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ccfA, the Genetic Determinant for the cCF10 Peptide Pheromone in *Enterococcus faecalis* OG1RF

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The nosocomial pathogen *Enterococcus faecalis* has a unique pheromone-inducible conjugative mating system. Conjugative transfer of the *E. faecalis* plasmid pCF10 is specifically induced by the cCF10 peptide pheromone (LVTLVFV). Genomic sequence information has recently allowed the identification of putative structural genes coding for the various enterococcal pheromones (D. B. Clewell et al., Mol. Microbiol. 35:246–247, 2000). The cCF10 pheromone sequence LVTLVFV was found within an open reading frame designated *ccfA*, encoding a putative lipoprotein precursor. Several other pheromone sequences were found in similar locations within other predicted lipoproteins. CcfA shows significant sequence relatedness to the *Escherichia coli* protein YidC, an inner membrane protein translocase, as well as to a large number of homologs identified in gram-positive and in gram-negative bacteria. Analysis of the deduced CcfA amino acid sequence suggested that mature cCF10 peptide could be formed from the proteolytic degradation of its signal peptide. Expression of the cloned *ccfA* gene with an inducible expression vector dramatically increased cCF10 production by *E. faecalis* and also resulted in cCF10 production by *Lactococcus lactis*, a non-pheromone producer. Site-directed mutagenesis of the *ccfA* sequence encoding the cCF10 peptide confirmed that *ccfA* was a functional genetic determinant for cCF10.

The medical importance of the nosocomial pathogen *Enterococcus faecalis* is in large part due to its remarkable ability to efficiently transfer genetic determinants (31). One extensively studied enterococcal mobile genetic element is the tetracycline resistance plasmid pCF10 (15). Comparative studies of enterococcal clinical isolates collected over 15 years from the same hospital suggest a possible scenario in which endemic pCF10-related plasmids selectively acquired different resistance determinants as they transferred horizontally among the enterococci in the hospital environment (15, 21).

pCF10 is one of numerous conjugative plasmids whose transfer is mediated by peptide pheromones excreted by recipient cells lacking the plasmid. Pheromone-induced conjugation is a highly specific process, dependent not only on the specialized plasmid-encoded transfer machinery, but also upon the distinct peptide sequence of the cognate hepta- or octapeptide pheromone (12, 17). These hydrophobic peptides are excreted into the culture supernatants in small amounts and can display biological activity at concentrations as low as $<5 \times 10^{-12}$ M (29).

One to five molecules of exogenously supplied cCF10 (LVTLVFV) are sufficient to specifically induce the conjugative transfer of pCF10 from a donor cell (29). The genetic determinant of cCF10 is chromosomal, and donor cells containing the pCF10 plasmid possess an intricate regulatory system composed of plasmid-encoded proteins to prevent self-induction of conjugation, since they are still capable of pheromone production (10).

It has been over 15 years since the amino acid sequences of the first enterococcal pheromones were reported (30, 33), yet their biosynthesis has remained a mystery. An obvious possibility is that the mature pheromones are derived from proteolytic processing of larger polypeptides. Support for this notion comes from physiological and genetic analysis of cCF10 production and control of endogenous pheromone by cells carrying pCF10 (10). In addition, An et al. (3) have identified a genetic determinant, *eep*, encoding a putative membrane peptidase which seems to enhance production of several enterococcal peptide pheromones.

Previous attempts to identify the pheromone genetic determinants have been unsuccessful due to the excessive probe degeneracy resulting from the high sequence homologies among the various peptides. Recently, the *E. faecalis* genome sequencing projects allowed the identification of putative genetic determinants of the peptide pheromones (13). In this study, we describe experimental evidence indicating that *ccfA* is the functional genetic determinant of peptide pheromone cCF10.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani (LB) broth (Gibco-BRL). *E. faecalis* strains were grown in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) or in M9-YE medium (18). *Lactococcus lactis* strains were grown in GM17-glucose medium (34).

Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 200 μg/ml for *E. coli* and 20 μg/ml for *E. faecalis* and *L. lactis*; fusidic acid, 25 μg/ml; rifampin, 25 μg/ml; and tetracycline, 10 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Gibco-BRL, Inc., Rockville, Md.) was used at a concentration of 100 μg/ml. Nisin (Sigma Chemical Co., St. Louis, Mo.) was used at a concentration of 25 ng/ml. Restriction enzymes used were purchased from Promega (Madison, Wis.) and Gibco-BRL (Rockville, Md.). Reactions were done as recommended by the manufacturer. Synthetic cCF10 (LVTLVFV) and its peptide analog (LATLVFV) were prepared by the microchemical facility at the University of Minnesota.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Enterococcus faecalis OG1RF	Wild type, Rif^r Fus^r	15
Lactococcus lactis LM2301	Nonproducer of cCF10	28
Escherichia coli DH5α		Gibco-BRL
Plasmids		
pGEM-T-Easy	Amp^r *lacZ*, cloning vector	Promega
PMSP3535	Erm^r *nisA nisR nisK*	9
pGEMmha	Amp^r *lacZ*, contains 880-bp *ccfA* fragment produced from PCR in pGEM-T-Easy vector	This study
pGEMLAT	Amp^r *lacZ*, contains 880-bp *ccfA* mutated fragment produced by PCR-based site-directed mutagenesis in pGEM-T-Easy vector	This study
pMHAnis	Erm^r, contains *Pst*I/*Sal*I *ccfA* fragment in pMSP3535	This study
pLAT	Erm^r, contains *Pst*I/*Sal*I *ccfA* mutated fragment in pMSP3535	This study
pCF10	Tet^r, conjugative plasmid	15

Detection of cCF10 activity. Clumping assays for the detection and determination of *ccfA*-related pheromone activity, including culture supernatants, were done as described previously (10). cCF10 activity in culture filtrates was confirmed by high-pressure liquid chromatography (HPLC) analysis as described previously (10). The elution time of the active fractions was compared with that of synthetic peptide.

General DNA techniques. Plasmid DNA from *E. coli* was prepared with a miniprep kit (Qiagen). Plasmid DNA from *E. faecalis* and *L. lactis* was prepared as described earlier (23, 26). Plasmid DNA was analyzed by restriction enzyme digestion on 0.9% Tris–borate–EDTA–agarose gels. Plasmid DNA was introduced into cells by electroporation (16). For Southern hybridization, *E. faecalis* OG1RF chromosomal DNA was digested with *Eco*RI, electrophoresed through agarose gels, and transferred to a positively charged nylon membrane (Boehringer, Mannheim, Germany) with a Genie apparatus (Idea Scientific Co., Minneapolis, Minn.). Digoxigenin-labeled probes were prepared and hybridized DNA was detected as specified in the user's guide (Boehringer).

PCR was done with a Perkin-Elmer apparatus under the conditions recommended by the manufacturer. Oligonucleotide primers were synthesized by the microchemical facility at the University of Minnesota.

To amplify the *ccfA* gene, the primers used were 6221 EcoF (5'-GCTAGAATTCTAGATGTAAGAGAGGGA-3'; incorporated *Eco*RI site underlined) and 6221 XhoR (5'-GTAGCTCGAGCATAAAATCGGCATCTGA-3'; incorporated *Xho*I site underlined). *E. faecalis* OG1RF chromosomal DNA served as the template for PCR amplification of *ccfA*. The DNA polymerase used in the amplification reaction was Bio-X-Act (Midwest Scientific, St. Louis, Mo.), which allowed the incorporation of A overhangs, facilitating the subsequent cloning of the PCR product in the pGEM-T-Easy cloning vector (Promega, Madison, Wis.); this resulted in pGEMmha.

Site-directed mutagenesis was done using the megaprimer method described by Barik (5), incorporating the following primers: 5'-TTAGGTCGCTAACCCAGCCCA-3' (LATR), 5'-GTTTTCCCAGTCACGAC-3' (M4), and 5'-CAGGAAACAGCTATGAC-3' (RV). Using pGEMmha as the template and PfuTurbo (Stratagene, La Jolla, Calif.) DNA polymerase, the first round of PCR was done using primers M4 and LATR, yielding the LAT megaprimer. This megaprimer was purified according to the methods of Barik (5) and then used in the second round of PCR along with the RV primer to yield the final PCR product containing the desired V14A mutation. Since PfuTurbo DNA polymerase lacks terminal deoxynucleotidyltransferase activity, a blunt-ended PCR product resulted.

In order to produce the A overhangs, the PCR product was subjected to an A-tailing protocol described by the manufacturer (Stratagene, La Jolla, Calif.). The reaction mixture contained purified PCR product, 25 mM $MgCl_2$, 0.2 mM dATP, 10× *Taq* buffer, and 5 U of *Taq*. The reaction was done at 70°C for 25 min. Subsequently the product was cloned into the vector pGEM-T-Easy; this resulted in pGEMLAT.

Cloning of *ccfA*. A nisin-inducible vector, pMSP3535 (9), was chosen as the expression vector for *ccfA*. An 880-bp *Spe*I/*Xho*I fragment containing *ccfA* from pGEMmha was ligated into pMSP3535 to yield pMHAnis. Similarly, the 880-bp *Spe*I/*Xho*I fragment from pGEMLAT was ligated into pMSP3535 to yield pLAT. Ligated products were transformed into *E. coli* DH5α competent cells by electroporation and then plated on LB plates supplemented with X-Gal and ampicillin to allow blue-white screening of transformants.

Potential white colonies were picked, and the isolated plasmid DNA was subjected to restriction enzyme digestion analysis to determine whether it contained the proper insert. The confirmed cloned plasmid DNAs were then transformed into *E. faecalis* OG1RF competent cells, resulting in strains MSPmha001 (with pMHAnis) and MSPmha002 (with pLAT). Resulting plasmid DNA isolated from *E. faecalis* was used to verify the nucleotide sequence of *ccfA*. Sequencing was performed by the microchemical facility at the University of Minnesota. pMHAnis and pLAT were introduced into *E. faecalis* OG1RF(pCF10) by conjugation with MSPmha001 and MSPmha002, respectively, as described previously (36). Plasmid pMHAnis was also electroporated into *L. lactis* competent cells (27).

Expression of *ccfA*. In order to check cCF10 production from either MSPmha001 or MSPmha002, a time course experiment was carried out. Parallel cultures (10 ml of THB, one tube for each time point) were inoculated with the appropriate strain and then induced with 25 ng of nisin per ml. Pheromone activity was determined at each time point as described above. Time course experiments were repeated thrice. *L. lactis* (pMHAnis) was also induced using 25 ng of nisin per ml .

Culture supernatant was collected by centrifugation at 8,000 rpm (Beckman J2-21 centrifuge, JA20 rotor) and subjected to a clumping assay (10) to detect pheromone activity. *E. faecalis* OG1RF/pMHAnis and *E. faecalis* OG1RF/pLAT in 20 ml of THB were induced with nisin at a concentration of 25 ng/ml, incubated at 37°C with shaking for 4 h, and then observed for any visible clumps formed at the bottom of the tubes.

Computer sequence analysis. Preliminary sequence data were obtained from the Institute for Genomic Research (TIGR) website (http://www.tigr.org). tBLASTn (1) searches were done at the TIGR Comprehensive Microbial Resource website at http://tigrblast.tigr.org/cmr-blast. Most of the sequence analyses were done using the Wisconsin package (version 10.2) of the Genetics Computer Group (Madison, Wis.). Multiple sequence alignment using ClustalW 1.81 (35) was done through the Biology Workbench (version 3.2; San Diego Supercomputer Center, University of California at San Diego) website at http://workbench.sdsc.edu.

RESULTS

Molecular characterization of *ccfA* gene of *E. faecalis* OG1RF. The amino acid sequence of the cCF10 pheromone, LVTLVFV (29), was used to perform a tBLASTn (4) search of the *E. faecalis* V583 genomic sequence, available from the TIGR database website at http://www.tigr.org. Initial results matched to two noncontiguous TIGR loci, EF2045 and EF3331. The former match showed the LVTLVFV sequence at amino acid positions 169 to 175 of a 348-amino-acid putative type II secretion system protein. In contrast, the EF3331 match mapped the pheromone sequence within an open reading frame (ORF) predicted to encode a prolipoprotein, with the LVTLVFV amino acid sequence encoded within the signal peptide of the lipoprotein precursor (Fig. 1). This gene organization seemed more likely to result in cCF10 expression, since sequence analysis predicts that the cAD1, cPD1, cOB1, and cAM373 peptide pheromones correspond to the carboxy-

FIG. 1. Partial nucleotide sequence of *ccfA*. Underlined is the hypothesized lipoprotein signal sequence. The amino acid sequence of the pheromone cCF10 is italicized. Sequencing of this particular locus in *E. faecalis* OG1RF verified its identity to that of strain *E. faecalis* V583 as determined by TIGR (http://www.tigr.org).

terminal residues of the signal sequences of putative prolipoproteins (13).

Unlike the tBLASTn search results for cCF10, the other pheromones only matched to a single locus in the genome. For lipoprotein synthesis, signal peptidase II cleaves the signal peptide at the conserved cysteine residue within the lipo-box processing site (14). In the case of cCF10, the LVTLVFV amino acid sequence is three residues upstream of this processing site; the other pheromone sequences are found at the C termini of the respective cleaved signal peptides (13).

A Blast (1) search of the microbial genome database revealed that CcfA is most closely related to the *Bacillus subtilis* gene SpoIIIJ (19). Sequence alignment analysis showed 65% similarity and 39% identity. The cCF10 LVTLVFV amino acid sequence, however, is not present within the *spoIIIJ* signal peptide. Furthermore, culture filtrates from *B. subtilis* do not exhibit any cCF10 pheromone activity (results not shown).

spoIIIJ appears to be in an operon coexpressed with a downstream gene, *jag*, of unknown function (Fig. 2). Examination of the surrounding nucleotide sequence around *ccfA* suggests a similar organization, whereby a *jag* homolog, *cjag*, is also present downstream. The function of SpoIIIJ is still unknown; however, it appears to be essential for sigma G activity in stage III of sporulation (19). The *jag* gene appears to be essential for SpoIIIJ activity (19).

CcfA also appears to be homologous to a protein of gram-negative bacteria, YidC. Comparative sequence alignment with *E. coli* YidC analysis shows 37% identity and 55% similarity. YidC is an inner membrane translocase which is essential for normal growth (32). The YidC protein is considerably larger than the homologs from gram-positive organisms, with the former proteins showing strong similarity to the carboxy-terminal segment of YidC (Fig. 2). It is interesting that although YidC does not appear to have a *jag* homolog, all three genes have a common genetic organization, having *rnpA* and *rpmH* genes directly upstream from them (Fig. 2). Although it can be speculated that the *jag* homologs might have the same function as the amino-terminal half of YidC, we did not find any amino acid sequence conservation that would support this notion.

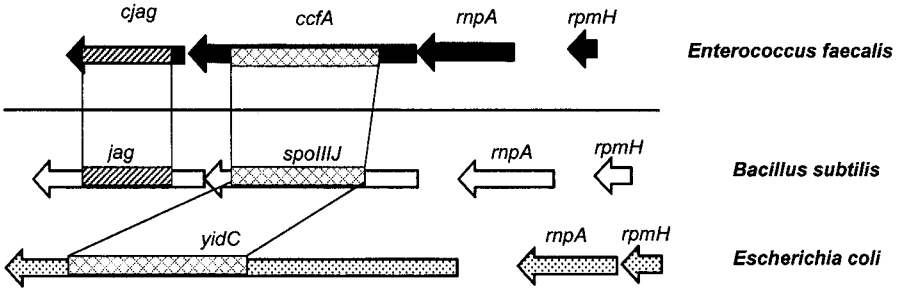

FIG. 2. Genetic map of the *ccfA* cluster in *E. faecalis*, in comparison to homologous loci in *B. subtilis* and *E. coli*. Regions of significant homology are shaded. *ccfA* is homologous to *spoIIIJ* and *yidC*. SpoIIIJ has an unknown function, whereas YidC is an inner membrane translocase. *cjag* is a homolog of *jag*, a *spoIIIJ*-associated gene of unknown function. RnpA is an RNase P protein involved in tRNA processing. *rpmH* encodes ribosomal protein component L34. Genomic sequence information and annotation were obtained from the NCBI Entrez Genomes website at http://www.ncbi.nlm.nih.gov.

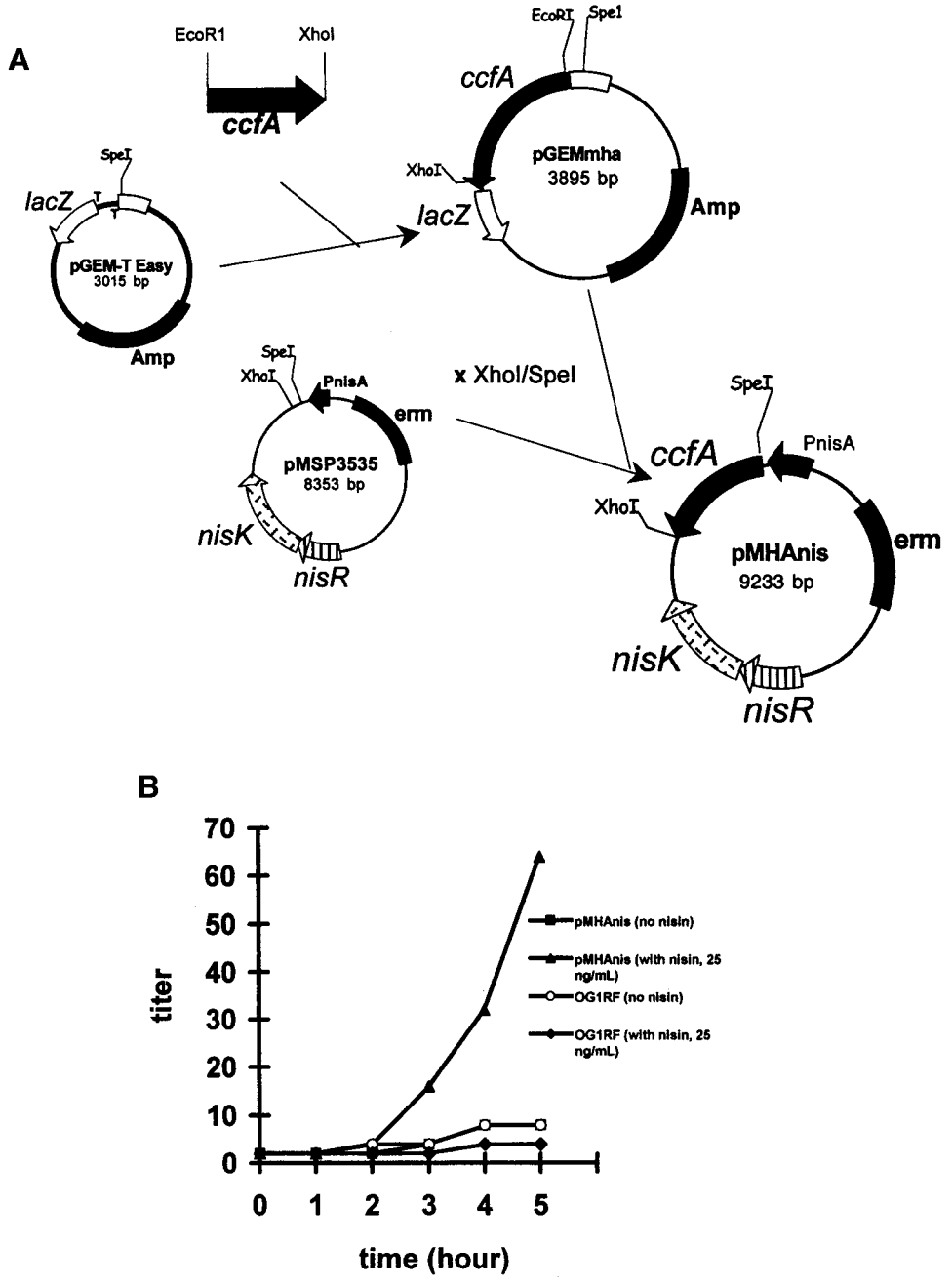

FIG. 3. (A) Construction of plasmid pMHAnis. (B) Detection of pheromone activity in culture supernatants. Cultures of *E. faecalis* OG1RF and *E. faecalis* OG1RF(pMHAnis) were grown overnight in the presence (25 ng/ml) or absence of nisin. Titer of cCF10 in the corresponding culture supernatants was determined.

Cloning and expression of *ccfA*. In order to confirm the existence of *ccfA* in *E. faecalis* OG1RF, primers 6221 EcoF and 6221 XhoR (Fig. 1) were used to amplify an 880-bp PCR product from genomic DNA. The PCR product was cloned into the nisin-inducible expression vector pMSP3535 (9). The sequence of the cloned PCR product and flanking vector sequences were determined (not shown) and found to be identical to the sequence from strain V543 in the TIGR database, confirming the identity of the two alleles of the gene.

A digoxigenin-labeled version of the same PCR product was used as a probe on Southern blots of *Eco*RI-digested genomic DNA. Analysis showed hybridization to a 10-kb fragment, confirming the presence of *ccfA* in *E. faecalis* OG1RF (results not shown).

E. faecalis secretes cCF10 into the medium at a low concentration (10^{-10} to 10^{-12} M), and pheromone activity can be detected in late logarithmic phase (29). Plasmid pMHAnis contains the *ccfA* gene cloned from OG1RF under control of

a nisin-inducible promoter, P_{NisA} (Fig. 3A). To determine whether *ccfA* is involved in cCF10 synthesis, the titer of pheromone activity was monitored from an *E. faecalis* OG1RF(pMHAnis) culture and compared to that from the wild type. The hypothesis tested is that increased expression of *ccfA* would result in pheromone activity released into the growth medium.

Culture supernatants were sampled at different time points, precipitated with trichloroacetic acid (TCA), and then subjected to a clumping assay to determine the titer of pheromone activity. In the absence of nisin induction, there was no significant difference in cCF10 titer compared to wild-type levels (Fig. 3B). However, upon nisin induction, pheromone activity was significantly increased, as much as eightfold. In our hands, expression of several other cloned enterococcal proteins under similar conditions resulted in a much higher level of induction (9, 22). It is thus possible that a posttranslational processing step involved in generation of mature cCF10 from the CcfA polypeptide may be rate limiting for cCF10 production.

Physiological effects of *ccfA* expression. *E. faecalis* OG1RF(pCF10) continues to produce peptide pheromone, and the plasmid encodes a complicated control system to prevent self-induction. Control of self-induction in donor cells involves several cell-associated regulators, as well as a precise stoichiometry between excreted inhibitor peptide (iCF10) and excreted cCF10 of endogenous origin (10). In the presence of recipient cells, exogenous cCF10 contributes to upsetting the "balance" in the iCF10-cCF10 ratio in the growth medium, leading to induction.

To test whether *ccfA* expression could result in higher production of endogenous cCF10, plasmid pMHAnis was transformed into *E. faecalis* OG1RF(pCF10). The resulting strain containing both plasmids was subjected to nisin induction (25 ng/ml). In the presence of nisin, after 2 h of growth with shaking at 37°C, the culture exhibited self-clumping (Fig. 4A). However, without nisin induction, no clumping was observed, and the culture appeared similar to the wild-type uninduced state.

In order to prove that CcfA was not simply playing a regulatory role in cCF10 synthesis, plasmid pMHAnis was transformed into a heterologous host. *L. lactis*, which does not normally produce the peptide pheromone. *L. lactis* carrying pMHAnis was grown in the presence and absence of nisin. The culture supernatant was collected and subjected to a clumping assay and HPLC analysis. Samples from nisin-induced cultures exhibited pheromone activity, whereas those from uninduced cultures and wild-type *L. lactis* did not (Fig. 4B). In C18 reverse-phase HPLC analysis of concentrated culture filtrates using the method described in detail by Buttaro et al. (10), the active fraction exhibited a retention time of 27 min, which was identical to that of synthetic cCF10.

Genetic evidence that the LVTLVFV sequence encoded by *ccfA* is responsible for pheromone activity. A synthetic peptide analog of cCF10, LATLVFV, was used in a clumping assay to determine whether this peptide could induce clumping in *E. faecalis* OG1RF(pCF10) cells. At physiological concentrations, this analog was incapable of inducing a pheromone response (Fig. 5A). To prove that mature peptide pheromone is processed proteolytically from the CcfA signal peptide, a single T→A mutation was introduced at nucleotide 40 of the *ccfA*

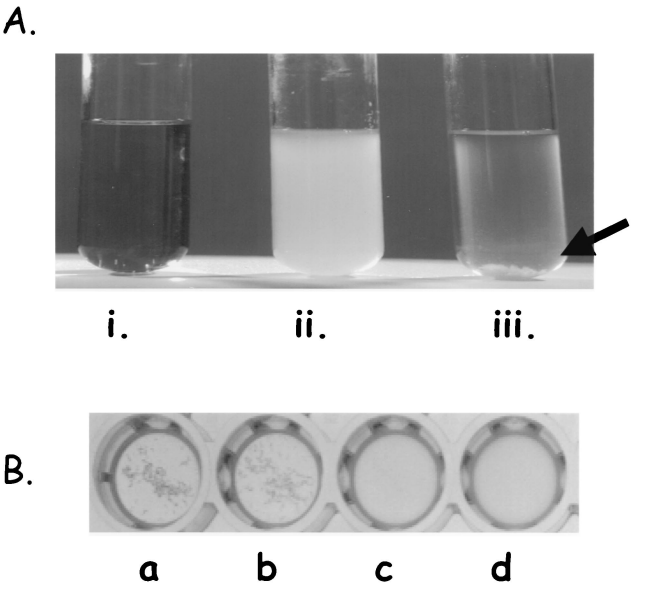

FIG. 4. (A) Overriding self-clumping control mechanism with nisin induction. (i) Uninoculated medium (THB); (ii) *E. faecalis*(pCF10/pMHAnis) 4-h culture; and (iii) *E. faecalis*(pCF10/pMHAnis) 4-h culture induced with nisin (25 ng/ml). The arrow points to the characteristic clumpy phenotype that results upon pheromone induction. (B) Heterologous expression of cCF10. Results of a clumping assay for pheromone activity in culture filtrates from the following cultures: *L. lactis*(pMHAnis) induced with nisin (25 ng/ml) (b) and *L. lactis*(pMHAnis) uninduced (c) in comparison with *E. faecalis*(pCF10/pMHAnis) induced with nisin (25 ng/ml) (a) and *E. faecalis*(pCF10/pMHAnis) uninduced (d). *E. faecalis* OG1RF(pCF10) was used as a responder strain for this assay.

gene, so that LATLVFV was the peptide produced in vivo from processing of the signal peptide (Fig. 5B). Site-directed mutagenesis of pGEMmha was done using the megaprimer method (5). The mutated gene was then cloned into the nisin-inducible expression vector to yield the construct pLAT.

Experiments were performed on batch cultures of *E. faecalis* OGIRF(pLAT), and the titer of secreted pheromone activity was determined at different time points. Unlike *E. faecalis* OG1RF(pMHAnis), the pheromone titer was comparable to wild-type levels whether or not *E. faecalis* OG1RF(pLAT) was induced with nisin. RNA dot blot analysis (not shown) indicated no significant difference in the levels of expression induced by nisin in cells carrying pLAT. Furthermore, pLAT was also transformed into *E. faecalis* OG1RF(pCF10). In contrast to the results obtained previously with *E. faecalis*(pCF10/pMHAnis), nisin induction of *E. faecalis*(pCF10/pLAT) did not result in self-clumping (data not shown).

DISCUSSION

E. faecalis has evolved numerous mechanisms of genetic exchange (11). Pheromone-induced mobilization of conjugative plasmids carrying various genetic determinants such as antibiotic resistance or virulence factors is perhaps one of the most widely studied systems (12, 17). Biochemical studies by Suzuki and coworkers and Clewell et al. led to the isolation and identification of several peptide pheromones which induce

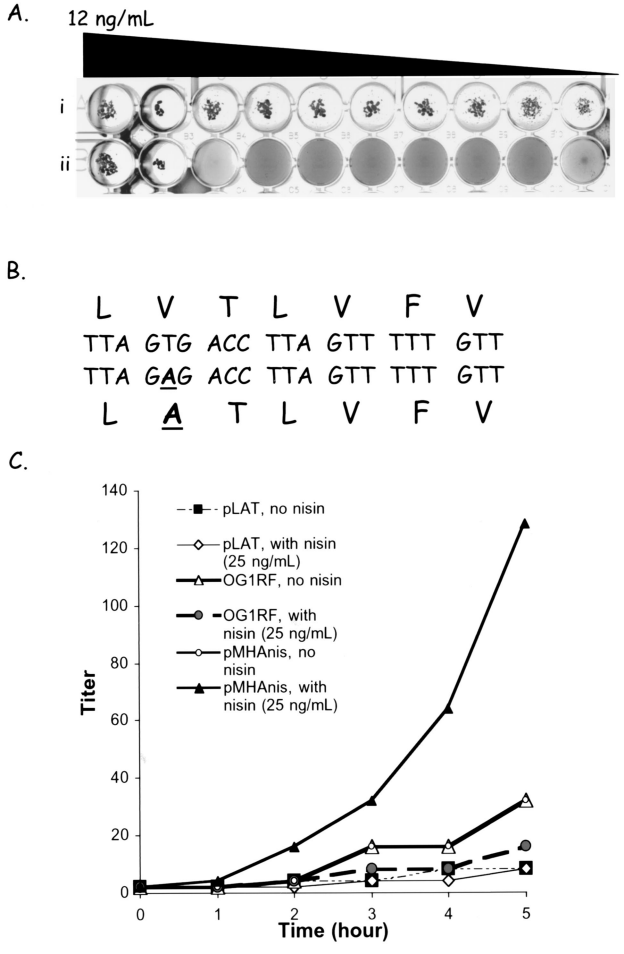

FIG. 5. (A) Clumping assay using synthetic (i) cCF10 peptide LVTLVFV and (ii) LATLVFV peptide. The starting concentration (12 ng/ml) was serially diluted (twofold) before it was inoculated with the indicator strain *E. faecalis* OG1RF(pCF10). At this starting concentration, cCF10 had a clumping titer of 2^{10} (i), whereas LATLVFV had a titer of only 2^2 (ii). (B) Nucleotide modification generated by site-directed mutagenesis to yield the appropriate V14A change with the prolipoprotein signal peptide. (C) Pheromone activity in culture supernatants. Cultures of *E. faecalis* OG1RF, *E. faecalis* OG1RF(pMHAnis), and *E. faecalis* OG1RF(pLAT) were grown for 5 h in the presence (25 ng/ml) or absence of nisin. Titer of cCF10 in the corresponding culture supernatants was determined.

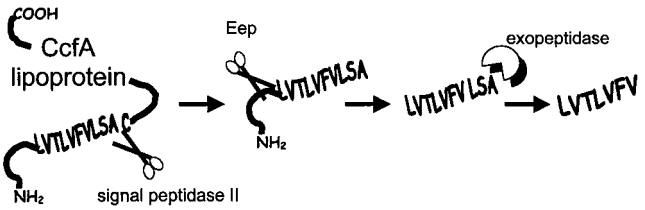

FIG. 6. Model for cCF10 biosynthesis. Signal peptidase II cleaves off the signal peptide of nascent prolipoprotein CcfA. The signal peptide is processed further by Eep, which cleaves at the amino-terminal end of the cCF10 peptide sequence. The decapeptide cCF10 precursor is further processed by an exopeptidase, resulting in mature cCF10. The scissors represent an endopeptidase; whereas the pie figure represents an exopeptidase.

conjugative transfer of plasmids with exquisite specificity (reviewed in reference 12).

The *E. faecalis* genome sequencing projects shed light on the origin of pheromone biosynthesis. As reported by Clewell et al. (13), computer sequence search and analysis identified various potential ORFs encoding prolipoproteins, each containing a specific pheromone amino acid sequence. In each case, the pheromone sequence was contained within the signal sequence of the prolipoprotein. This suggests that the prolipoprotein signal peptide is potentially a pheromone precursor, which could subsequently be proteolytically modified to yield the mature peptide pheromone. This model is substantiated by results from experiments reported by Buttaro et al. (10), which showed that mature cCF10 is predominantly cell wall associated, and pheromone activity generated by incubation of subcellular fractions can be significantly decreased by the addition of protease inhibitors. In addition, An et al. (3) identified Eep, believed to be involved in the processing of pheromone. The Eep protein has structural features characteristic of a membrane-associated zinc metalloprotease. Eep belongs to the family of RIP (regulated intramembrane proteolysis) proteins involved in intramembrane processing of nascent proteins, a mechanism which appears to be conserved from bacteria to humans (8).

The present study was undertaken to confirm the identity of the structural gene from which cCF10 pheromone is biosynthesized. Sequence analysis showed that the cCF10 LVTLVFV amino acid sequence, like that of other pheromones such as cAD1, cOB1, cPD1, and cAM373, corresponded to the carboxy-terminal end of a prolipoprotein signal peptide (13). Lipoprotein signal peptides are liberated upon signal peptidase II cleavage at the cysteine residue contained within the conserved lipobox processing site (7, 14). The fate of signal peptides released during protein biosynthesis is further degradation by signal peptide peptidases (14). In the case of cCF10, however, the LVTLVFV sequence is three bases upstream of the cysteine cleavage site. Assuming that Eep is involved in an endoproteolytic cleavage of the carboxy-terminal portion of the signal peptide, this suggests that another protease could be required during processing of the CcfA signal peptide to generate mature cCF10. An et al. showed that although the absence of the *eep* product significantly reduces cCF10 production, elevated expression of *eep* does not drastically increase cCF10 production, in contrast to the results observed with cAD1 (3). This suggests that exoproteolytic processing of the decapeptide pro-cCF10 molecule produced by Eep could be a rate-limiting step. A model for cCF10 production is shown in Fig. 6.

Increased expression of CcfA resulted in a significant increase in pheromone production by *E. faecalis* OG1RF(pMHAnis). Furthermore, when this protein was overexpressed in pCF10-containing cells, the strains were no longer capable of regulating self-induction. This observation concurs with the current model, in which regulation of self-induction by donor cells is based on maintenance of a proper ratio between inhibitor peptide and endogenous pheromone (10). In the presence of excess pheromone provided by neighboring recipient cells, or in this case when CcfA is overexpressed, the balance is tipped and leads to induction. When *ccfA* was expressed in a heter-

ologous host, *L. lactis*, it conferred pheromone production in the otherwise cCF10-nonproducing organism.

L. lactis is a fastidious organism with a wide array of proteases needed for proteolytic digestion of milk proteins to obtain amino acids essential for growth (24). Database search of the *L. lactis* genomic sequence at http://www.tigr.org identified YvjB, a putative zinc metalloprotease highly homologous to Eep. Therefore, it is not surprising that *L. lactis* carrying pMHAnis was capable of properly processing the CcfA signal peptide. The introduction of a single base change in the *ccfA* coding region (pLAT) of the cloned gene, which would result in substitution of an alanine for a valine within the hydrophobic portion of the signal peptide region of the protein, would be unlikely to affect transcription from the nisin promoter or processing and secretion of CcfA. However, this mutation abolished the enhancement of cCF10 production that was observed when the cloned wild-type gene was overexpressed. This result argues strongly that the observed biological effects are due to the change in amino acid sequence of the processed peptide.

Numerous efforts have been made to genetically inactivate the *ccfA* determinant in strain OG1RF. The strategies employed for these experiments included efforts to make *ccfA* "knockouts" by integration of replication-deficient plasmids carrying internal fragments of the gene by a single crossover. We also attempted to carry out replacement (via double crossover) of the wild-type gene with the "pLAT" allele (Fig. 5C), encoding the A→V substitution in the second position of the mature pheromone. In spite of the fact that the same methods have been used successfully in our laboratory to generate mutations in other *E. faecalis* genes, we have been unable to generate a stable clone carrying any type of *ccfA* mutation.

In one experiment (data not shown), in which allelic replacement was attempted, we obtained several very slow-growing clones which produced no cCF10 and which were confirmed (by pulsed-field genomic DNA analysis; not shown) to be strain OG1RF. Although these isolates did not maintain viability beyond one or two serial transfers, sequence analysis of amplified *ccfA* region DNA taken from the initial colonies indicated that several unintended point mutations of the DNA upstream of the *ccfA* coding region had apparently been generated during the recombination process, but the protein coding sequence had not been altered. These mutations may have abolished expression of the gene and generated the pheromone-negative, growth-defective phenotype, although it is also possible that these strains contained additional unlinked point mutations. Therefore, we cannot conclude with certainty that this gene is essential in *E. faecalis*. However, the available information suggests that either the mature lipoprotein, the processed cCF10 peptide, or both might be required for normal growth of strain OG1RF. To examine this question further, attempts are under way to construct a derivative of OG1RF in which *ccfA* expression from an ectopic locus can be controlled experimentally to allow inactivation of the wild-type determinant even if the gene is essential.

The function of SpoIIIJ is still unknown, yet much work has been done on the other CcfA homolog, YidC. YidC is a homolog of the eukaryotic Oxa1p protein found in mitochondria and chloroplasts (32). Like Oxa1p, YidC is believed to be a translocase required for the proper insertion of nascent proteins into the inner membrane of bacteria (32). YidC was shown to be required for membrane insertion of Sec-independent proteins and also appears to be involved to a lesser extent in the membrane insertion of some Sec-dependent proteins. More interestingly, Samuelson et al. (32) showed that YidC is essential for cell viability. The growth defect of a YidC deletion mutant was rescued by arabinose-controlled expression of *yidC* in *trans*. When arabinose-grown overnight cultures were reinoculated into minimal medium or arabinose-free rich medium, the growth defect was restored.

The work presented here shows that the peptide pheromone cCF10 can be produced from proteolytic processing of the signal sequence of the polypeptide encoded by the *ccfA* gene. Although the cCF10 sequence was also found within an internal sequence in the C-terminal portion of another polypeptide, our results indicate that the expression of the *ccfA* gene is responsible for the vast majority, if not all, of the cCF10 produced by *E. faecalis* OG1RF. Previous analysis of enterococcal genomic sequence data (13), along with the identification of the *eep* determinant encoding a putative membrane protease activity which enhances production of several pheromones, and recent analysis of production of pheromone cAD1 and the cognate inhibitor iAD1 (F. Y. An and D. B. Clewell, Abstracts, ASM Conference on Cell-Cell Communication in Bacteria, abstr. 66, 2001) suggest a common processing pathway for maturation of enterococccal pheromone and inhibitor peptides.

Since Eep-mediated cleavage of the signal peptides is believed to occur in the membrane (2, 8), and mature cCF10 is actually more hydrophobic than the signal peptide precursor, it seems likely that the cleavage process could be accompanied by a simultaneous active export of the peptide from the membrane to the outside environment. A similar process is believed to occur in the secretion of bacteriocin and bacteriocin-inducing factor peptides produced by numerous gram-positive bacteria (20).

It is interesting that the pheromone plasmids have apparently evolved to allow their host cells to use a by-product of normal metabolism which might be considered cellular waste as an indicator of the proximity of potential recipient cells. There are an extremely large number of additional functional peptides that could be generated in these organisms by similar mechanisms, as well as peptides that could be produced from expression of ORFs in the genome whose lengths are too short to be considered functional genes in standard computerized genomic analyses. Thus, it would not be surprising to find many more peptide-regulated physiological processes than those currently recognized.

The plasmid-determined detection system for the cCF10 molecule needs to be exquisitely sensitive because of the low levels of active signal produced. At the same time, the low levels of cCF10 production could make it easier to control the response to endogenous pheromone in donor cells. Interestingly, the levels of induction of pCF10 transfer genes observed in donor cells at physiologically relevant cCF10 concentrations are relatively modest (5- to 50-fold) and probably transient (6, 25, 29), but these are obviously sufficient to promote frequent transfer of the plasmid under favorable conditions, i.e., availability of recipient cells. When considered as autonomous ("selfish") DNA elements, the pheromone plasmids seem to

have adapted an economical evolutionary strategy to optimize their dissemination while minimizing their metabolic burden on the host cell.

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