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Genetic Structure of the *Enterococcus faecalis* Plasmid pAD1-Encoded Cytolytic Toxin System and Its Relationship to Lantibiotic Determinants

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Pheromone-responsive conjugative plasmids are unique to the species *Enterococcus faecalis*. Many pheromone-responsive plasmids, including those frequently isolated from sites of infection, express a novel cytolysin that possesses both hemolytic and bacteriocin activities. Further, this cytolysin has been shown to be a toxin in several disease models. In the present study, nucleotide sequence determination, mutagenesis, and complementation analysis were used to determine the organization of the *E. faecalis* plasmid pAD1 cytolysin determinant. Four open reading frames are required for expression of the cytolysin precursor (*cylL*$_L$, *cylL*$_S$, *cylM*, and *cylB*). The inferred products of two of these open reading frames, CylL$_L$ and CylL$_S$, constitute the cytolysin precursor and bear structural resemblance to posttranslationally modified bacteriocins termed lantibiotics. Similarities between the organization of the *E. faecalis* cytolysin determinant and expression units for lantibiotics exist, indicating that the *E. faecalis* cytolysin represents a new branch of this class and is the first known to possess toxin activity.

Enterococcus faecalis isolates derived from infection sites are more frequently hemolytic than isolates from the oral cavity or stools of healthy volunteers (22, 26, 48). The variable nature of the hemolytic phenotype results from the hemolysin determinant being located on highly transmissible, pheromone-responsive plasmids (e.g., pAD1; recently reviewed in references 8 and 9), although evidence has been obtained recently for its occasional residence on the *E. faecalis* genome (23). The observation of acute toxicity of hemolytic *E. faecalis*, when injected intraperitoneally in mice (25), suggested that the hemolysin may contribute to bacterial virulence in models of human disease. Hemolytic *E. faecalis* strains have been observed to cause a more rapid and fulminant endophthalmitis in a rabbit infection model than isogenic strains rendered nonhemolytic as the result of insertion of a transposon into various areas of the hemolysin determinant (29). Similar observations of an endocarditis model where hemolytic strains were found to be significantly more toxic than isogenic, nonhemolytic mutants have been made (6; for a recent review of enterococcal virulence, see reference 28).

Contributing to virulence is a common motif for bacterial hemolysins (3, 35, 46, 47). The *E. faecalis* hemolysin, however, is unique in that in addition to mediating lysis of erythrocytes, it also possesses antibacterial activity toward a broad range of gram-positive bacteria (4, 5, 27). The hemolysin/bacteriocin (or, generically, cytolysin) may contribute to enterococcal virulence through its toxic activity or by disrupting local ecology.

The cytolysin determinant encoded by pAD1 has been cloned and has been characterized by transposon mutagenesis and deletion analysis (24). Two functional domains of the operon were identified in these studies—one region encoding what had been identified previously as the toxin precursor (operationally defined as component L [18]) and the second region encoding an activator (termed component A [18]). Both component L and the activated hemolysin were observed to be heat stable, whereas the activator was observed to be heat labile (24).

The nucleotide sequence of the activator or component A gene (*cylA*) and characterization of the physical nature of the *cylA* gene product have been reported previously (41). Component A, or CylA, was observed to share physical and biochemical features with serine proteases of the subtilisin class. Interestingly, the cloned *cylA* gene was also observed to contribute to immunity to the related bacteriocin activity. A model was therefore proposed for the extracellular activation of the *E. faecalis* cytolysin precursor by limited proteolysis mediated by CylA. Further proteolysis under conditions of CylA excess, as was observed at the cell surface, may inactivate the cytolysin (41).

Immediately 5′ to *cylA* is *cylB*, whose nucleotide sequence has been reported (17). *cylB* is the first member of the HlyB family of ATP-binding transport proteins to have been identified in an operon from a gram-positive bacterium (15, 17). CylB was observed to be essential for externalization of the *E. faecalis* cytolysin precursor activity, component L.

Although a substantial amount of information describing (i) the protein that activates the *E. faecalis* cytolysin precursor extracellularly (41) and (ii) the dependence of cytolysin precursor externalization on CylB expression (17) has been obtained, little is known about the cytolysin precursor or its relationship to other toxins and bacteriocins of gram-positive bacteria. It was therefore of interest to determine the complete nucleotide sequence of the region of pAD1 DNA identified as being required for expression of the *E. faecalis* cytolysin and to use site-specific mutagenesis and complementation analysis to

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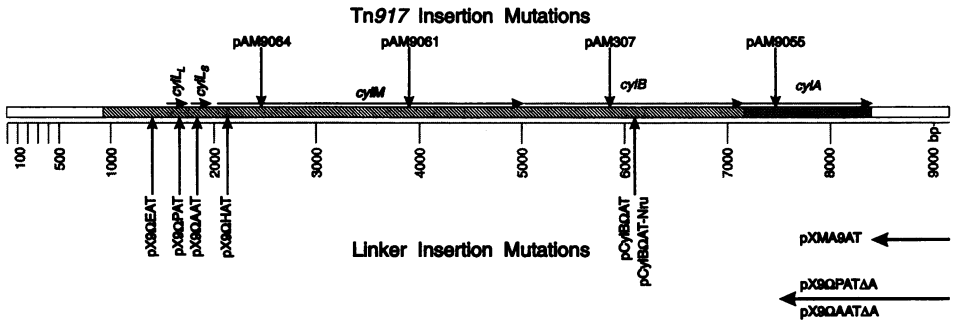

FIG. 1. Schematic representation of the cloned region of pAD1 that mediates expression of the cytolysin in *E. coli* in construction pRAS28-4 (24). The insert was subcloned as an *Sst*I-*Sal*I fragment into the vector pKIM2 (see Materials and Methods) to generate the construction pKIMCyl. Extraneous sequences at the right end as illustrated were removed (to the point indicated by the arrow), generating construction pXMA9. Insertion of mutagenic linkers (vertical arrows) into the *Pst*I and *Hin*dIII sites of pXMA9 at the positions indicated generated pX9ΩPAT and pX9ΩHAT, respectively, following transfer of the mutated inserts into shuttle vector pAT28 (44). pX9ΩEAT and pX9ΩAAT were generated by linker insertion mutagenesis at *Eco*RV and *Ava*II restriction sites, respectively, using an analogous procedure. pCylBΩAT and pCylBΩAT-Nru were derived from pRAS28-4Ω (17) as described in Results and possess linker insertion mutations where indicated. pRAS28-4Ω and pCylBΩAT mutations result in the in-frame insertion of the amino acid sequence MHRS (17) at the point indicated, whereas the mutation in pCylBΩAT-Nru results in a frameshift mutation. Deletion derivatives of pX9ΩPAT and pX9ΩAAT lacking most of the CylA reading frame were constructed by digestion with *Bal*I (which occurs within the *cylA* reading frame [41]) and *Sal*I (which defines the right end of the insertion diagrammed) and blunting of the protruding end with Klenow fragment of DNA polymerase and deoxynucleotide triphosphates prior to closure with ligase. This resulted in deletion of the C-terminal three-fourths of the CylA coding sequence to the position indicated by the horizontal arrow below the map, and the resulting deletion derivatives were designated pX9ΩPATΔA and pX9ΩAATΔA. The positions of Tn*917* insertion within the cytolysin determinant of pAD1, generating plasmids pAM9064, pAM9061, and pAM9055, were mapped previously (24), as was the position of Tn*917* insertion in the pAD1::Tn*917* derivative pAM307 (17, 42). The hatched area spanning coordinates 900 to 7200 represents that section of the operon observed to be related to expression of the cytolysin precursor previously termed component L (24). The cross-hatched area denotes the region of the determinant identified to be related to expression of the cytolysin activator component A (24, 41). The positions of ORFs $cylL_L$, $cylL_S$, *cylM* (solid horizontal arrows), *cylB* (17), and *cylA* (41) (shaded horizontal arrows) required for cytolysin expression, relative to transposon and mutagenic linker insertions, are indicated.

determine the involvement of observed reading frames in cytolysin expression. The results of this study provide evidence that the operon encoding the *E. faecalis* cytolysin is related to antibiotic operons found in other gram-positive bacteria. The *E. faecalis* cytolysin is the first relative of this class of antimicrobial agents known to possess toxin activity.

MATERIALS AND METHODS

Bacteria and culture media. *E. faecalis* FA2-2 (10) and UV202 (14, 49) strains were used as hosts to evaluate extracellular complementation and intracellular complementation, respectively. *E. faecalis* strains were cultivated routinely in brain heart infusion (Difco, Detroit, Mich.), whereas Luria-Bertani broth (37) was used for the cultivation of *Escherichia coli* strains. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) used for selection of *E. faecalis* strains included rifampin and fusidic acid at 25 μg/ml each, chloramphenicol and tetracycline at 10 μg/ml each, and streptomycin and spectinomycin at 500 μg/ml each (except for maintenance or selection of pAT28-based [44] clones, for which spectinomycin at 250 μg/ml was used). For maintenance of recombinant constructions in *E. coli*, ampicillin at 100 μg/ml, chloramphenicol at 25 μg/ml, or spectinomycin at 150 μg/ml was used where appropriate.

Blood agar plates were used for the qualitative detection of hemolytic activity. These plates contained brain heart infusion and 1.5% Bacto Agar (Difco) to which washed, human or bovine erythrocytes were added to a final concentration of 5%.

Nucleotide sequence determination. Nested deletions of the cloned pAD1 cytolysin determinant contained in plasmid pRAS28-4 (Fig. 1) were constructed as described previously (16, 20). Nucleotide sequence determinations were made with commercially available kits for single-stranded template-directed reactions (Sequenase 2.0; U.S. Biochemical, Cleveland, Ohio) or double-stranded template-containing reactions (fmol DNA Sequencing System; Promega, Madison, Wis.), as instructed by the manufacturers. ^{35}S incorporated into the dideoxynucleotide terminated chains from 5′-(α-thio)dATP (DuPont NEN; Wilmington Del.) precursors was detected by exposing the resulting, dried, 6 to 8% polyacrylamide gels to Kodak SB X-ray film overnight.

Restriction site mutagenesis. Restriction enzyme recognition sites occurring in each open reading frame (ORF) of the cytolysin operon were mutagenized by linker insertion. The hemolysin-encoding insert of pRAS28-4 (24) was subcloned into pKIM2 (a fusion of the *lacZ*α and multiple-cloning-site region of pUC18 and the *cat* gene and replication functions of pACYC184 [15a]) as an 8.3-kb *Sst*I-*Sal*I restriction fragment, yielding the construction pKIMCyl. This was the first step in generating a construction lacking vector *Hin*dIII and *Pst*I sites, so that those sites occurring in the $cylL_L$ and *cylM* reading frames would be unique and amenable to mutagenesis. Extraneous information encoded at the *Sal*I-proximal end of the cloned determinant, as well as vector *Hin*dIII and *Pst*I recognition sites, was then removed as follows. Five micrograms of pKIMCyl was linearized by *Sal*I digestion and degraded bidirectionally with 6 U of *Bal* 31 exonuclease for 1 to 30 min essentially as described previously (24). The DNA molecules were recircularized by ligation in the presence of 100 pmol of the self-complementary oligonucleotide 5′-pGCTCTAGAG COH-3′, which introduced a unique *Xba*I site at the new fusion point. Multiple linker insertions were eliminated by digestion of the ligation products with *Xba*I, ultrafiltration through a Centricon 100 ultrafiltration capsule (Amicon, Beverly, Mass.) to remove excess linker, and finally religation. JM109 was transformed by electroporation as described elsewhere (13).

The smallest deletion derivative that conferred the hemolytic phenotype to *E. coli* transformants was termed pXMA9

(Fig. 1). Lacking vector *Pst*I and *Hin*dIII restriction sites, pXMA9 was used for linker insertional mutagenesis of *Eco*RV (upstream ORF), *Pst*I (reading frame $cylL_L$, as described below), and *Hin*dIII (*cylM*) sites occurring within the cloned cytolysin determinant. Mutagenesis was accomplished by linearizing 1 μg of pXMA9 with either *Eco*RV, *Pst*I, or *Hin*dIII and religating in the presence of the self-complementary, nonphosphorylated oligonucleotide CCGAGCTCGG, GGTA CCTGCA, or AGCTGGTACC, respectively. The first oligonucleotide (CCGAGCTCGG) was used to introduce an *Sst*I recognition site into the interrupted *Eco*RV site. The second and third oligonucleotides introduced *Kpn*I recognition sites into interrupted *Pst*I and *Hin*dIII sites, respectively. Again, the chance of multiple insertions of linker sequences was minimized by heating each of the ligation products (with phosphodiester bonds introduced at the 3′ ends of the nonphosphorylated linkers only) to 65°C, ultrafiltration through Centricon 100 capsules, and reannealing at the ambient temperature prior to transformation.

A reading frame identified in sequencing and complementation studies, termed $cylL_S$, lacked a restriction enzyme recognition site that was unique within the operon. Therefore, a 7.3-kb *Bcl*I fragment of the cytolysin determinant (previously cloned and described as pBCL3 [24]) was used for mutagenesis. This fragment lacks *Ava*II recognition sites aside from that occurring within the $cylL_S$ reading frame and contains all of the cytolysin determinant except for the C-terminal half of the CylA-encoding region. A self-complementary, nonphosphorylated adapter of sequence GTCAGGATCCT was inserted into the *Ava*II site by ligation, heating, ultrafiltration, and annealing as outlined above. This adapter introduced a *Bam*HI site and a frame shift into the $cylL_S$ reading frame. The 3′ end of the operon containing the remainder of *cylA* was restored as described previously (24), resulting in an intact operon with $cylL_S$ selectively interrupted. A schematic representation of the insertion and deletion mutations constructed and tested in this study is provided in Fig. 1.

Insertionally mutagenized cytolysin determinants were subcloned into shuttle vector pAT28 (44), as either *Sst*I-*Xba*I or *Kpn*I-*Xba*I fragments, and introduced into *E. faecalis* by electroporation as previously described (11).

Complementation analysis. Intracellular and extracellular *trans*-complementation capabilities were assessed on blood agar plates. Previously mapped transposon insertions within the cytolysin operon of pAD1 (24) were complemented by restriction site-specific mutations cloned into the compatible vector pAT28 in the recombination-deficient strain UV202 (14, 49). These complementation pairs of plasmids within the same cell were examined for the ability to produce zones of hemolysis after incubation on blood agar for 24 to 48 h. Extracellular complementation was observed at the junction of cross-streaks on blood agar after 24 to 48 h of incubation as described previously (17, 24, 41).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the *E. faecalis* cytolysin operon is L37110.

RESULTS

Portions of the nucleotide sequence of the cloned pAD1 cytolysin determinant encoding the cytolysin activator (*cylA*) and an ATP-binding cassette transporter (*cylB*) have been reported previously (17, 41). The relationship between *cylA*, *cylB*, and the three new reading frames identified in this study (*cylM*, $cylL_L$, and $cylL_S$) is shown in Fig. 1.

Nucleotide sequence determination and characteristics of $cylL_L$ and $cylL_S$. The nucleotide sequence of $cylL_L$, $cylL_S$, and *cylM* is provided in Fig. 2. The nucleotide sequence is remarkable in containing a duplication with 70 identities over 78 bases (Fig. 2, residues 627 to 714 and 903 to 980), including a run of 47 consecutive identities. Moreover, these repeats are included within a region predicted to contain extensive secondary structure (not shown). Each of the repeat sequences occurs in separate small ORFs, termed $cylL_L$ (68 amino acids) and $cylL_S$ (63 amino acids). Alignment of the inferred amino acid sequences encoded by $cylL_L$ and $cylL_S$ reveals little sequence similarity outside of the acidic domain encoded by these repeat sequences. The repeats, which encode (with a single conserved substitution) at the amino acid sequence level PSFEELS(V or L)EEMEAIQGSGDVQAETTP, occur at subtly different locations within the $CylL_L$ and $CylL_S$ reading frames (Fig. 2). Within $CylL_L$, the repeat is located near the amino terminus and begins at the eighth amino acid. In $CylL_S$, however, the repeat is located in the center of the small protein, 19 residues from the amino terminus and 18 residues from the carboxyl terminus.

Aside from the highly conserved 26-amino-acid domain, bracketed at each end by proline residues, there are local similarities surrounding cysteine residues that occur within the inferred sequences of $CylL_L$ and $CylL_S$. Overlapping the C-terminal end of the large conserved domain is the sequence TTXXC in both $CylL_L$ and $CylL_S$. Additionally, $CylL_L$ possesses the related sequence SSXXC, nine residues further toward the C terminus. Finally, both $CylL_L$ and $CylL_S$ share a common sequence at the C terminus of SXKXC. As discussed below, these features are similar to those observed in the precursors of well-characterized lantibiotics (1, 19, 31, 32, 40). The overall organization of a hydrophilic N-terminal domain followed by a hydrophobic C-terminal domain is also similar to that observed for lantibiotic precursors (1, 30–32, 40). In contrast to well-characterized lantibiotics which are cationic, however, $CylL_L$ and $CylL_S$ both are predicted to possess substantial acidic character, with theoretical isoelectric points of 3.82 and 3.91, respectively.

$CylL_L$ and $CylL_S$ are required for cytolytic activity. To demonstrate that both $CylL_L$ and $CylL_S$ are required for both hemolytic and bacteriolytic activity, the following mutagenesis and complementation experiments were performed. The reading frame encoding $CylL_L$ contained within the clone pXMA9 was interrupted by insertion of a 10-nucleotide, partially self-complementary linker into a unique *Pst*I site, resulting in a frameshift mutation, as described in Materials and Methods. The resulting construction was designated pX9ΩP. When subcloned into the shuttle vector pAT28 (44), the clone pX9ΩPAT, which was capable of replication in *E. faecalis*, resulted (Fig. 1). Transformants of *E. faecalis* FA2-2 harboring pX9ΩPAT were phenotypically nonhemolytic and nonbacteriolytic, indicating that the targeted reading frame was essential for cytolysin activity. When these transformants were cross-streaked with FA2-2(pAM9055) (a strain harboring a Tn*917* insertion within the CylA gene of pAD1 [24] [Fig. 3]), however, hemolysis occurred at the junction, indicating that FA2-2(pX9ΩPAT), although deficient in expression of the inactive cytolysin precursor, remained capable of expressing and secreting the activator component, CylA.

The $CylL_S$ reading frame was inactivated by a similar strategy, which involved insertion of an 11-nucleotide partially self-complementary linker into an *Ava*II restriction site to generate the construction pX9ΩA. However, since additional *Ava*II restriction sites occurred within construction pXMA9, the insertion was initially made into a clone harboring only a portion of the cytolysin determinant and the complete deter-

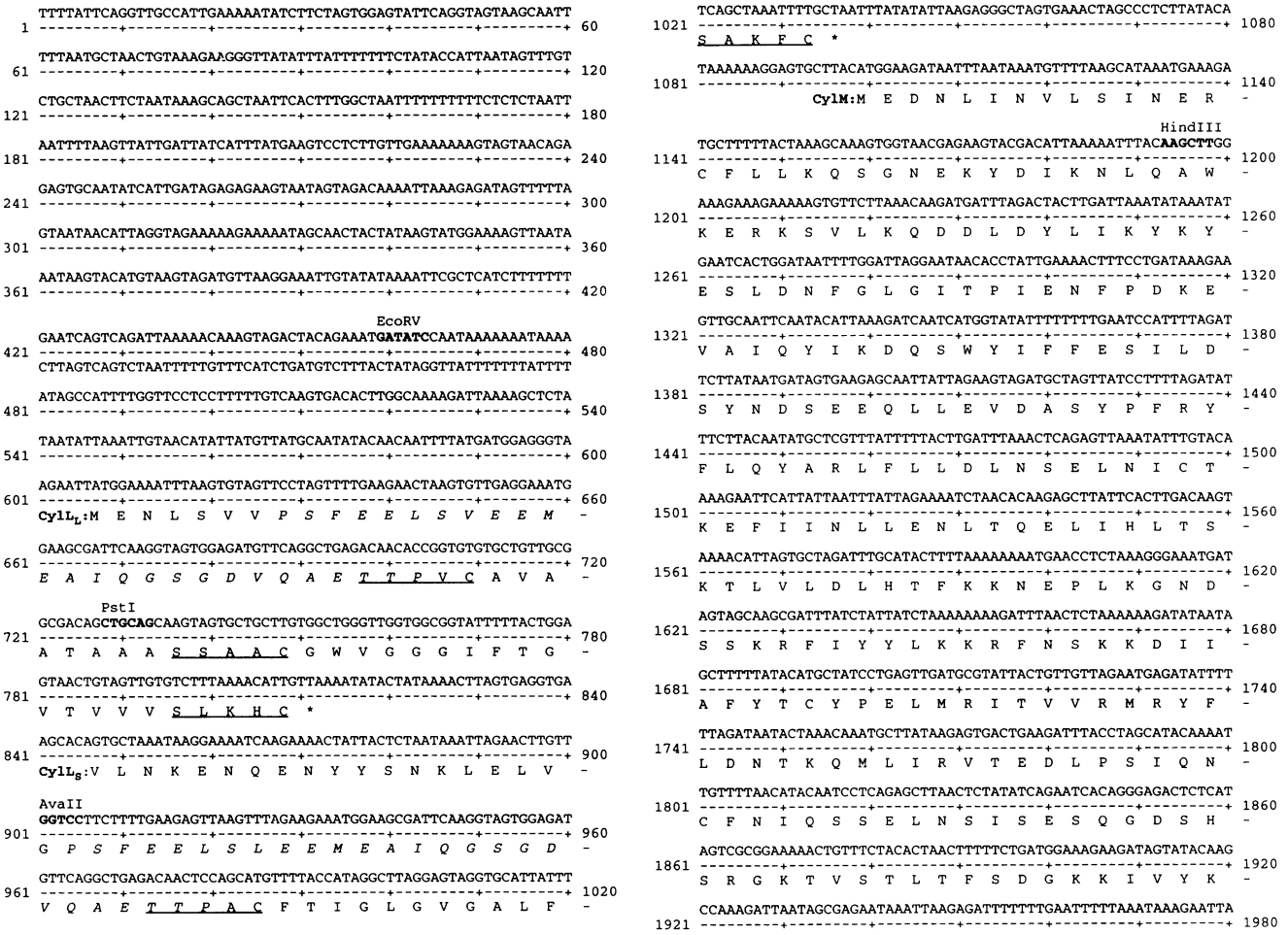

FIG. 2. Nucleotide sequence of the *E. faecalis* cytolysin operon. Restriction sites mutated by linker insertion are indicated in boldface letters. Conserved domains within $CylL_L$ and $CylL_S$ are shown in italics. Common motifs surrounding cysteine residues within $CylL_L$ and $CylL_S$ are underlined. The complete nucleotide sequence for the region of the cytolysin determinant that encodes $CylL_L$ (nucleotides 607 to 810), $CylL_S$ (nucleotides 847 to 1035), and CylM (nucleotides 1099 to 4077) is provided. The relative positions of the two previously reported nucleotide sequences encoding CylB (nucleotides 4092 to 6233 [17]) and CylA (6233 to 7468 [41]) are indicated.

minant was reassembled following mutagenesis as described in Materials and Methods. The reconstituted, mutated cytolysin determinant was subcloned as before into shuttle vector pAT28 and transformed into *E. faecalis*, yielding strain FA2-2(pX9ΩAAT) (Fig. 1). This construction also was observed to be noncytolytic for either erythrocytes or bacteria, suggesting a critical role for $CylL_S$ in the expression of cytolysin activity. As was the case for linker insertions within $cylL_L$, FA2-2(pX9ΩAAT) was observed to complement FA2-2(pAM9055) demonstrating the continued production of the CylA activator by FA2-2(pX9ΩAAT).

Although the observation that insertional inactivation of $cylL_L$ and $cylL_S$ resulted in noncytolytic transformants provided substantial evidence for the involvement of these components in hemolysin and bacteriocin activities, it was inconclusive since this phenotype would also be predicted to result from polar effects on ORFs located between $cylL_S$ and *cylA*. Unambiguous proof of the involvement of both $CylL_L$ and $CylL_S$ in cytolysis was obtained by cross-streaking FA2-2(pX9ΩAAT) ($CylL_S$ deficient) and FA2-2(pX9ΩPAT) ($CylL_L$ deficient) on blood agar plates. As shown in Fig. 4, hemolysis occurs at the junction of this cross-streak, indicating (together with previous observations on the activity of CylA [41]) that three extracellular factors participate in the cytolysis reaction—$CylL_L$, $CylL_S$, and CylA—even though $CylL_L$ and $CylL_S$ translation products lack obvious signals for secretion via the SecA-SecY pathway (2).

Nucleotide sequence and properties of *cylM*. Separated by 60 nucleotides 3′ to the terminator codon of $cylL_S$ is a large reading frame termed *cylM*. The putative start codon and ribosome binding site initiating *cylM* occur at the end of the potentially large secondary structure that includes most of the $cylL_L$ and $cylL_S$ reading frames referred to previously. *cylM* appears to encode a large cytoplasmic protein of 993 amino acids. Comparison of the inferred sequence of the *cylM* gene product to sequences within the GenBank database revealed sequence similarity in the C-terminal half (residues beyond position 450), with proteins encoded within lantibiotic operons (an observation brought to our attention by R. Siezen [42a]). Among related sequences associated with lantibiotic expression are NisC (414 amino acids; accession number S36737 [36]), which exhibited 45.96% similarity when aligned according to the default parameters for the University of Wisconsin Genetics Computer Group version 7.2 program Bestfit (12),

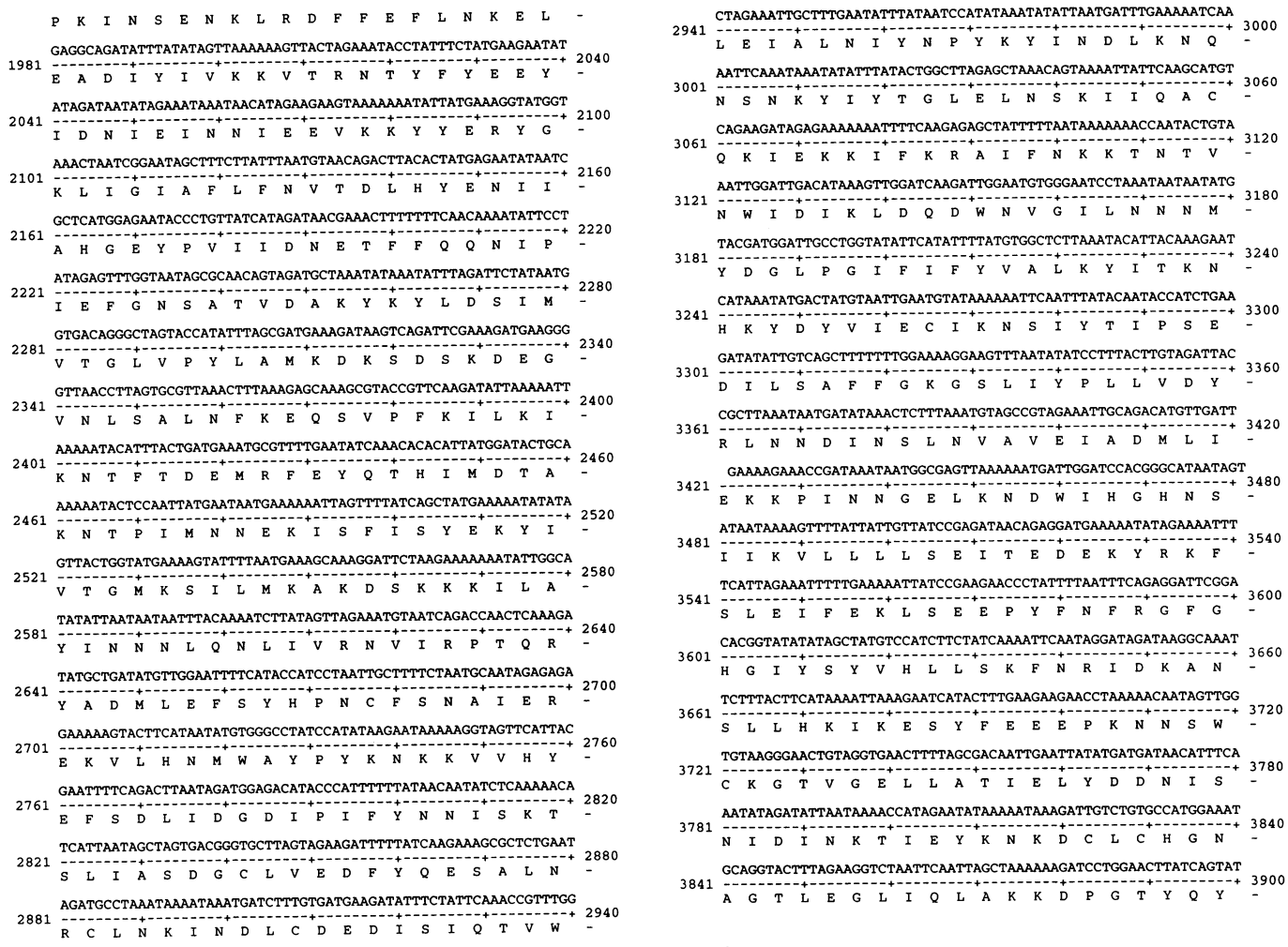

FIG. 2—*Continued.*

EpiC (455 amino acids; accession number P30196 [39]) which was 45.519% similar, and SpaC (441 amino acids; accession number C42655 [7]) which exhibited 47.529% similarity. Importantly, conserved residues include those putatively assigned to the active site of this class of proteins (42a).

Requirement of *cylM* for cytolysin expression. To determine whether *cylM* was essential for cytolysin expression, the *cylM* reading frame was interrupted by the insertion of a 10-nucleotide linker generating a frame shift. As described in Materials and Methods, the mutagenic linker was inserted into a unique *Hin*dIII restriction site within pXMA9, generating the plasmid pX9ΩH. The mutated cytolysin determinant was subcloned into shuttle vector pAT28, resulting in the construction pX9ΩHAT (Fig. 1), which was used to transform *E. faecalis* FA2-2. FA2-2(pX9ΩHAT) was observed to be noncytolytic, indicating that CylM expression was essential for cytolysin expression.

As was observed for the previous site-specific mutations, FA2-2(pX9ΩHAT) actively secreted CylA, as evidenced by hemolysis at the junction of a cross-streak made with FA2-2(pAM9055). When FA2-2(pX9ΩHAT) was streaked across cells harboring the previously described insertion mutations in $cylL_L$(pX9ΩPAT) and $cylL_S$ (pX9ΩAAT), however, no hemolysis was observed at either of the streak junctions (Fig. 3), indicating that expression, posttranslational modification, or secretion of both $CylL_L$ and $CylL_S$ from construction pX9ΩHAT depended upon expression of functional CylM. To prove that the block in externalization of functional $CylL_L$ and $CylL_S$ observed for the strain bearing the site-specific lesion in *cylM* did not result from unknown polar effects on the gene immediately downstream, the following experiment was performed. pX9ΩHAT was transformed into the recombination-deficient *E. faecalis* strain, UV202. A functional *cylM* determinant was introduced in *trans* by filter mating (21) UV202(pX9ΩHAT) with JH2SS(pAM307), a pAD1 derivative which harbors a Tn*917* insertion in the ORF immediately downstream of *cylM*, termed *cylB* (Fig. 1) (17). The precise location of this transposon insertion within *cylB* has been determined by nucleotide sequence analysis (17, 42). Transconjugants were identified by selecting for recipient chromosomal markers (rifampin and fusidic acid resistances) and donor plasmid markers (erythromycin resistance). Introduction of pAM307 harboring an intact *cylM* gene, but a Tn*917* insertionally inactivated *cylB* gene, restored the cytolytic phenotype to UV202(pX9ΩHAT), demonstrating that mutations within *cylM* and *cylB* were capable of *trans* complementation and proving unambiguously that expression, modification, or secretion of $CylL_L$ and $CylL_S$ is dependent upon expression of the *cylM* gene.

Polar effects of Tn*917* insertion into *cylM*. The above-described experiment demonstrated that linker mutagenesis

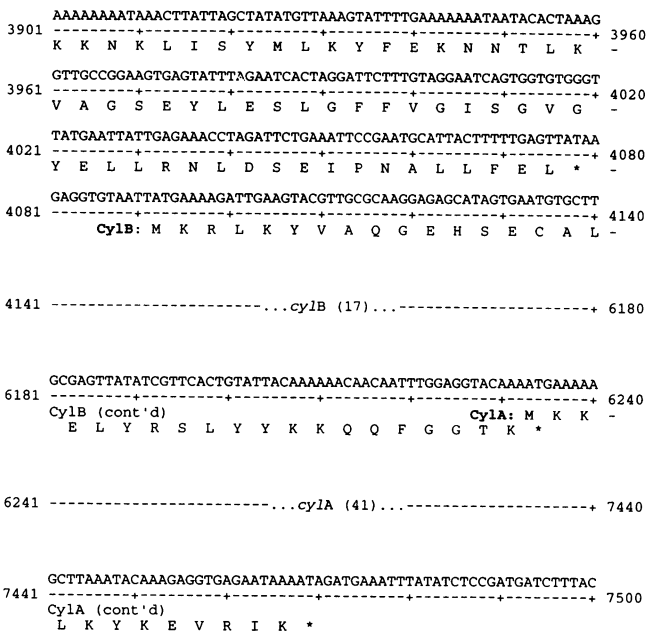

FIG. 2—*Continued.*

near the 5′ end of *cylM* did not have an observable polar effect on expression of downstream *cylB* and *cylA*, as determined by intracellular and extracellular *trans* complementation events, respectively, with Tn*917* insertion mutations. To clearly determine whether regulation of *cylM* expression was related or unrelated to expression of *cylB* and *cylA*, it was therefore of interest to determine whether the converse was true—that is, whether Tn*917* insertion within *cylM* exerts a polar effect on the expression of downstream *cylB* and *cylA* genes. Previous studies had shown that transposon insertion 5′ to *cylA* resulted in a noncytolytic but $CylA^+$ phenotype, with little discernible quantitative effect on expression of CylA (24, 41).

Among Tn*917* insertions into wild-type plasmid pAD1 previously examined for expression of CylA, two (pAM9064 and pAM9061 [24]) that mapped within the region determined in the present study to encode CylM (Fig. 1) were selected for complementation analysis. Each Tn*917* insertion derivative was introduced by conjugation into UV202 strains harboring each of the previously described linker insertion mutations in $cylL_L$ (pX9ΩPAT), $cylL_S$ (pX9ΩAAT), and *cylM* (pX9ΩHAT). Additionally, a previously described construction harboring a mutagenic *Cla*I linker inserted into the *cylB* reading frame (pRAS28-4Ω [17]) was tested in these complementation studies. Prior to testing in *E. faecalis* UV202, however, the mutated cytolysin operon carried by an *E. coli* replicon in the construction pRAS28-4Ω was subcloned into shuttle vector pAT28, as described above. This resulted in the construction pCylBΩAT, which was capable of replication in *E. faecalis* UV202.

The results of these intracellular *trans* complementation studies are summarized in Fig. 3. The only linker insertion mutations capable of complementing Tn*917* insertions within *cylM* were those with mutagenic linkers in $cylL_L$ or $cylL_S$. Linker insertions in *cylM* or *cylB* were not capable of complementing transposon insertions in *cylM*, regardless of where within the large *cylM* reading frame the transposon insertions occurred. The observation that Tn*917* insertion into *cylM* abrogates expression of *cylB*, whereas linker insertion does not, provides substantial evidence that *cylM* and *cylB* are transcribed within a polycistronic message which is terminated by Tn*917* insertion.

Externalization of $CylL_L$ but not $CylL_S$ requires the putative ATP-binding domain of CylB. Complementation in the form of hemolysis was observed at the junction of a cross-streak of FA2-2(pAM307) (a strain described above that harbors a Tn*917* insertion mutation in the *cylB* reading frame) and FA2-2(pX9ΩAAT), which is specifically defective in expression of $CylL_S$ (Fig. 1). This observation indicated that, despite a defect in *cylB*, FA2-2(pAM307) was capable of expressing and externalizing functional $CylL_S$ in a qualitatively undiminished capacity. No hemolysis was observed at the cross-streak junction of FA2-2(pAM307) and FA2-2(pX9ΩPAT) (which is specifically defective in $CylL_L$ expression), confirming that FA2-2(pAM307) was incapable of externalizing $CylL_L$ although capable of externalizing $CylL_S$. This observation suggested that perhaps a second secretion mechanism for $CylL_S$ externalization existed. The experiments were repeated using FA2-2(pCylBΩAT) (Fig. 1), which possesses an in-frame insertion of four additional codons in *cylB*. The linker insertion in pCylBΩAT occurs approximately 250 bp 3′ to the point of Tn*917* insertion in plasmid pAM307 as shown in Fig. 1. Surprisingly, complementation analysis, as described above, revealed that FA2-2(pCylBΩAT) was defective in externalizing both $CylL_L$ and $CylL_S$. This observation provided evidence that CylB mediates externalization of both $CylL_L$ and $CylL_S$, in apparent conflict with observations that cells harboring the Tn*917* insertion 250 bp upstream (pAM307) remain capable of secreting $CylL_S$.

On the basis of the known nucleotide sequence of Tn*917* at the point of insertion in pAM307 (17, 42), a modified, truncated CylB of 293 total residues, with the 14 C-terminal residues being derived from read-through into the end of Tn*917*, would be predicted. The linker mutagenesis in pCylBΩAT results in the insertion of amino acids M, H, R, and S at the point of modification but otherwise is predicted to result in a full-length CylB (17). It was therefore hypothesized that the additional amino acids inserted into CylB in the derivative encoded by pCylBΩAT result in a misfolded protein, or a protein otherwise incapable of orienting or organizing within the bacterial membrane in a structure capable of $CylL_L$ and $CylL_S$ recognition and/or externalization. In contrast, the Tn*917* truncated form of CylB expressed from pAM307 would lack the C-terminal ATP-binding domain (17) but otherwise may be capable of proper folding and organization into the membrane and therefore may be capable of secreting the smaller $CylL_S$ but not $CylL_L$.

To test this hypothesis, a frameshift mutation was introduced at the site of linker mutagenesis of pCylBΩAT. Briefly, pCylBΩAT was digested at the *Cla*I site engineered into the mutagenic linker used in pCylBΩAT construction (17) and the two-base protruding ends were filled in with the Klenow fragment of DNA polymerase I and with dGTP and dCTP. The addition of 2 nucleotides at the *Cla*I site within pCylBΩAT resulted in creation of a novel *Nru*I recognition site (in construction pCylBΩAT-Nru), which would be predicted to result in a truncated CylB derivative, lacking the C-terminal ATP-binding domain and approximately 80 amino acid residues larger than that expressed from pAM307. The prediction was that this frameshift and protein truncation (in the absence of aberrantly folded domains C-terminal to the point of modification) would restore normal folding and organization in the membrane of the amino-terminal half of CylB and therefore restore competence for secretion of $CylL_S$ but not $CylL_L$ (as observed for cells harboring pAM307). Hemolysis was in fact observed at the junction of a cross-streak of

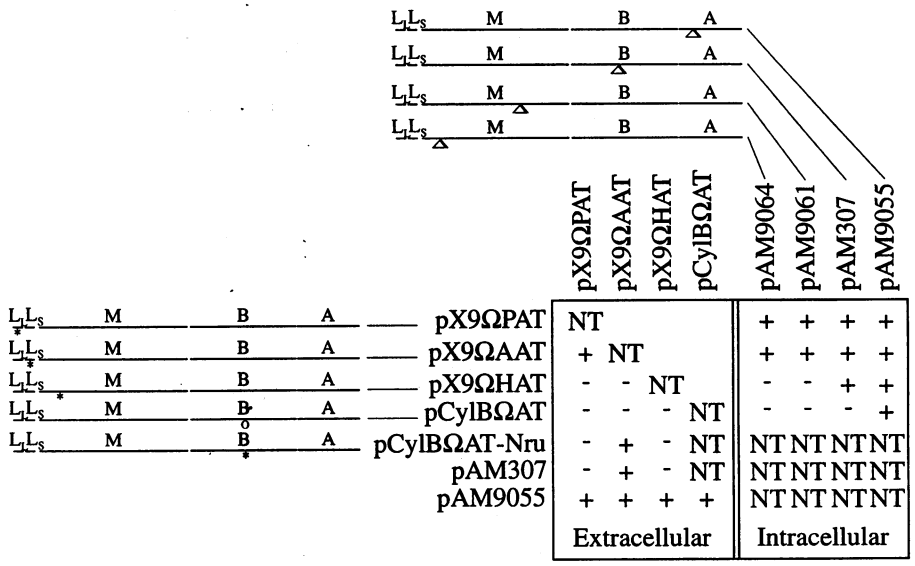

FIG. 3. Extracellular and intracellular *trans*-complementation analysis. NT, not tested. For extracellular complementation, a plus sign indicates the formation of a hemolysis zone at the junction of a cross-streak of *E. faecalis* FA2-2 harboring separately each of the plasmids indicated and a minus sign indicates no hemolysis zone at the junction of the cross-streak. For intracellular complementation, a plus sign indicates the formation of a hemolysis zone around colonies of the recombination-deficient *E. faecalis* UV202 when harboring both plasmids indicated and a minus sign indicates those combinations of plasmids that failed to confer a hemolytic phenotype. *, approximate positions of linker insertion mutations that result in a shift in reading frame; ∘, approximate position of a linker insertion mutation that, although introducing new codons, preserved the reading frame; Δ, approximate locations of Tn*917* insertions.

FA2-2(pCylBΩAT-Nru) and FA2-2(pX9ΩAAT) but not at the junction of FA2-2(pCylBΩAT-Nru) and FA2-2(pX9ΩPAT). Therefore, truncation of the defective CylB encoded by pCylBΩAT, resulting from the introduction of a frameshift in pCylBΩAT-Nru, restored competence for externalization of $CylL_S$ but not $CylL_L$.

Linker mutagenesis of an ORF 5′ to *cylL*$_L$. To demonstrate that a divergent, upstream ORF that initiates 118 bp 5′ to *cylL*$_L$ is not detectably related to cytolysin expression or immunity, a mutagenic linker was inserted into a unique *Eco*RV recognition site that occurs within this ORF. Insertion of the 10-bp mutagenic linker, as described in Materials and Methods, and transformation of the recombinant molecule into *E. coli* resulted in a hemolytic phenotype that was indistinguishable from those of transformants of the parental pXMA9 plasmid. Subcloning of the cytolysin operon with the inserted mutagenic linker into shuttle vector pAT28 (44) as a *Kpn*I-*Xba*I restriction fragment resulted in plasmid pX9ΩEAT. Electrotransformation of pX9ΩEAT into FA2-2 resulted in transformants with qualitatively normal hemolytic and bacte-

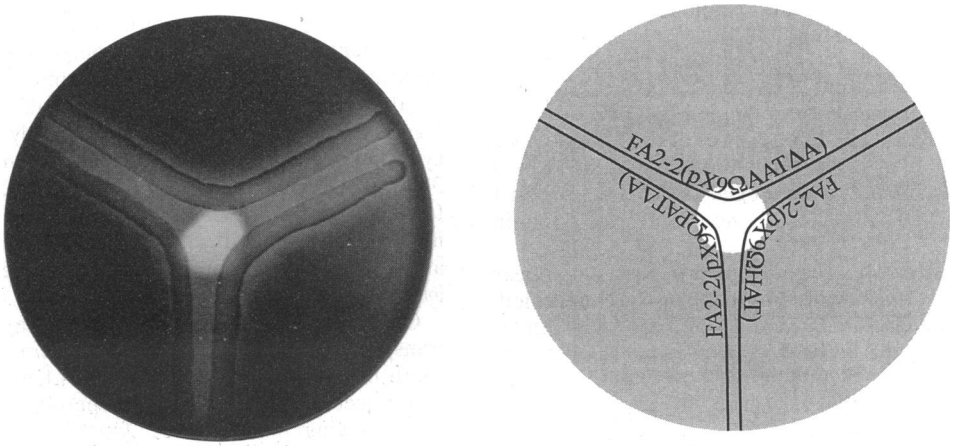

FIG. 4. Cytolytic activity requires three independently expressed and secreted components. Cytolytic activity occurs only at the junction of a three-way streak of FA2-2(pX9ΩPATΔA) ($CylL_L^-$ $CylL_S^+$ $CylM^+$ $CylB^+$ $CylA^-$ [which contributes $CylL_S$; Fig. 1]); FA2-2(pX9ΩAATΔA) ($CylL_L^+$ $CylL_S^-$ $CylM^+$ $CylB^+$ $CylA^-$ [which contributes $CylL_L$]); and FA2-2(pX9ΩHAT) ($CylL_L^+$ $CylL_S^+$ $CylM^-$ $CylB^+$ $CylA^+$ [which is phenotypically $CylL_L^-$ and $CylL_S^-$ but contributes CylA]). No activity occurs with any combination of two secreted factors, as can be seen along the parallel legs of the streaks.

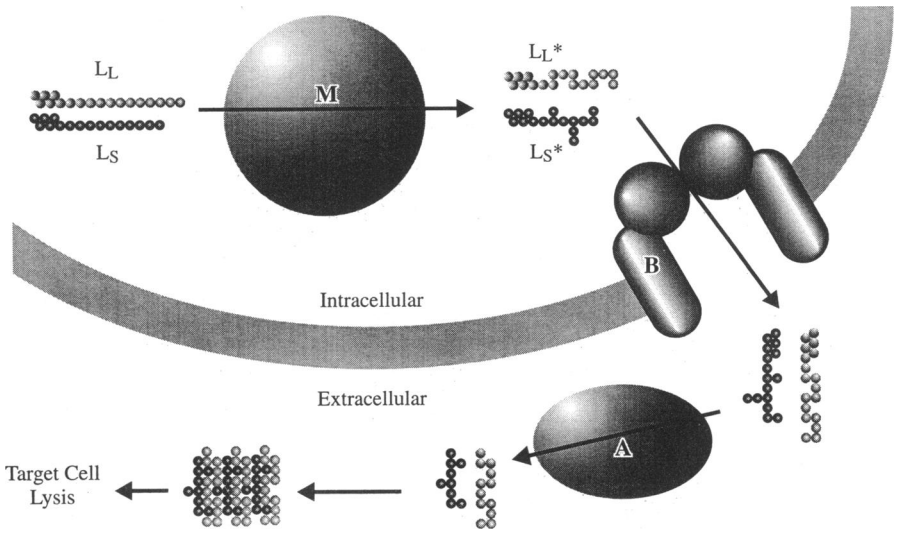

FIG. 5. Model of *E. faecalis* cytolysin maturation, externalization, and activation. Secretion of $CylL_L$ (L_L) and $CylL_S$ (L_S) in a state that can be activated extracellularly by CylA requires expression of a functional CylM (M) gene product, indicating that CylM is involved in maturation of both $CylL_L$ and $CylL_S$ (putatively to $CylL_L$* [L_L*] and $CylL_S$ [L_L*]). Following direct or indirect interaction with CylM, $CylL_L$ and $CylL_S$ are secreted via the CylB ATP-binding cassette transporter, CylB (15, 17), although $CylL_S$* export does not require the ATP-binding domain of CylB. Once outside of the cell, the cytolysin precursor components are activated by CylA (A) (a protein resembling subtilisin class serine proteases that is secreted independently of *cylB*, presumably by a conventional *secA-secY* type process [41]) and target cell lysis is effected by a process that may involve $CylL_L$ and $CylL_S$ interaction (either in solution or on the membrane) since neither is active individually.

riolytic phenotypes. This evidence indicates that the divergent, upstream ORF is not detectably related to expression of the cytolysin.

DISCUSSION

We have shown by mutagenesis and complementation that production of cytolytic activity by *E. faecalis* requires expression of five reading frames: *cylL*$_L$, *cylL*$_S$, *cylM*, *cylB*, and *cylA*. Expression of all five reading frames is required for production of both hemolytic and bacteriolytic activities, and it is now unambiguous that these represent two manifestations of the same activity.

CylA has been shown previously to activate the cytolysin precursor(s) extracellularly (18, 24, 41). Furthermore, CylA possesses structural and biochemical similarity to subtilisin class serine proteases, suggesting that the extracellular activation mechanism is proteolysis (41). CylB was reported previously to bear structural resemblance to members of the large class of bacterial toxin transporters typified by HlyB of the *E. coli* α-hemolysin operon (17). On the basis of this resemblance and the dependence of cytolysin precursor externalization on a functional *cylB* determinant, it was suggested that the product of *cylB* mediates externalization of the *E. faecalis* cytolysin precursor (17).

In the present study, two inferred gene products that appear to represent the *E. faecalis* cytolysin precursor were identified. Each of these products, $CylL_L$ and $CylL_S$, can be complemented extracellularly, as can be demonstrated at the junction of a cross-streak (Fig. 4). A number of observations suggest that $CylL_L$ and $CylL_S$ are related to a rapidly expanding class of bacteriocins of gram-positive origin—the lantibiotics. The inferred primary amino acid sequence of each of the products is hydrophilic toward the amino-terminal end and becomes hydrophobic toward the carboxyl terminus as is typical for lantibiotics (1, 30, 31, 40). The small sizes of the inferred products of *cylL*$_L$ and *cylL*$_S$ (68 and 63 amino acids, respectively) are also similar to those reported for lantibiotic precursors (1, 31, 40). Each of the inferred products is relatively rich in cysteine, and the cysteine residues are flanked by hydroxylated amino acids, which in lantibiotics are combined to form lanthionine and lanthionine derivatives.

Common features with lantibiotics and their genetic determinants extend beyond the structural similarities that occur between lantibiotic precursors and $CylL_L$ and $CylL_S$. Where lantibiotic genes have been studied, each has been observed to exist as an integral part of a complex operon. Common among lantibiotic operons are dedicated lantibiotic secretion proteins related to the HlyB family (7, 33, 39), as was first reported for CylB (17). A recent comparison of these ATP-binding cassette transporters revealed that the closest relatives of *cylB* were in fact those encoded within lantibiotic operons (15). A subtilisin class serine protease activator, analogous to CylA (41), has been identified as an integral component in the maturation pathway for the lantibiotics epidermin (39) and nisin (45). Further, analogs to the C-terminal half of the *cylM* reading frame, which was observed in this study to be essential for cytolysin expression, also occur within lantibiotic operons (7, 36, 39). Although little evidence that precisely defines a role for analogs of CylM exists, they have been hypothesized to be involved in the modification of the ribosomally incorporated amino acids cysteine, serine, and threonine into the characteristic lanthionine residues (33, 39).

On the basis of the results of deletion analysis, linker and transposon insertional mutagenesis, *trans* complementation (both intra- and extracellular), and identification of parallels and precedents observed within operons encoding lantibiotics, the following model for *E. faecalis* cytolysin expression is proposed (Fig. 5).

(i) Genes related to intracellular maturation (*cylM*) and secretion (*cylB*) of the cytolysin precursors ($CylL_L$ and $CylL_S$) are transcribed as a unit, perhaps including the coding information for the cytolysin precursors as well. This deduction is

based on the observation of a strong polar effect of Tn*917* insertion within various regions of *cylM* on the expression of *cylB*, as detected by intracellular complementation. The *cylA* gene, which encodes the extracellular activator of the cytolysin, appears to be transcribed independently, since previous studies showed that Tn*917* insertions within regions of the cytolysin operon 5′ to the *cylA* structural gene do not detectably affect its expression (24, 41) (even though expression of a gene immediately 3′ to some transposon insertions [i.e., *cylB*] is abrogated). The independently expressed *cylA* gene product appears to be secreted via a conventional signal peptide-mediated event, since previous studies found that inactivation of the putative cytolysin secretory protein, CylB, did not affect externalization of CylA (17). Further, CylA appears to possess a conventional signal peptide sequence (41).

(ii) CylM appears to modify the cytolysin precursors $CylL_L$ and $CylL_S$ (a theory based on similarity between the structure and inferred function of CylM and analogs occurring in lantibiotic operons [7, 36, 39] and based on the observation in the present study that secretion of either $CylL_L$ or $CylL_S$ in an activatable form requires a functional *cylM* determinant). This posttranslational modification may involve the fusion of dehydrated hydroxyl amino acids and cysteines within $CylL_L$ and $CylL_S$ to lanthionine bridges or related structures, since both cytolysin precursors contain multiple potential modification sites of sequence (S/T)XXXC, similar to the modification signature observed within other lantibiotic precursors (1, 31, 40).

(iii) Following modification, the cytolysin precursors $CylL_L$ and $CylL_S$ appear to be secreted through CylB. However, since it was observed in the present study that truncation of the C-terminal half of CylB permitted externalization of activatable $CylL_S$, but not $CylL_L$, $CylL_S$ appears to be passively externalized through the putative CylB channel, whereas $CylL_L$ appears to require the coupled energetics provided by the CylB C-terminal ATP-binding domain. The signal that targets $CylL_L$ and $CylL_S$ for secretion remains to be identified experimentally. $CylL_L$ and $CylL_S$ share in common a run of 25 of 26 identities, with few other similarities in primary amino acid sequences. Similar amino-terminal hydrophilic sequences appear to target other lantibiotics for secretion by CylB-related proteins encoded by other lantibiotic operons (1, 31, 40). If the conserved domain is related to $CylL_L$ and $CylL_S$ targeting, then positional effects may determine its dependence on CylB energetics for export, since the conserved peptide sequence occurs near the amino terminus of $CylL_L$ but is centrally located within $CylL_S$. Once outside the cell, CylA appears to activate one or both cytolysin precursors ($CylL_L$ and/or $CylL_S$) by limited proteolysis as discussed previously (41). Previous studies have suggested that immunity to the activated cytolysin for the producing bacterium may be achieved by further proteolysis near the surface of the cell, where CylA occurs at high levels (41).

Taken together, the evidence that the *E. faecalis* cytolysin represents a new class of cytolytic agent related to lantibiotics is compelling. The actual presence of posttranslationally modified amino acids within purified $CylL_L$ and $CylL_S$ remains to be demonstrated and is the aim of current studies. Despite considerable similarity at the genetic level, the *E. faecalis* cytolysin differs from the class of lantibiotics typified by nisin (30, 34) in several respects. First, the *E. faecalis* cytolysin consists of two dissimilar precursors, both of which are required to effect target cell lysis. A second important difference is that the *E. faecalis* cytolysin is not limited in target cell specificity to only prokaryotic cells. In fact, the *E. faecalis* cytolysin, originally identified as a hemolysin (43), has been shown to make an important contribution to the severity of disease in endophthalmitis (29) and endocarditis (6) models and the cytolytic phenotype is enriched among clinical isolates of the organism (22, 26). The observation of an association between the *E. faecalis* cytolysin and bacterial virulence and the finding of similarities between the *E. faecalis* cytolysin and lantibiotics such as nisin (which has been approved for use in food in several countries [38]) indicate that the potential for engineering lantibiotics past some threshold where undesired toxic or cytolytic activities may arise exists. Further comparisons between the chemical, structural, and biological properties of the *E. faecalis* cytolysin and classical lantibiotics will help to define these limits.

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REFERENCES

1. **Bannerjee, S., and J. N. Hansen.** 1988. Structure and expression of a gene encoding the precursors of subtilisin, a small protein antibiotic. J. Biol. Chem. **263:**9508–9514.
2. **Benson, S. A., M. N. Hall, and T. J. Silhavy.** 1985. Genetic analysis of protein export in *Escherichia coli* K-12. Annu. Rev. Biochem. **54:**101–134.
3. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect. Immun. **57:**2037–2040.
4. **Brock, T. D., and J. M. Davie.** 1963. Probable identity of a group D hemolysin with a bacteriocin. J. Bacteriol. **86:**708–712.
5. **Brock, T. D., B. Peacher, and D. Pierson.** 1963. Survey of the bacteriocines of enterococci. J. Bacteriol. **86:**702–707.
6. **Chow, J. W., L. A. Thal, M. B. Perri, J. A. Vazquez, S. M. Donabedian, D. B. Clewell, and M. J. Zervos.** 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. **37:**2474–2477.
7. **Chung, Y. J., M. T. Steen, and J. N. Hansen.** 1992. The subtilin gene of *Bacillus subtilis* ATCC 6633 is encoded in an operon that contains a homolog of the hemolysin B transport protein. J. Bacteriol. **174:**1417–1422.
8. **Clewell, D. B.** 1993. Bacterial sex pheromone-induced plasmid transfer. Cell **73:**9–12.
9. **Clewell, D. B.** 1993. Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*, p. 349–368. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York.
10. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152:**1220–1230.
11. **Cruz-Rodz, A., and M. S. Gilmore.** 1990. High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. Mol. Gen. Genet. **224:**152–154.
12. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
14. **Dybvig, K., S. K. Hollingshead, D. G. Heath, D. B. Clewell, F. Sun, and A. Woodard.** 1992. Degenerate oligonucleotide primers for

enzymatic amplification of *recA* sequences from gram-positive bacteria and mycoplasmas. J. Bacteriol. **174:**2729–2732.
15. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
15a. **Gaither, K., and M. S. Gilmore.** Unpublished data.
16. **Gilmore, M. S., K. S. Gilmore, and W. Goebel.** 1985. A new strategy for ordered DNA sequencing based on a novel method for the rapid purification of near-milligram quantities of a cloned restriction fragment. Gene Anal. Tech. **2:**108–114.
17. **Gilmore, M. S., R. A. Segarra, and M. C. Booth.** 1990. An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. Infect. Immun. **58:**3914–3923.
18. **Granato, P. A., and R. W. Jackson.** 1969. Bicomponent nature of lysin from *Streptococcus zymogenes*. J. Bacteriol. **100:**865–868.
19. **Hansen, J. N.** 1993. Antibiotics synthesized by posttranslational modification. Annu. Rev. Microbiol. **47:**535–564.
20. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates target breakpoints for DNA sequencing. Gene **28:**351–359.
21. **Hershfield, V.** 1979. Plasmids mediating multiple drug resistance in group B *Streptococcus*: transferability and molecular properties. Plasmid **2:**137–149.
22. **Huycke, M. M., C. A. Spiegel, and M. S. Gilmore.** 1991. Bacteremia caused by hemolytic high-level gentamicin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35:**1626–1634.
23. **Ike, Y., and D. B. Clewell.** 1992. Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. J. Bacteriol. **174:**8172–8177.
24. **Ike, Y., D. B. Clewell, R. A. Segarra, and M. S. Gilmore.** 1990. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: insertional mutagenesis and cloning. J. Bacteriol. **172:**155–163.
25. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. **45:**528–530.
26. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1987. High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. J. Clin. Microbiol. **25:**1524–1528.
27. **Jett, B. D., and M. S. Gilmore.** 1990. The growth-inhibitory effect of the *Enterococcus faecalis* bacteriocin encoded by pAD1 extends to the oral streptococci. J. Dent. Res. **69:**1640–1645.
28. **Jett, B. D., M. M. Huycke, and M. S. Gilmore.** 1994. Virulence of enterococci. Clin. Microbiol. Rev. **7:**462–478.
29. **Jett, B. D., H. G. Jensen, R. E. Nordquist, and M. S. Gilmore.** 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. **60:**2445–2452.
30. **Jung, G.** 1991. Lantibiotics—ribosomally synthesized biologically active polypeptides containing sulfide bridges and α-β-didehydroamino acids. Angew. Chem. Int. Ed. Eng. **30:**1051–1068.
31. **Kaletta, C., and K.-D. Entian.** 1989. Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of this peptide product. J. Bacteriol. **171:**1597–1601.
32. **Kaletta, C., K.-D. Entian, R. Kellner, G. Jung, M. Reis, and H.-G. Sahl.** 1989. Pep5, a new lantibiotic: structural gene isolation and prepeptide sequence. Arch. Microbiol. **152:**16–19.
33. **Klein, C., C. Kaletta, N. Schnell, and K.-D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **58:**132–142.
34. **Kolter, R., and F. Moreno.** 1992. Genetics of ribosomally synthesized peptide antibiotics. Annu. Rev. Microbiol. **46:**141–163.
35. **König, W., Y. Faltin, J. Scheffer, H. Schöffler, and V. Braun.** 1987. Role of cell-bound hemolysin as a pathogenicity factor for *Serratia* infections. Infect. Immun. **55:**2554–2561.
36. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. De Vos.** 1993. Characterization of the nisin gene cluster *nis*ABTCIPR of *Lactococcus lactis*: requirement of expression of the *nis*A and *nis*I genes for development of immunity. Eur. J. Biochem. **216:**281–291.
37. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Molitor, E., and H.-G. Sahl.** 1991. Applications of nisin: a literature survey, p. 434–439. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
39. **Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, V. Ungermann, F. Gotz, and K. D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic epidermin. Eur. J. Biochem. **204:**57–68.
40. **Schnell, N., K. D. Entian, U. Schneider, F. Gotz, H. Zahner, R. Kellner, and G. Jung.** 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. Nature (London) **333:**276–278.
41. **Segarra, R. A., M. C. Booth, D. A. Morales, M. M. Huycke, and M. S. Gilmore.** 1991. Molecular characterization of the *Enterococcus faecalis* cytolysin activator. Infect. Immun. **59:**1239–1246.
42. **Shaw, J. H., and D. B. Clewell.** 1985. Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **164:**782–796.
42a. **Siezen, R.** Personal communication.
43. **Todd, E. W.** 1934. A comparative serological study of streptolysins derived from human and from animal infections, with notes on pneumococcal haemolysin, tetanolysin, and staphylococcus toxin. J. Pathol. Bacteriol. **39:**299–321.
44. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1990. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in Gram-positive bacteria. Nucleic Acids Res. **18:**4296.
45. **van der Meer, J. R., J. Polman, M. M. Beerthuyzen, R. J. Siezen, O. P. Kuipers, and W. M. De Vos.** 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. **175:**2578–2588.
46. **Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Hemolysin contributes to virulence in extraintestinal *Escherichia coli* infections. Nature (London) **294:**665–667.
47. **Welch, R. A., and S. Falkow.** 1984. Characterization of *Escherichia coli* hemolysins conferring quantitative differences in virulence. Infect. Immun. **43:**156–160.
48. **Williams, N. B., M. A. Forbes, E. Blau, and C. F. Eickenberg.** 1950. A study of the simultaneous occurrence of enterococci, lactobacilli, and yeasts in saliva from human beings. J. Dent. Res. **29:**563–570.
49. **Yagi, Y., and D. B. Clewell.** 1980. Recombinant-deficient mutant of *Streptococcus faecalis*. J. Bacteriol. **143:**966–970.