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Generation of Auxotrophic Mutants of *Enterococcus faecalis*

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A 22-kb segment of chromosomal DNA from *Enterococcus faecalis* OG1RF containing the pyrimidine biosynthesis genes *pyrC* and *pyrD* was previously detected as complementing *Escherichia coli pyrC* and *pyrD* mutations. In the present study, it was found that the *E. faecalis* pyrimidine biosynthetic genes in this clone (designated pKV48) are part of a larger cluster resembling that seen in *Bacillus* spp. Transposon insertions were isolated at a number of sites throughout the cluster and resulted in loss of the ability to complement *E. coli* auxotrophs. The DNA sequences of the entire *pyrD* gene of *E. faecalis* and selected parts of the rest of the cluster were determined, and computer analyses found these to be similar to genes from *Bacillus subtilis* and *Bacillus caldolyticus* pyrimidine biosynthesis operons. Five of the transposon insertions were introduced back into the *E. faecalis* chromosome, and all except insertions in *pyrD* resulted in pyrimidine auxotrophy. The prototrophy of *pyrD* knockouts was observed for two different insertions and suggests that *E. faecalis* is similar to *Lactococcus lactis*, which has been shown to possess two *pyrD* genes. A similar analysis was performed with the *purL* gene from *E. faecalis*, contained in another cosmid clone, and purine auxotrophs were isolated. In addition, a pool of random transposon insertions in pKV48, isolated in *E. coli*, was introduced into the *E. faecalis* chromosome en masse, and an auxotroph was obtained. These results demonstrate a new methodology for constructing defined knockout mutations in *E. faecalis*.

Enterococci, which have been recognized as a cause of many infections, including infectious endocarditis (26), are the second- to third-most-common pathogens found in hospital-acquired infections. Because of an alarming increase in resistance to different antibiotics, therapy of enterococcal infections has become increasingly difficult. Thus, there is a need for more basic knowledge of these organisms in order to understand how to control or prevent enterococcal infections through developing either therapeutics or vaccines. In a previous report, we described the physical map of the genome of *Enterococcus faecalis* OG1RF as well as several cosmid clones that complemented *Escherichia coli* auxotrophs (27). In this study, the possibility of generating enterococcal *pyr* and *pur* auxotrophs by allelic replacement was tested. This methodology is important for developing genetic approaches for studying enterococcal virulence. We also describe the further characterization of a clone containing *pyr* biosynthesis genes and the determination of the arrangement of the *pyr* gene cluster by mapping, sequencing, and complementation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. The bacterial strains used in this work are described in Table 1. The cosmid vector pLAFRx is a derivative of pLAFR which contains *oriT* of RK2 and a polylinker for cloning (12, 17). pKV48 and pKV53 are cosmids from the previously constructed enterococcal genomic libraries from *E. faecalis* OG1RF (Table 1) which complement *E. coli pyrC* and *purL* auxotrophs, respectively (27). The vector used for subcloning was pBluescript II SK+ phagemid (36). Transposon mutagenesis was performed by using Tn*phoA* (22) from a λTn*phoA* derivative that also carried the *c*I857 and *P*(Am)80 mutations and mini-γδ-200 (mγδ) from strain CBK884, obtained from Michael G. Caparon (11). Lambda *c*I857 was used for transduction in *E. coli.*

Media. *E. coli* transformants and transconjugants were grown on LB agar (25) with appropriate antibiotics. M63 salts (25) supplemented with 0.2% glucose, thiamine (100 μg/ml), $MgSO_4$ (1 mM), and 1.5% agar was used as the minimal medium for growth of *E. coli*. For testing of possible enterococcal auxotrophs, Davis minimal medium with supplements (DMMS) as described by Murray et al. (27) was used as the defined synthetic enterococcal broth medium. DMMS agar contains 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Uracil or adenosine was added at a concentration of 40 μg/ml for the growth of auxotrophs in defined media. The media for electroporation included brain heart infusion (BHI; Difco), BYGT (BHI, yeast extract, glucose, Tris) (8), and SR (tryptone, yeast extract, sucrose, glucose, gelatin, agar) (7). Concentrations of antibiotics used for selection were as follows: tetracycline at 12.5 μg/ml, chloramphenicol at 20 to 40 μg/ml, ampicillin at 150 μg/ml, nalidixic acid at 20 μg/ml, and kanamycin at 25 μg/ml for *E. coli* and rifampin at 100 μg/ml, fusidic acid at 25 μg/ml, and kanamycin at 2,000 μg/ml for OG1RF.

Routine DNA techniques. Plasmid DNA was isolated by a slightly modified alkaline sodium dodecyl sulfate protocol (4): solution I contained no lysozyme, and solution III was made of 3 M potassium acetate and 5 M glacial acetic acid. Preparation of competent cells and transformation of plasmid DNA were performed as described previously (6) or by electroporation (5) using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, Calif.). Transducing lysate preparation and transduction were performed by using standard procedures (34). Southern transfer and hybridizations were carried out as described previously (27). PCR was performed by using a PCR Optimizer Kit (Invitrogen, San Diego, Calif.) and a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.).

Complementation. The cosmid pKV48 was transferred into *E. coli* pyrimidine auxotrophic mutants by conjugative mobilization, transformation, or transduction. For conjugative mobilization, *E. coli* MG1655 containing pKV48, one of the *E. coli* chloramphenicol-resistant pyrimidine auxotrophs (GE1721 or GE1728; Table 1), and the mobilizing strain GE3422, which contains pRK2013 (10), were each grown in LB broth to log phase (about 4 h); 200-μl aliquots of the cultures were combined and incubated at 37°C for 2 h without shaking. The mating mixture was diluted and spread onto LB-tetracycline-chloramphenicol. For *E. coli pyrAa*, *pyrAb*, *pyrB*, *pyrF*, and *pyrE* auxotrophs (Table 1), pKV48 was introduced by transformation or transduction, selecting for tetracycline resistance. The *E. coli pyr* auxotrophs containing pKV48 were scored for the ability to grow on the M63 minimal agar. pKV48 derivatives containing transposon insertions were also transduced into the seven *E. coli pyr* auxotrophs, selecting for both kanamycin and tetracycline resistance. Complementation was tested by comparing colony formation on the following media: M63 agar (30 and 37°C), M63-kanamycin and M63-uracil. pKV53 and its derivatives were tested similarly in an *E. coli purL* mutant (GE1726), with or without adenosine, for complementation.

Transposon mutagenesis. To identify the positions of the *pyr* genes, pKV48 was mutagenized by using the transposons Tn*phoA* and mγδ. For mutagenesis with Tn*phoA*, a fresh λTn*phoA* stock was prepared in LE392. *E. coli* MG1655 containing pKV48 was grown for about 3 h, and λTn*phoA* was added at a multiplicity of infection of approximately 1. Following incubation at 30°C for 15 min, the cells were diluted 1:10 into LB broth to allow outgrowth for 4 h at 37°C with aeration. Aliquots of 200 μl were plated on LB-kanamycin with 40 μg of XP

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference(s) and/or source
Strains		
E. coli		
MG1655	F^- λ^-	16
LE392	*e*14-(*mcrA*) *hsdR514 supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	34
KK2186	*endA1 hsdR4 supE sbcI5 thi-1 strA*	18
GE3422	LE392 containing pRK2013, Kan^r	10
W1485	F^+ λ^-	2
GE1728	MG1655 *pyrC*::Tn*10*dCamNS Cam^r	27
GE1721	MG1655 *pyrD*::Tn*10*dCamNS Cam^r	27
GE1726	MG1655 *purL*::Tn*10*dCamNS Cam^r	27
RC50	*carA50 thi-1 malA xyl-7 strA135* λ^r λ^- *tsx-273* (also referred to as *pyrAa*)	24; CGSC[a]
Hfr3000YA289	*pyrB289* λ^- *relA1 spoT1 thi-1*	CGSC
X82	*pyrF287 lacZ53*(Am) λ^- *trpC60 hisG1 rpsL8*	CGSC
AT2538	*pyrE60* λ^- *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 supE44 galK2 hisG4 fbD1 rpsL31 xyl-5 mtl-1 argE3*	CGSC
JEF8	*carB8 thr-31 crelA1 metB1* (also referred to as *pyrAb*)	CGSC
CBK884	mγδ mutagenesis donor strain containing pMGD5 and pXRD4043, Kan^r Cam^r	11
LW49	mγδ mutagenesis recipient strain, Nal^r	11
E. faecalis		
OG1RF	OG1 Rif^r Fus^r (spontaneous)	27
TX5063a	OG1RF*purL*::mγδ5062, generated by allelic replacement with pTX5602; auxotroph; Kan^r Rif^r Fus^r	This study
TX5066a	OG1RF*pyrD*::mγδ220, generated by allelic replacement with pTX5601; no auxotrophic phenotype; Kan^r Rif^r Fus^r	This study
TX5077	OG1RF*pyrE*::mγδ217 generated by allelic replacement with pBEM217; auxotroph; Kan^r Rif^r Fus^r	This study
TX5078	OG1RF*pyrC*::mγδ219, generated by allelic replacement with pBEM219; auxotroph; Kan^r Rif^r Fus^r	This study
TX5079	OG1RF*pyrR*::mγδ218, generated by allelic replacement with pBEM218; auxotroph; Kan^r Rif^r Fus^r	This study
TX5082	OG1RF*pyrD*::mγδ221, generated by allelic replacement with pBEM221; no auxotrophic phenotype; Kan^r Rif^r Fus^r	This study
TX5081	OG1RF*pyr*::mγδC22, generated by allelic replacement with a pool of pKV48::mγδ; auxotroph; Kan^r Rif^r Fus^r	This study
Plasmids		
pBluescript	2,958-bp phagemid derived from pUC19, Amp^r	36
pLAFRx	21.6-kb cosmid vector with *mob* site and *oriT* of RK2, Tet^r	27
pKV48	pLAFRx containing a 22-kb fragment from OG1RF with *pyr* gene cluster, Tet^r	27, this study
pKV53	pLAFRx containing a 24-kb fragment from OG1RF; *purL* complementing clone; Tet^r	27, this study
pBEM201-pBEM213	pKV48 with Tn*phoA* insertion at positions 201–213, Tet^r Kan^r	This study
pBEM214	3-kb *Eco*RI-*Bam*HI fragment of pKV48 cloned into pBluescript, Amp^r	This study
pBEM215	Deletion of pBEM207, Tet^r Kan^r	This study
pBEM216	2.5-kb *Not*I-*Hin*dIII fragment of pBEM207 cloned into pBluescript, Amp^r	This study
pBEM217	pKV48*pyrE*::mγδ217 Tet^r Kan^r	This study
pBEM218	pKV48*pyrR*::mγδ218 Tet^r Kan^r	This study
pBEM219	pKV48*pyrC*::mγδ219 Tet^r Kan^r	This study
pBEM220	pKV48*pyrD*::mγδ220 Tet^r Kan^r (also called pTX5061)	This study
pBEM221	pKV48*pyrD*::mγδ221 Tet^r Kan^r	This study
pBEM222	pKV48*pyrAa*::mγδ222 Tet^r Kan^r	This study
pTX5062	pKV53*purL*::mγδ5062 Tet^r Kan^r	This study

[a] CGSC, *E. coli* Genetic Stock Center, Yale University.

(5-bromo-4-chloro-3-indolyl phosphate) per ml and incubated for 2 to 3 days at 37°C. Blue colonies were saved separately, and then all colonies were pooled. Cosmids contained in the pool were mobilized to the chloramphenicol-resistant auxotrophic *E. coli* recipients by triparental mating with W1485 as the helper strain. Strains containing cosmids with a Tn*phoA* insertion(s) were selected on LB-tetracycline-chloramphenicol-kanamycin plates, and then the transposon insertion in the cosmid was confirmed by restriction endonuclease digestion. Thirteen cosmids (named pBEM201 to pBEM213) with Tn*phoA* insertions at different positions in pKV48 were used for subsequent analysis.

To make knockout mutations for construction of subsequent enterococcal mutants with insertions in *pyr* or *pur* genes, the transposon mγδ was chosen to mutagenize pKV48 and pKV53. The kanamycin resistance determinant in mγδ is expressed in both gram-positive and gram-negative hosts and can thus be used as a selective marker for introducing insertions into enterococci. The mutagenesis strain, CBK884(pMGD5, pXRD4043), which contains mγδ on a conjugative donor plasmid and a source of γδ transposase, was transformed with pKV48 or pKV53 with selection on LB-tetracycline-kanamycin. A single transformant colony was then grown in 3 ml of LB-chloramphenicol (40 μg/ml) with 0.5 mM IPTG (isopropylthiogalactoside) for 3 h to induce transposase production; the recipient strain, LW49, was grown in LB-nalidixic acid. Donor cells (0.5 ml) and 0.2 ml of recipient cells were mixed in an 18-mm-diameter test tube and incubated for 30 min in a 37°C water bath without shaking. Then 5 ml of prewarmed LB broth containing IPTG was added, and the mixture was incubated for 3 h without agitation. Exconjugants were selected on LB-tetracycline-kanamycin-nalidixic acid. The pools of exconjugants containing pKV48 or pKV53 with mγδ insertions were transduced into several *E. coli pyr* auxotrophs or the *pur* auxotroph and purified for single colonies. Purified single colonies were then transferred with toothpicks onto both M63 agar and LB-tetracycline-kanamycin. Strains with mutant cosmids that no longer complemented the relevant *E. coli* auxotroph were saved for subsequent sequencing and allelic replacement studies. Transposon insertions were also mapped according to restriction endonuclease digestion patterns.

Restriction mapping and subcloning. pKV48 and selected derivatives with Tn*phoA* insertions were analyzed by digestion with *Eco*RI, *Bam*HI, *Cla*I, and *Hin*dIII (Promega, Madison, Wis.), using buffers supplied with the enzymes or KGB reaction buffer (34). Digested plasmid DNA was electrophoresed in 0.6% *ultra*PURE electrophoresis-grade agarose (GIBCO BRL, Life Technologies Inc., Gaithersburg, Md.) in 1× TBE (0.09 M Tris base, 0.09 M boric acid, 0.002 M EDTA). The 1-kb DNA ladder and *Hin*dIII-digested λ DNA fragments (GIBCO BRL) were used as molecular weight markers. Determination of fragment sizes by measurements of photographed gels and comparisons of endonuclease restriction patterns were used to generate a restriction map of pKV48.

To generate subclones of pKV48, both pBluescript and pKV48 were double digested with 10 U each of *Eco*RI and *Bam*HI (Promega) for 2 to 4 h in KGB

buffer. Digested DNA was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (Amresco, Solon, Ohio), and the supernatant was precipitated with 2 volumes of 95% ethanol. DNA fragments excised from agarose gels were purified by electroelution (38). Digested pBluescript and pKV48 were then mixed and ligated with 40 U of T4 DNA ligase (New England Biolabs, Beverly, Mass.), using the reaction buffer supplied. All subclones were transformed into *E. coli* KK2186. Insertions in pBluescript were detected on LB-ampicillin with 2 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) per plate. White colonies were randomly selected for plasmid extraction and digestions.

DNA sequencing and analyses. One-pass DNA sequencing was performed for the *pyr* and *purL* genes, with the exception of *pyrD*, whose DNA sequence was determined until all ambiguities were resolved. The nucleotide sequence of part of the *pyr* cluster was determined by the dideoxy-chain termination method (35), using automated sequencing (32). Some of the cluster and most of the *pyrD* gene were also manually sequenced with ^{35}S-labeled nucleotides. The Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc. [ABI], Foster City, Calif.) was used for PCR amplification according to the protocol provided with the kit. Samples were analyzed using an ABI model 373A DNA sequencer. Data were processed with a sequencing analysis program from ABI. The primers used for sequencing included pBluescript T7 and T3 primers (Stratagene, Menasha, Wis.), a primer which is complementary to both inverted repeat sequences of Tn*phoA* (GW26, 5′-ACG GGA AAG GAT CCG TCC AGG-3′), a primer complementary to one end of mγδ (mγδ-R, 5′-GAT TTA GGA TAC ACG GAA TTT CG-3′), a primer complementary to the *phoA* gene in Tn*phoA* (5′-CTG AGC AGC CGG GTT TTC CAG AAC AG-3′), and primers specific to sequences generated in this study. Both the *phoA* primer and the mγδ-R primer, which is complementary to the mγδ right-arm sequence, were used for sequencing DNA flanking the transposon. Cosmids pBEM220 (also called pTX5061), pTX5062, pBEM201, pBEM206, pBEM208, pBEM209, pBEM217 to pBEM219, pBEM221, and pBEM222 were used for direct sequencing without subcloning, using the *phoA* primer or the mγδ-R primer. The GW26 primer was used when only one of the Tn*phoA* inverted repeats was present such as in pBEM215 and pBEM216. Double-stranded cosmid DNA was prepared for automated sequencing using a Magic Minipreps DNA Purification System Sample Kit (Promega) or by equilibrium centrifugation in CsCl-ethidium bromide gradients (34).

DNA sequence analysis was performed with the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis package, version 7.2. DNA and protein homology searches were performed with either the BLAST or the FASTA/TFASTA sequence comparison algorithms (30). Both searches were performed via the GenEMBL and SWISS-PROT databases.

Allelic replacement. Cosmids with mγδ insertions that had lost the ability to complement an auxotroph of *E. coli* (including pBEM220, pTX5062, pBEM217 to pBEM219, and pBEM221) were transformed into OG1RF by using a modification of the electroporation protocol described previously (9). For preparation of competent cells, OG1RF was grown overnight in BYGT with various glycine concentrations. A concentration of around 6% glycine led to optimal growth inhibition (70 to 90% reduction in optical density at A_{660}). The overnight culture was diluted 10-fold into fresh medium with the same concentration of glycine and incubated for 1 h at 37°C. Cells were chilled on ice, harvested by centrifugation at 5,000 × *g* for 12 min, and washed twice with 1/3 of the original volume of chilled electroporation solution (0.625 M sucrose–1 mM $MgCl_2$ adjusted to pH 4.0 with 1 N HCl). The washed cells were resuspended in 1/30 of the original volume of electroporation solution and then incubated on ice for 30 to 60 min (or saved at −70°C for later use). Cosmid DNA was prepared by equilibrium centrifugation in CsCl-ethidium bromide gradients. Competent cells (100 to 200 μl) were mixed with 2 to 5 μg of DNA (in <20 μl of distilled H_2O or low-salt buffer), added to a chilled 0.2-cm cuvette, and electroporated immediately with a Bio-Rad Gene Pulser apparatus at a capacitance of 25 μF, resistance of 200 Ω, and peak voltage of 2.5 kV (field strength of 8,750 to 10,000 V/cm for *E. faecalis*). Cells were then incubated in 1 ml of BYGT with 0.25 M sucrose for 90 to 120 min at 37°C and plated on SR agar plates containing kanamycin (2,000 μg/ml).

After electroporation, colonies growing on the selective plates were further tested by colony hybridization and by Southern hybridization to show the physical structure expected from allelic replacement by homologous recombination. To determine if pLAFRx sequences were present, colony hybridization was carried out as described previously (3), using the whole pLAFRx as a probe. The presence of mγδ was verified by using a 2-kb *Bam*HI fragment from mγδ as a probe. The probe used for Southern hybridization of TX5066a chromosomal DNA was a 1-kb PCR product containing the *pyrD* gene generated with primers just upstream and downstream of *pyrD*. The probe for Southern hybridization of TX5063a chromosomal DNA was a 4-kb *Eco*RI fragment excised from pKV48.

To simplify the process of screening inactivated *pyr* genes in *E. coli* and to identify possible mutations in regions other than structural genes, we tested the possibility of generating enterococcal auxotrophs with allelic replacement by electroporating a pool of different pKV48::mγδ insertions. Insertions of mγδ into pKV48 were selected as described above, and several hundred of the mutants were pooled. Cosmid DNA was prepared from the pooled cells as described above and used for electroporation of OG1RF. Transformants were selected on BHI-kanamycin (2,000 μg/ml) and saved for further analysis, including growth in DMMS broth and agar. Southern blotting was performed to map the insertions in OG1RF, with pKV48 as the probe.

Growth curves. The phenotype of the OG1RF::mγδ derivatives created by allelic replacement was first screened on DMMS agar as described above and then tested in DMMS broth. For growth in broth, OG1RF strains were grown overnight in BHI broth, harvested, washed twice with 0.9% NaCl, and then added to the DMMS broth at a final inoculum of 10^7 CFU/ml. The cell densities of OG1RF and the OG1RF transposon insertion mutants were determined by measuring the optical density at A_{660} and Klett units. Growth of cultures with and without adenosine or uracil was assessed by Klett units and colony counts at various times over 18 h of incubation (shaken at 300 rpm) at 37°C.

Nucleotide sequence accession numbers. The sequences reported have been submitted to GenBank and assigned accession numbers as follows: *pyrR*, U25091; *pyrP*, U25095; *pyrB*, U25092; *pyrC*, U25093; *pyrAb*, U25090; *pyrD*, U24692; *pyrF*, U25094; *pyrE*, U24682; and *pyrAa*, U36195.

RESULTS

Complementation and transposon mutagenesis. Previous studies have shown that a *pyrC* mutant of *E. coli* was complemented by the cosmid clone pKV48 (27). The possibility that pKV48 could complement other mutations in the pyrimidine biosynthesis pathway was tested by introducing this cosmid into various *E. coli pyr* mutants and scoring for their abilities to grow on minimal media (Table 2). Prototrophy was restored for *E. coli pyrAa*, *pyrAb*, *pyrB*, *pyrC*, *pyrD*, *pyrF*, and *pyrE* auxotrophs, indicating that genes encoding analogous products were contained in the cosmid. The *E. coli pyrAa* and *pyrAb* (also referred to as *carA* and *carB*) mutants require both arginine and uracil. These auxotrophs were complemented by pKV48 on minimal media in the presence of arginine but not in its absence or when only uracil was present. Although the pyrimidine biosynthesis functions are spread throughout the *E. coli* chromosome, they are clearly clustered in *E. faecalis*. This result is similar to that found for *Bacillus* species (14, 33).

Mapping *pyr* genes by transposon mutagenesis. Thirteen Tn*phoA* insertions and six mγδ insertions were isolated and mapped in pKV48 (Fig. 1 and Table 1). Cosmids with representative insertions were tested for complementation in seven *E. coli pyr* gene mutants (Table 2). Among the Tn*phoA* insertion derivatives of pKV48, pBEM207, pBEM208, and pBEM 209 (with insertions at positions 207, 208, and 209, respectively) had lost the ability to complement the *pyrC*, *pyrAa*, and *pyrAb* mutations. pBEM205 and pBEM206 had lost the ability to complement the *E. coli pyrAa* and *pyrAb* auxotrophs. pBEM 202 and pBEM203 had lost the ability to complement the *pyrD* mutation, while pBEM204 complemented *pyrD* and therefore may lie outside this gene. pBEM201 failed to complement the *pyrF* mutant. pBEM210 complemented *pyrC*, suggesting that the insertion at position 210 and the upstream insertions at positions 211, 212, and 213 lie outside the pyrimidine gene cluster. This was verified by subsequent sequencing from the insertion at position 209 (see below). Colonies of *E. coli* carrying pBEM207 and pBEM208, with Tn*phoA* insertions in *pyrP* (see below), were blue on LB agar containing XP, suggesting that the transposon insertions at positions 207 and 208 were in a gene encoding a protein exported in *E. coli.*

Among the mγδ insertion derivatives, pBEM218 and pBEM 219 did not complement *pyrC*, *pyrAa*, or *pyrAb*, while pBEM 220 and pBEM221 did not complement *pyrD*. pBEM217 did not complement the *pyrE* mutant, and pBEM222 did not complement the *pyrAa* mutant, the only strain with which it was tested.

Since all insertion mutants complemented the *E. coli pyrB* mutant, there was a possibility that pKV48 contained a *pyrB* function outside the pyrimidine gene cluster. To test this, plasmid pBEM215 was used. This plasmid contains the left side of the insert in pKV48 and lacks most of the pyrimidine gene cluster, including the region found to encode *pyrB* (see below). Plasmid pBEM215 did not complement the *E. coli pyrB* mutant

TABLE 2. Complementation of *E. coli* auxotrophs by pKV48 and its transposon insertion derivatives

Plasmid	Insertion	Complementation of *E. coli* mutant carrying[a]:						
		pyrB	*pyrC*	*pyrAa*	*pyrAb*	*pyrD*	*pyrF*	*pyrE*
KV48	None	+	+	+	+	+	+	+
pBEM210	pKV48::Tn*phoA*210	NT	+	NT	NT	+	NT	NT
pBEM209	pKV48::Tn*phoA*209[b]	+	−	−	−	+	+	+
pBEM218	*pyrR*::mγδ218	+	−	−	−	+	+	+
pBEM207	*pyrP*::Tn*phoA*207	+	−	−	−	+	+	+
pBEM208	*pyrP*::Tn*phoA*208	+	−	−	−	+	+	+
pBEM219	*pyrC*::mγδ219	+	−	−	−	+	+	+
pBEM222	*pyrAa*::mγδ222	NT	NT	−	NT	NT	NT	NT
pBEM206	*pyrAb*::Tn*phoA*206	+	+	−	−	+	+	+
pBEM205	*pyrAb*::Tn*phoA*205	+	+	−	−	+	+	+
pBEM204	*orf2*::Tn*phoA*204	NT	NT	NT	NT	+	NT	NT
pBEM203	*pyrD*::Tn*phoA*203	+	+	+	+	−	+	+
pBEM220	*pyrD*::mγδ220	+	+	+	+	−	+	+
pBEM221	*pyrD*::mγδ221	+	+	+	+	−	+	+
pBEM202	*pyrD*::Tn*phoA*202	+	+	+	+	−	+	+
pBEM201	*pyrF*::Tn*phoA*201	+	+	+	+	+	−	+
pBEM217	*pyrE*::mγδ217	+	+	+	NT	+	+	−

[a] Complementation was performed by introducing pKV48 or its transposon insertion derivatives into *E. coli* auxotrophs and scoring for the ability to grow on M63 minimal agar. +, growth observed; −, no growth; NT, not tested. *pyrAa* and *pyrAb* are also called *carA* and *carB*, respectively, in *E. coli.*

[b] This insertion is about 40 nucleotides upstream from the *pyrP* gene.

(data not shown), making it unlikely that a *pyrB* gene is encoded outside of the pyrimidine gene cluster on pKV48.

Restriction mapping and sequencing of pKV48. The restriction map of pKV48 was generated by using *Eco*RI, *Bam*HI, *Cla*I, or *Hin*dIII digestion of pKV48 and the 13 Tn*phoA* insertion derivatives (Fig. 1). In most cases, both single and double digestions, and occasionally triple digestions, were performed. To facilitate sequencing and ordering of the genes contained in pKV48, three subclones, pBEM214, pBEM215, and pBEM 216, were constructed (Fig. 1). Subclone pBEM214 contains a 3-kb *Eco*RI-*Bam*HI fragment of pKV48 spanning the Tn*phoA* insertions at positions 202 and 203 which had resulted in loss of *pyrD* complementation. Subclone pBEM215 was generated by deleting two adjacent *Bam*HI fragments (11 and 4.7 kb) from pBEM207, which is pKV48 with a Tn*phoA* insertion at position 207. The 11-kb deleted *Bam*HI fragment includes one arm of the inserted Tn*phoA*. The remaining large fragment (about 37 kb) containing the other arm of Tn*phoA*, including its kanamycin resistance gene, was self-ligated. An additional clone was constructed by excising and purifying the 11-kb *Bam*HI fragment from pBEM207 and further digesting it with *Not*I and *Hin*dIII. A 2.5-kb *Not*I-*Hin*dIII fragment was ligated into appropriately digested pBluescript to produce pBEM216. Both pBEM215 and pBEM216 contain one of the Tn*phoA*207 inverted repeats and thus contain sequences complementary to the GW26 primer, which was used to prime DNA sequencing reactions. Enterococcal sequences contained in pBEM216 and pBEM215 are contiguous to each other at position 207.

The subclone pBEM214 was first sequenced from one end, by using the pBluescript T3 primer. The first 225 nucleotides demonstrated homology with the *orf2* (open reading frame 2) sequence from *Bacillus subtilis* and *B. caldolyticus*, whose *pyr* genes are organized in similar clusters (14, 33). New primers were designed on the basis of the DNA sequence obtained and were used to extend the sequencing. In this way, both strands of the entire *pyrD* gene as well as part of the *pyrF* gene were sequenced. Most of the *pyrD* sequence was also obtained by manual sequencing of both strands. The *pyrD* gene is 936 nucleotides long in *B. subtilis*, while the enterococcal *pyrD* sequence is either 939 or 936 nucleotides long, depending on which of two adjacent ATG codons is used for initiation of translation. The predicted *E. faecalis* PyrD amino acid sequence showed 79 to 81% similarity to PyrD from *B. subtilis* and *B. caldolyticus* and 53% similarity to PyrD from *E. coli*, reflecting the closer phylogenetic relationship among gram-positive organisms. Recently, another gram-positive organism, *Lactococcus lactis*, was shown to contain two *pyrD* genes (1). The *E. faecalis* PyrD protein shows 88% similarity (73% identity) to PyrDb of *L. lactis.*

The initial 270-nucleotide sequence from the subclone pBEM216, obtained by using the GW26 primer, revealed homology with *pyrP* of *B. subtilis* and *B. caldolyticus* (13, 37). Using a primer based on the *pyrP* sequence, we found that the next 300-nucleotide sequence had homology with the *pyrB* gene of these organisms. Sequencing from the other end of the 2.5-kb insert in pBEM216 by using the pBluescript T7 primer revealed a region of sequence similarity to *pyrC*, located about 5.5 kb upstream from *pyrD* in pKV48. Sequencing of about 200 nucleotides from pBEM215 by using the GW26 primer verified the expected *pyrP* sequence, which was continuous with the downstream *pyrP* sequence in pBEM216. The *pyrP* sequence was also obtained from direct sequencing of pBEM208 as described above. Using the *phoA* primer to sequence the region flanking Tn*phoA* in cosmid pBEM206, which had lost the ability to complement *pyrAb*, we obtained 254 nucleotides of sequence similar to *pyrAb* of *B. subtilis* and *B. caldolyticus*. Similarly, sequence from the mγδ insertion in cosmid pBEM222, which had lost the ability to complement *pyrAa*, provided a 310-nucleotide sequence that was similar to *pyrAa* of *B. subtilis* and *B. caldolyticus*. The *pyrR* homolog was sequenced from the insertion at position 209 by using the *phoA* primer and from the downstream insertion at position 218 by using the mγδ-R primer. Computer analysis showed that the insertion at position 209 is about 40 nucleotides upstream of the predicted *pyrR* gene. Sequences flanking mγδ insertions at positions 217 and 219 revealed similarity to *pyrE* and *pyrC*, respectively, of *Bacillus* spp. (Fig. 1). Insertions at positions 220 and 221 were about 120 nucleotides apart. In all, the lengths of the 10 sequenced regions (*pyrR*, *pyrP*, *pyrB*, *pyrC*, *pyrAa*, *pyrAb*, *orf2*, *pyrD*, *pyrF*, and *pyrE*) varied from 180 (*pyrF*) to 939 (*pyrD*)

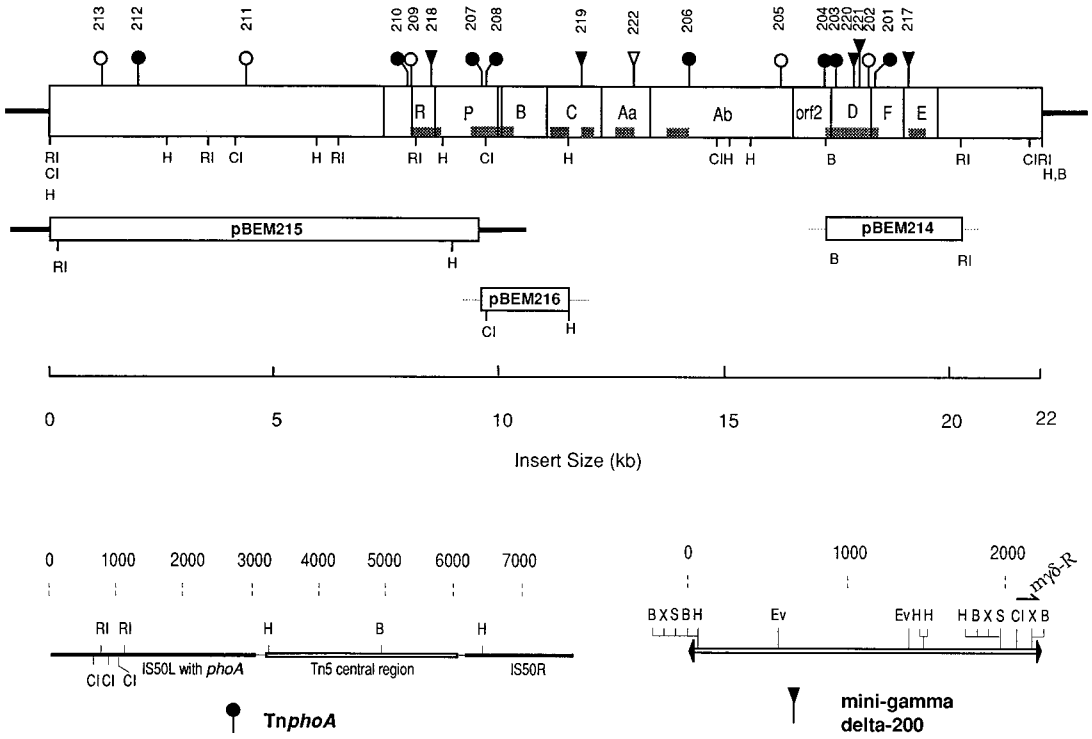

FIG. 1. Analysis of pKV48. The large rectangle indicates 22 kb of enterococcal DNA, which contains the pyrimidine gene cluster. The *pyr* genes are indicated in the rectangle (R = *pyrR*, P = *pyrP*, etc.) by analogy with the pyrimidine gene cluster of *B. subtilis*. The pyrimidine pathway and enzymes encoded by these genes are shown in Fig. 3. Subclones are indicated by the smaller rectangles. pBEM215 was constructed by deleting two adjacent *Bam*HI fragments from pBEM207. A 2.5-kb *Not*I-*Hin*dIII fragment from pBEM207 was subcloned into pBluescript to generate pBEM216. pBEM214 contained a 3-kb *Bam*HI-*Eco*RI fragment from pKV48. The thick lines at the ends of pKV48 and pBEM215 represent cosmid pLAFRx. The thin lines flanking pBEM214 and pBEM215 represent pBluescript. The shaded bars within the pKV48 rectangle indicate regions that have been sequenced. Circles labeled 201 to 213 refer to Tn*phoA* insertions, with relative orientations represented by open and filled circles. The structure of the Tn*phoA* transposon is shown at the bottom. The orientation of the transposon shown is that corresponding to the filled circles. Triangles labeled 217 to 222 represent positions of mγδ insertions, with relative orientations represented by open and filled triangles. The structure of the mγδ transposon is shown at the bottom. The orientation of the transposon shown is that corresponding to the filled triangles. The location of the mγδ-R primer is shown. Restriction enzyme cleavage sites: RI, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; CI, *Cla*I; Ev, *Eco*RV; X, *Xba*I.

nucleotides, and their DNA sequence similarity to the corresponding genes of *B. subtilis* and *B. caldolyticus* varied from 50 to 69%. The partial *pyrP* nucleotide and predicted peptide sequences were compared with the *E. coli* uracil permease sequence; the 296-nucleotide sequences showed 53% identity, and the predicted peptide sequences showed 78.3% similarity (data not shown).

According to the map and sequence information, the distance from *pyrB* to *pyrD* in pKV48 is approximately 7.5 kb, while that between *pyrB* and *pyrD* in *B. subtilis* and *B. caldolyticus* is 7.2 kb. The distance between *pyrC* and *pyrD* and the distance between *pyrAb* and *pyrD* in *E. faecalis* were also very close to those in *B. subtilis* and *B. caldolyticus*. The alignment of the *pyr* gene cluster of these three organisms demonstrated that the organization in *E. faecalis* is very similar to that in *B. subtilis* and *B. caldolyticus*. Using the complete sequences from *Bacillus* spp., we determined the possible positions of the insertions in the *pyr* genes (Table 3).

Isolation and characterization of insertions in the *purL* gene. The cosmid pKV53 was previously shown to complement an *E. coli purL* auxotroph (27). Using mγδ, we performed mutagenesis with this cosmid. When the pool of pKV53 with mγδ insertions was transduced into an *E. coli purL* auxotroph and selected as before, a colony with a mutant cosmid, designated pTX5062, that no longer complemented the *E. coli* auxotroph was isolated. Insertion of the transposon into *purL* of pTX5062 was confirmed by sequence analysis using the mγδ-R primer to prime the sequencing reaction. The *purL* gene of *B. subtilis* encodes a 228-amino-acid-long polypeptide. The transposon insertion in the *E. faecalis purL* gene was at a position corresponding to codon 21 (Table 3).

Allelic replacement. We next tested the possibility of gener-

TABLE 3. Locations of transposon insertions in *pyr* and *pur* genes and growth of OG1 mutants

Gene	Transposon insertion	Protein size[a] (amino acids)	Location of insertion[a,b] (codon)	Phenotype of insertion in OG1RF
pyrR	mγδ218	182	134	$Pyr^{+/-}$
pyrP	Tn*phoA*208	435	390	NA
pyrC	mγδ219	429	288	Pyr^{-}
pyrAa	mγδ222	367	213	NA
pyrAb	Tn*phoA*206	1,072	300	NA
pyrD	mγδ220	313	197	Pyr^{+}
pyrD	mγδ221	313	238	Pyr^{+}
pyrE	mγδ217	217	27	Pyr^{-}
purL	mγδ5062	228	21	Pur^{-}
pyr cluster	mγδC22	NA	NA	Pyr^{-}

[a] Based on the *B. subtilis* pyrimidine biosynthesis operon.
[b] Predicted by comparing sequences with *pyr* genes from *B. subtilis*. Pyr^{-} or Pur^{-}, *pyr* or *pur* auxotrophic phenotype (optical density at 12 h, 0.03 to 0.07); Pyr^{+}, prototrophic phenotype (optical density at 12 h, 0.21 to 0.26); $Pyr^{+/-}$, slow growth in defined medium (optical density at 12 h, 0.12); NA, not available.

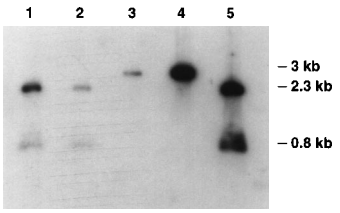

FIG. 2. Hybridization of genomic DNA from enterococcal transposon insertion mutants. A Southern blot of *Bam*HI-*Eco*RI-digested DNA from TX5066b (lane 1), TX5066a (lane 2), OG1RF (lane 3), pKV48 (lane 4) and pBEM220 (lane 5) was hybridized with a 1-kb *pyrD* PCR product generated with primers just outside *pyrD*. TX5066b is a transposon insertion mutant generated by electroporating linearized pBEM220 DNA into OG1RF.

ating *pyr* or *pur* auxotrophs of enterococci by using the insertion mutants described above. Cosmids containing mγδ insertions in the *pyrR*, *pyrC*, *pyrD* (two different insertions), *pyrE*, and *purL* genes (Table 2) were electroporated into OG1RF competent cells. Transformants that survived on kanamycin (2,000 μg/ml) were expected to result from recombination between the cosmid and the host chromosome because pLAFRx cannot replicate in enterococci. Southern blot analysis confirmed the locations of the transposon insertions in the chromosome of *E. faecalis* and showed that only a single insertion was present.

Transformants were tested for auxotrophy by measuring growth on DMMS with or without uracil or adenosine supplements (Table 3). The transposon insertion mutants from *pyrC*, *pyrE*, and *purL* mutants grew poorly on DMMS agar, forming microcolonies, as well as in DMMS broth unless uracil or adenosine was added. The *pyrR* mutant showed intermediate growth under these conditions, possibly because of an incomplete polar effect on downstream genes. In contrast, both *pyrD* mutants showed growth that was comparable to that of the wild-type parent on DMMS agar or in broth.

The insertion mutations in *E. faecalis* were stable, as no revertants were observed for *pyrE* or *purL* (frequency of $<10^{-8}$) when these mutants were plated on DMMS agar. A single revertant of the *pyrC* insertion (TX5078), which grew well on DMMS agar, was detected at a frequency of less than 10^{-8}. The revertant was still resistant to 2,000 μg of kanamycin per ml, suggesting that the mγδ transposon was still present. Presumably a rare second-site mutation was responsible for the phenotype.

Because the *pyrD* insertions did not produce auxotrophy in enterococci, the mγδ220 insertion mutant was analyzed in more detail. Thirteen putative OG1RF*pyrD*::mγδ220 isolates hybridized with a probe from mγδ but not with pLAFRx, indicating that kanamycin-resistant cells arose from a double crossover between the cosmid and the host chromosome (data not shown). Southern blots of DNA from two *pyrD*::mγδ220 mutants (strains TX5066a and TX5066b) digested with *Bam*HI plus *Eco*RI were hybridized with the 1-kb *pyrD* probe, and the results confirmed the correct allele replacement (Fig. 1 and 2). Finally, PCR of TX5066a was performed with a primer derived from the end of *pyrD* and a primer from within mγδ. The amplified fragment was subjected to DNA sequence analysis, which verified that the mγδ insertion was located in the *pyrD* gene. These results allowed us to conclude that insertion of mγδ in the *pyrD* gene does not create an auxotrophic phenotype in *E. faecalis.*

Chromosome replacement en masse. We wished to determine if a more efficient method for identification of biosynthetic genes (or any phenotypic class) could be performed in enterococci. To this end, we pooled several hundred mγδ insertions in pKV48, isolated DNA, and electroporated the DNA into *E. faecalis* OG1RF. Twenty-three kanamycin-resistant colonies were analyzed, and one was found to have a pyrimidine requirement (Table 3). Southern blot analysis of this mutant (TX5081) showed it to have an insertion in the *pyr* gene cluster (data not shown), although the specific gene location was not determined. Thus, one can identify specific *E. faecalis* functions (e.g., metabolic genes) by this procedure without relying on heterologous complementation or other laborious procedures.

DISCUSSION

We show in this report that following transposon mutagenesis in *E. coli*, it is possible to construct auxotrophic knockout mutants of *E. faecalis*. Both pyrimidine- and purine-requiring auxotrophs, the first auxotrophic mutants of an enterococcal strain, were generated by this procedure. Clearly, mutations in any gene giving a phenotype that is detectable in *E. coli* could be isolated by this procedure. This should have particular applicability to genes involved in infection or other host interactions. Such genes could be detected in *E. coli* by using antisera from patients or other procedures.

The genes coding for de novo pyrimidine biosynthesis have been extensively studied in *E. coli* and *B. subtilis*. In *E. coli*, the *pyr* genes are scattered around the chromosome and are not coordinately regulated (28). Pyrimidine-mediated regulation of expression of *pyrBI* and *pyrE* in *E. coli* has been shown to occur primarily by transcriptional attenuation (20). In *B. subtilis*, the genes encoding enzymes in the pyrimidine biosynthesis pathway are clustered on the chromosome and appear to be coordinately regulated (29, 31, 37). Expression is believed to occur from a promoter upstream from *pyrR*, producing a single transcript, that is negatively regulated by *pyrR* (37). At least one of these genes, *pyrB*, which encodes aspartate transcarbamylase, is regulated developmentally (23).

Little was previously known about the organization of *pyr* genes or their regulation in enterococci. In this study, we used a defined medium, developed previously in this laboratory (27), to identify auxotrophs. It is clear that *E. faecalis* resembles *Bacillus* spp. with respect to organization of the *pyr* gene cluster. This resemblance is supported by the functional analysis of several genes and by complementation of *E. coli* mutants. It should also be noted that two Tn*phoA* insertions into the *pyrP* gene, thought to encode a permease, gave blue colonies on XP medium, indicating export of the enterococcal *pyrP* gene product in *E. coli*. Thus, it is likely that this function is also conserved.

The regulation of expression of the *pyr* gene cluster in *E. faecalis* also appears to share features with the same regulation in *Bacillus* spp. Thus, an insertion in *pyrR* (mγδ218) showed reduced growth in DMMS, consistent with a polar effect of this insertion on downstream *pyr* genes. If *pyrR* is a negative regulator as in *Bacillus* spp., we would expect overexpression in addition to polar effects on downstream genes. Perhaps this explains the partial auxotrophic phenotype.

Results of complementation in *E. coli* are more difficult to interpret, presumably because of the heterologous nature of the system. Insertions in *pyrR*, *pyrP*, or *pyrC* are deficient in complementation for downstream functions (except for *pyrB*) down to the *pyrAa* and *pyrAb* genes (Table 2). This finding is consistent with expression in *E. coli* of these enterococcal genes from a single promoter, upstream from *pyrR*. Note that complementation by these genes is also defective in the mutant with Tn*phoA*209, which is inserted upstream from *pyrR*, con-

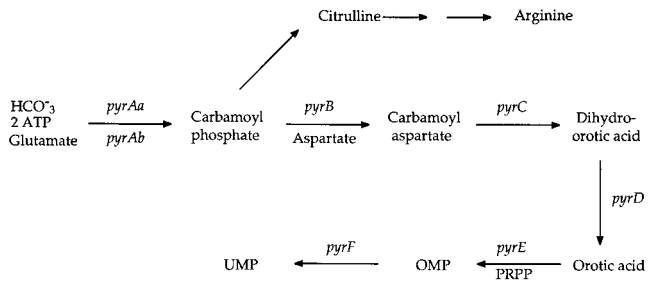

FIG. 3. De novo synthesis of UMP. Genes referred to in Fig. 1 are shown and encode the following enzymes: *pyrAa* and *pyrAb*, glutaminase of carbamoylphosphate synthetase and carbamoylphosphate synthetase, respectively (also known as *carAB* [carbamoylphosphate synthase] in *E. coli*); *pyrB*, aspartate transcarbamylase; *pyrC*, dihydroorotase; *pyrD*, dihydroorotate dehydrogenase; *pyrE*, OMP-PRPP transferase; and *pyrF*, OMP decarboxylase.

sistent with the upstream location of the promoter. The *pyrB* gene in pKV48 derivatives containing upstream transposon insertions, however, complements an *E. coli* mutant despite the fact that its expression should be reduced in some of these mutants. The reason for this anomaly is not known. Another unusual result is the loss of complementation for *pyrAa* in an insertion mutant in the *pyrAb* gene (Tn*phoA*206; Table 2). Effects of *pyrAb* knockout mutations on *pyrAa* have been reported previously (15) and may reflect the subunit nature of this enzyme.

Another anomaly in the heterologous system is the complementation of the *E. coli pyrD*, *pyrF*, and *pyrE* mutants by these mutant cosmids (Table 2). Similarly, *E. coli pyrF* and *pyrE* mutants are complemented by cosmids with insertions in the *pyrD* gene (Table 2). Assuming that mγδ and Tn*phoA* insertions show polarity, these results are not consistent with a single transcript being produced in *E. coli*. Perhaps there are internal promoters in the *pyr* gene cluster that function in *E. coli*. Whether such promoters are physiologically significant in *E. faecalis* is not known. It is also possible that the anomalous complementation of *pyrB*, *pyrD*, *pyrF*, and *pyrE* reflects a requirement for smaller amounts of these gene products than for the other *pyr* functions.

The observation that two independent *pyrD* knockout mutants, located centrally in the coding sequence, were not auxotrophs suggests that the PyrD function in *E. faecalis* may be duplicated, as in *L. lactis*. The two proteins in *L. lactis* show only 24% sequence identity, with PyrDa being related to the enzyme from *Saccharomyces cerevisiae* (70% identity) and PyrDb being related to the enzyme from *B. subtilis* (64% identity) (1). If this situation held for *E. faecalis*, it would explain why we did not detect the second gene by either Southern blot or PCR analysis, since the sequences of the *E. faecalis pyrD* and the *L. lactis pyrDa* genes are significantly different. Inspection of the pathway for de novo UMP synthesis (Fig. 3) shows that auxotrophs were isolated for steps before and after the reaction performed by the *pyrD*-encoded enzyme. The observation that auxotrophs were obtained for steps preceding the *pyrD*-dependent reaction suggests that there is no alternative pathway for synthesizing orotic acid.

Auxotrophs of *E. faecalis* may also be useful in studies of virulence. For example, in other systems, auxotrophs have been used to develop attenuated live vaccine strains (19). In addition, auxotrophic mutants were used to identify *Salmonella* genes that were expressed during infection (21). Such an in vivo expression procedure could be used for selection of enterococcal virulence genes that are specifically induced in host tissues.

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