerases that makes a sequence-specific single stranded nick in the origin of replication. Plasmid REPs, phage REPs (RstAs) and transposon REPs (Cro/CI transcriptional regulators) belong to this family. Long-range PCRs confirmed linkage of these genes with *ccrAB* and conservation of gene synteny surrounding *ccrAB* with the exception of isolates 64/F99/H6, 399/F99/A10, 399/F99/H8, and S399/F99/A14, for which linkage of *tnp*-orf1 and orf1–*ccrB* was not confirmed. Furthermore, a *ccrB*–*ccrA* linkage was not shown in 64/F99/H6 (Table 3 and Fig. 1a). The inability to link genes that were positive on gene-specific PCRs may indicate that the region between these genes is larger than expected or that the specific genes are located at other regions in the genome. The transposase of the IS30 family is, for instance, located at more than one site in *E. faecium* DO. Annotation of contig 655 (http://maple.lsd.ornl.gov/cgi-bin/JG1_microbial/contig_viewer.cgi?org=eaec&chr=08jun04&contig=Contig655&sort=left_bp, on 21 June 2010) also indicates that the *ccrAB* genes are located in a region containing several transposases. The
regions surrounding ccrAB in staphylococci contain highly variable genes encoding ORFs of unknown functions. These variable regions are called J1 and J2, and variations in these regions are used to define the SCCmec subtypes (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009), and so our results from enterococci are in line with these observations of highly variable regions surrounding ccrAB in staphylococci. CcrA and CcrB have roles in the excision and integration of SCCmec in staphylococci (Wang & Archer, 2010) and we have showed that the ccrAB Ent genes are expressed in E. faecium DO. It has been postulated that SCCmec may carry the genes conferring methicillin resistance but may also enable genetic exchange of other genes among staphylococcal species (Katayama et al., 2003). However, to our knowledge, no studies have provided direct experimental evidence for intercellular transfer of SCC between staphylococci.

DNA sequencing of the ccrABEnt, trp and orfI of the 15 ccrABEnt-positive isolates showed 94–100% and 96–100% sequence identity in ccrAEnt and ccrBEnt genes (GenBank accession nos FJ572967–FJ573039). Upper case letters in parentheses represent the origin of the isolate: (C), chicken; (H), human; (D), dog; (P), pig; and (A), unknown animal origin. Outgroups are represented by S. aureus N315 ccrA and ccrB (GenBank accession no. D86934). The asterisks indicate isolates in which ccrAEnt and ccrBEnt belong to different clades. All sequences were aligned using CLUSTAL W. The neighbour-joining phylogenetic tree was made with MEGA4.0 using 2000 bootstrap replicates and the P-distance model. Bootstrap values higher than 80% are shown at the branches. The scale bar indicates genetic distance in substitutions per site. The two main clades of ccrAEnt and ccrBEnt are indicated. The dataset consisted of 547 nt with 484 parsimony-informative sites for ccrAEnt and 513 nt with 227 parsimony-informative sites for ccrBEnt.

Fig. 3. Phylogram for ccrAEnt and ccrBEnt genes. The nucleotide sequences for ccrAEnt and ccrBEnt genes from E. faecium (n = 15), E. hirae (n = 10/11), E. durans (n = 10), E. casseliflavus (n = 1) and E. gallinarum (n = 1) were used (GenBank accession nos FJ572967–FJ573039). Upper case letters in parentheses represent the origin of the isolate: (C), chicken; (H), human; (D), dog; (P), pig; and (A), unknown animal origin. Outgroups are represented by S. aureus N315 ccrA and ccrB (GenBank accession no. D86934). The asterisks indicate isolates in which ccrAEnt and ccrBEnt belong to different clades. All sequences were aligned using CLUSTAL W. The neighbour-joining phylogenetic tree was made with MEGA4.0 using 2000 bootstrap replicates and the P-distance model. Bootstrap values higher than 80% are shown at the branches. The scale bar indicates genetic distance in substitutions per site. The two main clades of ccrAEnt and ccrBEnt are indicated. The dataset consisted of 547 nt with 484 parsimony-informative sites for ccrAEnt and 513 nt with 227 parsimony-informative sites for ccrBEnt.
Investigation of possible association between ccrAB_{Ent} and ST within *E. faecium* of human origin

MLST analyses of *E. faecium* isolates (n=39) revealed that the ccrAB_{Ent} genes are dispersed among different STs (Table 1). Ten of 29 (34%) hospital-related *E. faecium* isolates were ccrAB_{Ent}-positive, while 4 of 10 (40%) non-hospital-related isolates were positive. Furthermore, specific STs within hospital-related strains were represented by both ccrAB_{Ent}-positive and -negative isolates (Table 1), suggesting that ccrAB_{Ent} genes are acquired and not a part of the core genome.

Concluding remarks

Cassette chromosome recombinases may be important in recombination and genome plasticity in enterococci. Expression analyses indicate that the recombinase genes are active in *E. faecium* DO and thus, may play a role in the recombination or movement of genetic elements. Further investigation of the ccrA_{Ent} and ccrB_{Ent} will be essential to reveal the contribution of these genes for recombination and mobilization events in enterococci.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Norwegian Research Council (projects no. 165997 and 183653/519), Northern Norway regional Health Authority Medical Research Program and the University of Tromsø. This work was supported by research grants from the Norwegian Research Council (projects no. 165997 and 183653/S19), Northern Norway regional Health Authority Medical Research Program and the University of Tromsø. Furthermore, specific STs within hospital-related strains were represented by both ccrAB_{Ent}-positive and -negative isolates (Table 1), suggesting that ccrAB_{Ent} genes are acquired and not a part of the core genome.

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Edited by: K. E. Weaver