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Inefficient Translation Renders the *Enterococcus faecalis fabK* Enoyl-Acyl Carrier Protein Reductase Phenotypically Cryptic

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Enoyl-acyl carrier protein (ACP) reductase catalyzes the last step of the bacterial fatty acid elongation cycle. *Enterococcus faecalis* is unusual in that it encodes two unrelated enoyl-ACP reductases, FabI and FabK. We recently reported that deletion of the gene encoding FabI results in an unsaturated fatty acid (UFA) auxotroph despite the presence of *fabK*, a gene encoding a second fully functional enoyl-ACP reductase. By process of elimination, our prior report argued that poor expression was the reason that *fabK* failed to functionally replace FabI. We now report that FabK is indeed poorly expressed and that the expression defect is at the level of translation rather than transcription. We isolated four spontaneous mutants that allowed growth of the *E. faecalis* Δ*fabI* strain on fatty acid-free medium. Each mutational lesion (single base substitution or deletion) extended the *fabK* ribosome binding site. Inactivation of *fabK* blocked growth, indicating that the mutations acted only on *fabK* rather than a downstream gene. The mutations activated *fabK* translation to levels that supported fatty acid synthesis and hence cell growth. Furthermore, site-directed and random mutagenesis experiments showed that point mutations that resulted in increased complementarity to the 3′ end of the 16S rRNA increased FabK translation to levels sufficient to support growth, whereas mutations that decreased complementarity blocked *fabK* translation.

The type II or dissociated fatty acid synthesis (FAS II) pathway is responsible for membrane fatty acid synthesis in most bacteria. Fatty acid synthesis is carried out by a series of discrete proteins encoded by separate genes (1, 2) and differs significantly from the type I mammalian and fungal systems, in which multifunctional polypeptides catalyze fatty acid synthesis (3–5). This distinguishing characteristic makes the type II system a prime target for antibacterial agents. Enoyl-acyl carrier protein (ACP) reductases (ENRs) catalyze the last step of the fatty acid elongation cycle, the reduction of *trans*-2-acyl-ACPs (an enoyl-ACP) to the fully saturated acyl-ACP species. Unlike the other FAS II enzymes, ENRs display an unusual diversity. In bacteria, four ENR isozymes have been identified: the products of the *fabI* (6, 7), *fabL* (8), *fabV* (9, 10), and *fabK* (11, 12) genes. FabI, FabL, and FabV are diverse members of the short-chain dehydrogenase/reductase (SDR) superfamily, whereas FabK is a TIM barrel flavoprotein (13). Genes encoding homologues of FabI are readily identified in most bacteria. These genes are about 40% identical to *Escherichia coli* FabI and contain a conserved Tyr-156–$(Xaa)_6$–Lys-163 (*E. coli* numbering) catalytic dyad (10, 14). For example, *E. coli* and *Francisella tularensis* contain a single ENR encoded by *fabI* that is essential for bacterial viability (7, 15). In contrast, two SDR superfamily ENRs, FabI and FabV, have been characterized in *Pseudomonas aeruginosa*. Each of the encoding genes can support growth and functionally replace *E. coli fabI* (10), as also found for the two SDR superfamily ENRs of *Bacillus subtilis* (8). Thus, it seems that some bacteria have only a single ENR whereas others have two, either of which suffices for growth in the laboratory. However, this is not the case for *Enterococcus faecalis*, a Gram-positive commensal bacterium that normally inhabits the gastrointestinal tract of humans and other mammals (16, 17). Recently, we characterized two ENRs, FabI and FabK, that play different roles in *E. faecalis*. FabI is the major ENR of this bacterium, in that deletion of *fabI* results in unsaturated fatty acid (UFA) auxotrophy, whereas deletion of *fabK* has no effect on growth, although the traces ENR activity remaining in the Δ*fabI* strain do alter the cellular fatty acid composition (18). Both FabI and FabK are fully functional *in vitro* and functionally replace *E. coli fabI*. Moreover, when expressed from a plasmid construct, FabK restores growth of the Δ*fabI* strain, indicating that it is poorly expressed in its native chromosomal location.

In this report we demonstrate that the inability of *fabK* to support growth of *E. faecalis* is due to inefficient translation. We found that upon plating of *E. faecalis fabI* null mutants on fatty acid-free medium, colonies arose that no longer required oleate supplementation. The mutations were found to extend the complementarity between the *fabK* ribosome binding region and the 3′ end of the 16S rRNA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. faecalis* strains (Table 1) were all derived from FA2-2, a plasmid-free strain (30). Primers used in this study are given in Table 2. Generally, *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter; pH 7.0), whereas *E. faecalis* strains were maintained at 37°C or 42°C in GM17 medium (31). Antibiotics were added as required at the following concentrations (in mg/liter): sodium ampicillin at 100 for *E. coli*; kanamycin sulfate at 25 for *E. coli*; erythromycin at 150 for *E. coli* and 5 for *E. faecalis*. Bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}). Oligonucleotide primers were synthesized by Integrated DNA Technologies, and

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
E. coli strains		
DH5α	Δ(*argF-lac*)169 ϕ80d*lacZ58*(M15) Δ*phoA8 glnV44 deoR481 gyrA96 recA1 endA1 hsdR17*	Lab stock
EC1000	Em^r; MC1000 derivative carrying single copy of pWV01*repA* in *glgB*	Lab stock
E. faecalis strains		
FA2-2	Rifampin/fusidic acid-resistant mutant derived from plasmid-free strain JH2	30
FAZL1	Δ*fabI* of *E. faecalis* FA2-2	18
BHK260	Spontaneous RBS mutant S1 of *FAZL1*, C-12 to G	This work
BHK261	Spontaneous RBS mutant S2 of *FAZL1*, C-12 deletion	This work
BHK262	Spontaneous RBS mutant S3 of *FAZL1*, A-9 to G	This work
BHK263	Spontaneous RBS mutant S4 of *FAZL1*, T-19 to G	This work
FAZL6	FA2-2 *fabK* (disruption)	This work
FAZL7	FAZL1 *fabK* (disruption)	This work
FAZL8	S1 *fabK* (disruption)	This work
FAZL9	S2 *fabK* (disruption)	This work
FAZL10	S3 *fabK* (disruption)	This work
FAZL11	S4 *fabK* (disruption)	This work
Plasmids		
pTRKL2	Erm^r, low-copy-no. *E. coli/Lactococcus* shuttle vector	42
pCR2.1	Amp^r Kan^r, TOPO TA cloning vector	Invitrogen
pZL52	Amp^r Kan^r, pCR2.1 carrying *E. faecalis* FAZL1 *fabK* plus 275 bp upstream and 288 bp downstream	This work
pZL53	Amp^r Kan^r, pCR2.1 carrying *E. faecalis* mutant S1 (strain BHK260) *fabK* plus 275 bp upstream and 288 bp downstream	This work
pZL54	Amp^r Kan^r, pCR2.1 carrying *E. faecalis* mutant S2 (strain BHK261) *fabK* plus 275 bp upstream and 288 bp downstream	This work
pZL55	Amp^r Kan^r, pCR2.1 carrying *E. faecalis* mutant S3 (strain BHK262) *fabK* plus 275 bp upstream and 288 bp downstream	This work
pZL56	Amp^r Kan^r, pCR2.1 carrying *E. faecalis* mutant S4 (strain BHK263) *fabK* plus 275 bp upstream and 288 bp downstream	This work
pZL157	Erm^r, EcoRV and BamHI-digested *fabK* fragment of pZL52 inserted into same sites of pTRKL2	This work
pZL158	Erm^r, EcoRV- and BamHI-digested *fabK* fragment of pZL53 inserted into same sites of pTRKL2, C-12 to G	This work
pZL159	Erm^r, EcoRV- and BamHI-digested *fab* fragment of pZL54 inserted into same sites of pTRKL2, C-12 deletion	This work
pZL160	Erm^r, EcoRV- and BamHI-digested *fabK* fragment of pZL55 inserted into same sites of pTRKL2, A-9 to G	This work
pZL161	Erm^r, EcoRV- and BamHI-digested *fabK* fragment of pZL56 inserted into same sites of pTRKL2, T-19 to G	This work
pBHK368	Erm^r, *fabK* A-9T of pZL157	This work
pBHK369	Erm^r, *fabK* A-9C of pZL157	This work
pBHK370	Erm^r, *fabK* C-12A of pZL157	This work
pBHK371	Erm^r, *fabK* $C_{-12}T$ of pZL157	This work
pBHK372	Erm^r, *fabK* T-19A of pZL157	This work
pBHK373	Erm^r, *fabK* T-19C of pZL157	This work
pMC1871	Tet^r, promoterless *lacZ* reporter plasmid	32
pBHK322	Erm^r, *lacZ* from pMC1871 cloned into BamHI site of pTRKL2, p(lacZ)	This work
pBHK323	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, p(WT)	This work
pBHK394	Erm^r, pTRKL2 *fabK* (−275 to +35)::*lacZ*	This work
pBHK324	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, C-12 to G, p(S1)	This work
pBHK325	Erm^r, pTRKL2 P32::*fabK* (−136 to + 35)::*lacZ*, C-12 deletion, p(S2)	This work
pBHK326	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, A-9 to G, p(S3)	This work
pBHK327	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::lacZ, T-19 to G, p(S4)	This work
pBHK334	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, A-9 to T	This work
pBHK335	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, A-9 to C	This work
pBHK336	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*,C-12 to A	This work
pBHK337	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, C-12 to T	This work
pBHK338	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, T-19 to A	This work
pBHK339	Erm^r, pTRKL2 P32::*fabK* (−136 to +35)::*lacZ*, T-19 to C	This work

TABLE 2 Oligonucleotide primers used in this study

Oligonucleotide	Sequence[a]
fabKup275	ATTAGACGCGGACAGCTTAG
fabKdo288	TACGACATCAGGCGTTAGTC
P32-f	ACTCGC*CTGCAG*AGATTAATAG TTTTAGCTA
fabKfusion-r	ATACTT*GTCGAC*TTGATTCCAAGCAACTCACA
P32FabK-f	CTAGGTAGGTAAAAAAATATTACATGAAAATAGGGGGCT
P32FabK-r	AGCCCCCTATTTTCATGTAATATTTTTTTACCTACCTAG
fabKup275-PstI	AATAT*CTGCAG*ATTAGACGCGGACAGCTTAG
fabK-random-f	TAGT*GGATCC*ATTAGACGCGGACAGCTTAGA
fabK-random-r	ATAT*CTGCAG*TTCACTTAGCCCCAACGCTGAT
Ef16S-f	CATCATGCCCCTTATGACCTGG
Ef16S-r	CCTGCAATCCGAACTGAGAGA
lacZ-f	ATGAACGGTCTGGTCTTTGC
lacZ-r	ATCGCTATGACGGAACAGGT
fabK-RT-f	GGCAATCCAGCCAAATACAT
fabK-RT-r	ATGTCCACCAGCTTCCATTC
c-12a-f	GAAGTGTACTTATCTTAGAACTAAAGGAAGTATAAAATC AATGAATCAACAGTTATG
c-12a-r	CATAACTGTTGATTCATTGATTTTATACTTCCTTTAGTTC TAAGATAAGTACACTTC
c-12t-f	GAAGTGTACTTATCTTAGAACTAAAGGATGTATAAAATC AATGAATCAACAGTTATG
c-12t-r	CATAACTGTTGATTCATTGATTTTATACATCCTTTAGTT CTAAGATAAGTACACTTC
a-9c-f	GTACTTATCTTAGAACTAAAGGACGTCTAAAATCAATGA ATCAACAGTTATGT
a-9c-r	ACATAACTGTTGATTCATTGATTTTAGACGTCCTTTAGT TCTAAGATAAGTAC
a-9t-f	GTACTTATCTTAGAACTAAAGGACGTTTAAAATCAATGA ATCAACAGTTATGT
a-9t-r	ACATAACTGTTGATTCATTGATTTTAAACGTCCTTTAGT TCTAAGATAAGTAC
t-19a-f	CTTGAATGAAGTGTACTTATCTTAGAACAAAAGGACGTA TAAAATCAATGAATC
t-19a-r	GATTCATTGATTTTATACGTCCTTTTGTTCTAAGATAAG TACACTTCATTCAAG
t-19c-f	CTTGAATGAAGTGTACTTATCTTAGAACCAAAGGACGTA TAAAATCAATGAATC
t-19c-f	GATTCATTGATTTTATACGTCCTTTGGTTCTAAGATAAG TACACTTCATTCAAG
fabK inside For	ATGC*GAGCTC*TGAAGAACAGGTTCCTGTCG
fabK inside Rev	ATCG*CTGCAG*CTGCATCTAAATCACGTGCC
pBVGh For (P1)	GTACCTACGTAGGATCGATC
pBVGh Rev (P2)	GCTTCCAAGGAGCTAAAGAG
fabK check Up (P3)	ATTAGACGCGGACAGCTTAG
fabK check Down (P4)	TACGACATCAGGCGTTAGTC

[a] The underlined italic sequences are the introduced restriction sites.

cloned genes were verified by sequencing performed by ACGT, Inc. Gene sequences were amplified using *Pfu* Turbo DNA polymerase (Stratagene) according to the manufacturer's recommendations. The pCR2.1-TOPO vector (Invitrogen) was applied for PCR cloning, and *E. coli* strain DH5α was the corresponding recipient host (Table 1). Primers fabKup275 and fabKdo288 were used to amplify *fabK* fragments from *E. faecalis* FA2-2 and four spontaneous mutants, and the PCR products were cloned to the pCR2.1-TOPO vector to give plasmids pZL52, pZL53, pZL54, pZL55, and pZL56 for sequencing. These five plasmids were digested with EcoRV and BamHI and inserted into plasmid pTRKL2 to yield plasmids pZL157, pZL158, pZL159, pZL160, and pZL161, respectively, which were linearized and used to transform strain FAZL1. Qiagen provided plasmid isolation and PCR product purification kits.

Construction of translational fusions. The BamHI-digested DNA fragment containing a promoterless *lacZ* from the plasmid pMC1871 (32) was ligated to the shuttle plasmid pTRKL2 cut with the same enzyme to give the plasmid pBHK322. The insertion direction was confirmed by DNA sequencing. Overlapping PCR was used to generate the fusion of the promoter P32 from *Lactococcus lactis* to *fabK* (−136 to +35 relative to the *fabK* ATG initiation codon). The promoter P32 from *L. lactis* and *fabK* (−136 to +35) from *E. faecalis* FA2-2 were first amplified from the genomic DNAs by using primer sets P32-f/P32fabKfusion-r and P32fabKfusion-f/fabKfusion-r, respectively. The first-step PCR fragments were purified from agarose gels and mixed to serve as templates to amplify chimera-P32fabK with primer set P32-f/fabKfusion-r. The PCR products after digestion with PstI and SalI were ligated into the plasmid pBHK322 to produce pBHK323. For the construction of the other four translational fusion plasmids, each containing a spontaneous mutation in the ribosome binding site (RBS) region, *fabK* (−136 to +35) was first amplified from the genomic DNAs of spontaneous mutants BHK260, BHK261, BHK262,

and BHK263 and used as the first-step PCR template. The following procedure was the same for the construction of the plasmid pBHK323, and finally the fusion plasmids pBHK324, pBHK325, pBHK326, and pBHK327 were obtained.

Construction of *E. faecalis* strains having disrupted *fabK* genes. *E. faecalis* strains having disruptions of *fabK* were constructed using the method described previously (33). A centrally located segment of *fabK* was obtained by amplification from the genomic DNA of *E. faecalis* FA2-2 strain with *Pfu* DNA polymerase and primers (Table 2) containing designed restriction sites (a SacI site in the forward primer and a PstI site in the reverse primer). This fragment was then used to construct plasmid pZL235 by insertion of the PstI/SacI-digested PCR fragment into plasmid pBVGh cut with the same enzymes. The disruption plasmid was transformed into competent cells of *E. faecalis* wild-type strain FA2-2. After recovery in SGM17 medium at 30°C, the transformed cells were plated on SGM17 agar containing 5 μg/ml of erythromycin plus 100 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and incubated for 24 h at 30°C. The resulting blue colonies were cultured overnight at 30°C in GM17 liquid medium containing erythromycin, and then the cultures were plated on GM17 agar medium containing 5 μg/ml of erythromycin and 100 μg/ml of X-Gal and incubated for 24 h at 42°C. To construct *fabK* disruptions in strain carrying the Δ*fabI* mutation, 0.1% Tween 40, 0.1% Tween 80, and 0.01% oleate were added to the growth medium. The single-crossover homologous recombinant colonies were screened for disruption of the *fabK* gene by PCR analysis with pBVGh primers (P1 and P2) and *fabK* check primers (P3 and P4) and verified by sequencing.

Transformation of *E. faecalis*. Electrocompetent cells were prepared as previously described (31) with minor changes. A single colony of *E. faecalis* was grown overnight in GM17 medium and then diluted 1:100 into 100 ml of SGM17 medium (GM17 with 0.5 M sucrose and 8% glycine) in a 500-ml flask with vigorous shaking at 37°C. When the OD_{600} reached 0.4 to 0.6, the cultures were chilled on ice for 30 min and then collected by centrifugation at 3,600 × *g* for 12 min at 4°C. The harvested pellets in each tube were suspended in 100 ml of ice-cold electroporation buffer (0.5 M sucrose and 10% glycerol) and then centrifuged at 3,800 × *g* for 16 min at 4°C. Following three rounds of washing the cells with electroporation buffer, the bacterial pellets were suspended in 1 ml of ice-cold electroporation buffer and divided into small aliquots, which were frozen on dry ice and kept at −80°C until use. For the preparation of strain FAZL1 (the Δ*fabI* mutant strain of *E. faecalis* FA2-2) electrocompetent cells, 0.1% Tween 40, 0.1% Tween 80, and 0.01% oleate were added to the growth medium.

Prior to electroporation, the frozen cell suspensions were thawed on ice, mixed with plasmid DNA (50 to 100 ng for delivery of plasmids), and then placed on ice for no more than 30 min prior to transfer into a chilled 2-cm gap cuvette (Bio-Rad). Electroporation parameters for the Gene Pulser apparatus (Bio-Rad) were 25-μF capacitance, 200-Ω resistance, and a 2.5-kV electrical pulse to give a field strength of 12.5 kV cm^{-1}, with an exponential decay constant of approximately 4 to 5 ms. After immediate placement on ice for 5 min, the pulsed cells were quickly diluted with 1 ml of SGM17MC medium (31), grown at 37°C for 2 h, and plated on SR agar plates containing the appropriate antibiotics. The plates were incubated at 37°C for 18 to 36 h. Transformants containing plasmids were verified by plasmid isolation.

Acyl-ACP preparations. The ACP thioester of *trans*-2-decenoic acid was synthesized as described previously (34). Briefly, a typical reaction mixture consisted of 25 μM holo-ACP, 200 μM fatty acid, and 170 nM *Vibrio harveyi* AasS in a buffer containing 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM tris(2-carboxyethyl) phosphine HCl, and 10 mM ATP in a reaction volume of 1 ml. The reaction mixtures were incubated at 37°C for 4 h, and reactions were stopped by addition of 2 volumes of acetone; the proteins were allowed to precipitate at −20°C overnight. The precipitates were pelleted at 20,000 × *g* for 30 min at 4°C and then washed twice with 3 volumes of acetone. The pellets were air dried and resuspended in 20 mM Tris-HCl (pH 7.4) containing 1 mM Tris(2-carboxyethyl) phosphine HCl. The final samples were concentrated with an Amicon ultracentrifugation filter device from Millipore (5,000 molecular weight cutoff). Acyl-ACP synthesis was verified by electrophoresis on 18% gels containing 2.5 M urea (2, 35) and by electrospray mass spectrometry. Note that the ACP preparations retained the N-terminal methionine.

NADH oxidation assay. ENR activity was monitored spectrophotometrically based on the decrease in the absorbance at 340 nm and using an NADH extinction coefficient of 6,220 M^{-1} (9). Cultures grown to mid-log phase in 100 ml of GM17 medium were collected by centrifugation, washed twice with 0.9% KCl, repelleted, and treated for 30 min with lysozyme (15 mg/g, wet weight) in an osmotically protected medium consisting of 0.4 M NaCl, 0.05 M phosphate buffer (pH 7.2), and 0.5 M sucrose. The cyclopropane fatty acid *cis*-9,10-methylenehexadecanic acid (0.005%) was added to GM17 medium to support the growth of the Δ*fabI* strain FAZL1. After lysozyme treatment, the cells were resuspended in 1 ml of 100 mM Tris-HCl (pH 8.0) containing 400 mM NaCl and lysed by passaging three times through a French pressure cell press at 11,000 lb/in^2. The crude extract was collected on ice and cleared by centrifugation, and the protein concentration was determined by the Bio-Rad Bradford assay, using a standard curve of bovine serum albumin (36). Crude extract protein preparations (1 to 10 μg) were added directly to UV-transparent microcuvettes containing the assay reaction mixture (volume, 100 μl) consisting of 0.1 M sodium phosphate buffer (pH 7.0), 100 μM *trans*-2-decenoyl-ACP, 150 μM NADH, and 0.1 M LiCl. Kinetic constants were determined using GraphPad Prism software, version 4.

Radioactive labeling, phospholipid extraction, and fatty acid analysis. Fatty acid biosynthesis was analyzed based on [1-^{14}C]acetate incorporation. *E. faecalis* strains were cultured in GM17 medium (containing 0.005% *cis*-9,10-methylenehexadecanic acid for growth of the Δ*fabI* mutant strain FAZL1) at 37°C overnight. Cells were washed twice with water and resuspended in the same volume of GM17 medium, and 0.1 ml of this suspension was added to 5 ml of GM17 containing 5 μCi of sodium [1-^{14}C]acetate. These cultures were grown at 37°C to mid-log phase, and the cells were collected by centrifugation. The phospholipids were extracted by using the method of Bligh and Dyer (37), and labeled fatty acid methyl esters were prepared and analyzed by using thin-layer chromatography and autoradiography as described previously (38).

β-Galactosidase assays. The cultures were grown overnight, then diluted 1:100 into fresh medium having the same composition, and grown to the mid-log phase before β-galactosidase assays were performed. Mid-log-phase cultures were collected by centrifugation, washed twice with Z buffer, and assayed for β-galactosidase activity after lysis with sodium dodecyl sulfate-chloroform (39). The data were obtained in triplicate in more than three independent experiments.

RNA isolation and reverse transcription-PCR. Total RNA preparations were isolated from the mid-log-phase cells of *E. faecalis* FA2-2 and its derivatives grown in GM17 medium by using the RNeasy bacterial RNA isolation kit (Qiagen). The contaminating DNA was digested using RNase-free DNase set (Qiagen) according to the manufacturer's protocol. The isolated RNA samples were analyzed by agarose gel electrophoresis to assess the quality of the rRNA preparations. To further rule out the possibility of trace DNA contamination of the RNA, a general PCR-based detection was also conducted by using total RNA as the template with primers Ef16S-f plus Ef16S-r (Table 2). The concentration and purity of RNA were determined by using a NanoDrop 2000C spectrophotometer (Thermo Scientific). RNA preparations were stored at −80°C until use. Synthesis of cDNA was performed in 20 μl of a mixture and using the Omniscript reverse transcription (RT) kit (Qiagen) according to the manufacturer's protocol. The resulting cDNA (0.5 μl) served as the template for PCR amplification of the fatty acid synthesis genes (*fab* genes), using specific primers (Table 2) and an Eppendorf thermal cycler.

Real-time quantitative RT-PCR. To test if the mRNA levels of *fabK* were altered in the spontaneous mutants, real-time quantitative PCR (qPCR) assays were conducted by using the SYBR green dye method as

previously reported (40). The qPCR mixture (20 μl) contained the following components: 10 μl of iQ SYBR green supermix, 1 μl each of the primers, 1 μl of 50-fold-diluted cDNA sample, and 8 μl of sterile water. Data analyses were done in triplicate on a Mastercycler ep realplex instrument (Eppendorf), using a program of a denaturing cycle at 95°C for 2 min, 45 cycles comprising 94°C for 20 s, 53°C for 20 s, and 72°C for 20 s, and a final step in which the temperature was elevated on a gradient from 60°C to 90°C for dissociating double-stranded DNA products. The mRNA levels relative to those of the wild type were calculated using the method of Livak and Schmittgen (41) with the 16S rRNA as an internal control gene, and water functioned as a blank control to monitor cross-contamination of the cDNA samples.

Site-directed mutagenesis of *fabK*. Plasmids pBHK368, pBHK369, pBHK370, pBHK371, pBHK372, and pBHK373, each carrying a single mutation within the RBS sequence, were obtained by using the QuikChange site-directed mutagenesis kit (Stratagene) with pZL157 as the PCR template, following the manufacturer's instructions. The primers used in PCRs and in mutagenesis are listed in Table 2. The constructed plasmids were transformed into *E. coli* DH5α by $CaCl_2$ treatment. Plasmids pBHK334, pBHK335, pBHK336, pBHK337, pBHK338, and pBHK339, each carrying a single mutation within the RBS sequence, were obtained by using the QuikChange site-directed mutagenesis kit (Stratagene) with pBHK323 as the PCR template, following the manufacturer's instructions. All constructs were verified by DNA sequencing.

Random mutagenesis of *fabK* and isolation of mutant plasmids for complementation. Random mutagenesis of the *fabK* gene plus 275 bp upstream was performed with the GeneMorph II random mutagenesis kit using primers fabK-random-f and fabK-random-r (Table 2) and plasmid pZL157 as the template at a concentration of 500 ng in a total reaction volume of 50 μl. The mutagenesis condition designed to give a low mutation frequency was used, because we expected that single mutations would restore growth of *E. faecalis fabI* mutants in the absence of oleate. The PCR products of the mutagenesis reactions were then digested with BamHI and PstI and inserted into vector pTRKL2 digested with the same enzymes. The ligation products were then transformed into *E. coli* DH5α with selection on brain heart infusion plates for resistance to erythromycin. The transformants of about 15,000 clones were collected, and the mixture of mutant plasmids was purified using the Qiagen Spin miniprep kit. The plasmids were electroporated into the *E. faecalis fabI* mutant strain FAZL1 as described above. The 10,000 clones obtained were screened for growth on GM17 medium without fatty acid supplementation. The plasmids of the growth-complemented strains were then isolated and again transformed to the strain FAZL1 to confirm the growth complementation. Finally, the confirmed complementing plasmids were sequenced to identify the mutations.

RESULTS

Spontaneously arising mutants that restore fatty acid synthesis to the Δ*fabI* strain contain point mutations within the *fabK* ribosome binding site region. The two *E. faecalis* proteins catalyzing the ENR reaction (Fig. 1A) are located in different genome neighborhoods. The *fabK* gene is found within a putative operon responsible for saturated fatty acid synthesis, whereas *fabI* is located in a smaller cluster with two genes involved in unsaturated fatty acid synthesis (19) (Fig. 1B). The Δ*fabI* strain FAZL1 is severely defective in fatty acid synthesis and is unable to grow on GM17 medium unless the medium is supplemented with an unsaturated fatty acid, such as oleic acid (18). A culture of strain FAZL1 was plated on GM17 medium lacking oleate supplementation at 37°C. Colonies appeared at a frequency of approximately 10^{-9}, and purified isolates were shown to retain the Δ*fabI* mutation. Four isolates, strains BHK260, BHK261, BHK262, and BHK263, were chosen for further study.

Since overproduction of FabK alleviates the growth defect of a *fabI* null mutant strain (18), the most likely explanation for the spontaneous mutations was increased FabK expression. We first investigated how *fabK* is transcribed. The cluster of fatty acid synthesis genes in which *fabK* is located appears to be an operon, although there is an unusually large intergenic region (136 bp) between the *acpP* and *fabK* genes. To test if *acpP* and *fabK* are cotranscribed together with the upstream genes, we performed RT-PCR analyses using primers bracketing each intergenic region (Fig. 1C). The same patterns were seen in both RT-PCR and PCR analyses, indicating that at least these four genes are cotranscribed. ACP is generally produced at much higher levels than the Fab proteins, because it acts as a coenzyme rather than an enzyme. Hence, *acpP* would be expected to be transcribed at a higher level than the other genes, which we did not observe. However, *E. faecalis* has a second *acpP* gene (Fig. 1) that could be expressed at high levels. We are currently studying the relative contributions of the two *acpP* genes.

We also investigated if the *acpP-fabK* intergenic region contained a promoter by using the intergenic sequence to drive expression of a *fabK-lacZ* translational fusion carried on a plasmid (Fig. 1D). The intergenic sequence gave no increase in β-galactosidase activity over that seen with the reporter construct lacking a promoter, whereas a construct driven by the *Lactococcus lactis* P32 promoter gave high levels of β-galactosidase activity.

Given the results of these background experiments, we asked if growth of these four spontaneous mutants (Fig. 2A) was mediated by FabK. This was first tested by insertion of the *fabK* gene (plus about 300 bp of upstream and downstream sequence) from each of the spontaneous mutants into the low-copy-number shuttle vector pTRKL2. All four plasmids allowed the Δ*fabI* mutant strain to grow on GM17 medium, whereas the plasmid carrying the wild-type genomic segment failed to support growth (Fig. 2B). We then sequenced the *fabK* regions of each of the spontaneous mutants. All four mutant strains were found to carry a unique point mutation within the *fabK* ribosome binding region, each of which would extend base pairing with the 3′ end of the 16S rRNA (Fig. 2C). The mutations were C-12G (strain BHK260), a ΔC12 single base deletion (strain BHK261), A-9G (strain BHK262), and T-19G (strain BHK263) (Fig. 2C). These data raised the question of whether our wild-type strain FA2-2 is representative of *E. faecalis*. It seemed possible that our strain had acquired mutations in this region that resulted in decreased *fabK* translation and that these were overcome or reversed by the spontaneous mutations. However, the sequence of the strain FA2-2 genomic segment located between the end of the *acpP* coding sequence and the beginning of the *fabK* sequence is either completely or almost completely conserved in the 295 complete and draft *E. faecalis* genome sequences currently available. Those few sequences that do not completely match the strain FA2-2 sequence have only a single base change located 63 bp upstream of the *fabK* initiation codon and hence are well removed from the ribosome binding region. Moreover, the strain FA2-2 *fabK* coding sequence is at least 99% conserved at both the nucleotide and protein levels in the 295 genome sequences.

Inactivation of *fabK* eliminates growth of the mutant strains. Although it seemed likely that growth of the spontaneously arising mutants of the Δ*fabI* strain was due to increased *fabK* expression *per se*, it was possible that the effects of the mutations were indirect. If the entire cluster of genes in which *fabK* is located is indeed an operon, then binding of ribosomes to the *fabK* sequence of the

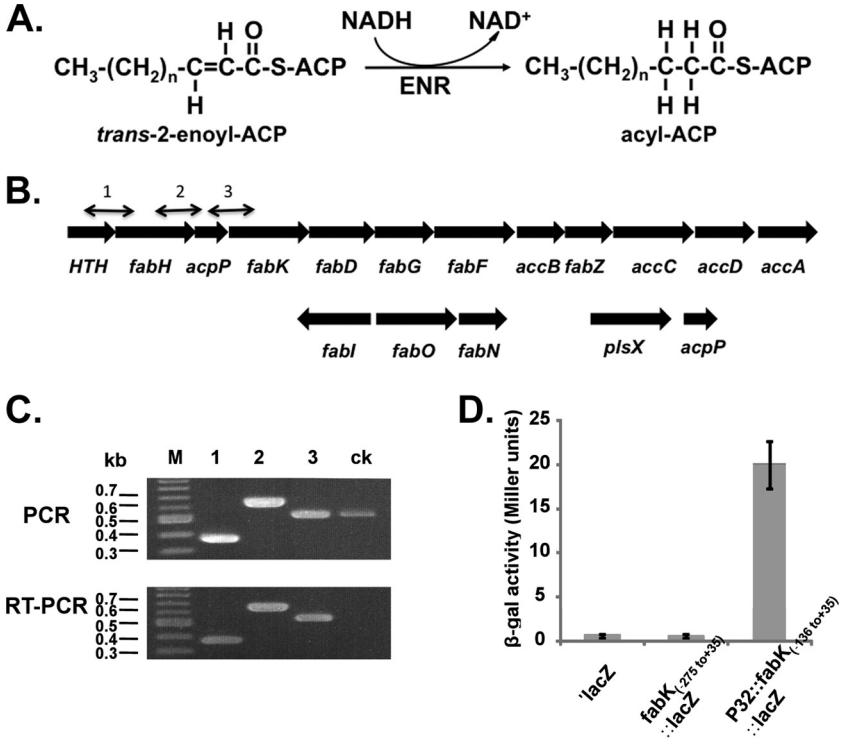

FIG 1 The ENR reaction, the *fabK* and *fabI* genome neighborhoods, and transcriptional analyses of the operon containing *fabK*. (A) The enoyl-acyl-ACP reductase reaction. (B) Genetic organization of the *E. faecalis* fatty acid biosynthetic genes (*fab*). The numbered short lines (1, 2, and 3) represent the specific PCR amplicons observed in the PCR and RT-PCR assays shown in panel C. The second *acpP* gene (EF_3111 in strain V583) located downstream of *plsX* is conserved in all extant *E. faecalis* genome sequences. (C) PCR and RT-PCR analyses of the *fab* operon. The results for the four genes at the 5′ end of the operon are shown. The helix-turn-helix-encoding gene probably encodes a homologue of the FabT transcription factor found in other *Firmicutes* bacteria (43, 44). The primer numbering system is the same as that for panel B. Both PCR products obtained from genomic DNA and those obtained by RT-PCR were separated by electrophoresis on a 1.5% agarose gel. The ck designation denotes two neighboring genes transcribed from the opposite strand that were included as controls. (D) β-Galactosidase activities of *fabK*::*lacZ* translational fusions. The wild-type strain FA2-2 carrying each of the fusion plasmids was grown in GM17 medium to mid-log phase, and β-galactosidase activities were measured in more than three independent experiments. The error bars indicate standard deviations. The plasmids carried a promoterless *lacZ* (′*lacZ*), the −275 to +35 fragment, which includes the first 35 bp of the *fabK* coding sequence fused to *lacZ* (*fabK-lacZ*), or the −136 to +35 fragment driven by the P32 promoter. The fusion plasmids were pBHK322, pBHK394, and pBHK323, respectively.

polycistronic mRNA might protect the mRNA, resulting in increased expression of the downstream genes. We tested this possibility by nonpolar disruption of the *fabK* coding sequence. If increased FabK ENR activity were responsible for growth, then *fabK* disruption would block growth in the absence of oleate, whereas if increased downstream expression were the important factor, growth should proceed. A suicide plasmid carrying an internal *fabK* fragment was inserted into *fabK* by single-crossover homologous recombination events, resulting in two disrupted copies of *fabK* bracketing the inserted plasmid (Fig. 3A and B). Upon insertion of the disruption plasmid, all four spontaneous mutants lost the ability to grow without oleate (Fig. 3C), whereas the wild-type strain grew well (due to the presence of FabI), thereby demonstrating the lack of a polar effect on expression of the downstream fatty acid synthetic genes.

Strains carrying a chromosomal or plasmid-borne mutant allele contain increased ENR activity. Our previous results showed that *E. faecalis* FabI catalyzes an essential step in fatty acid biosynthesis, and deletion of *fabI* results in almost complete loss of ENR activity (18). To directly test if growth of the spontaneous mutant strains was due to restoration of ENR activity, the ENR activities in cells from cultures grown on GM17 medium were assayed. The four spontaneous mutant strains had ENR activities that were 26 to 38% that of the FabI-containing wild-type strain FA2-2, whereas the ENR activities of the derivatives of the Δ*fabI* strain carrying the plasmid-borne mutant segments were restored to 9% to 16% that of the wild-type strain (Fig. 4A). We also used [1-^{14}C]acetate labeling and argentation thin-layer chromatographic analysis of the *de novo*-synthesized fatty acids to demonstrate ENR activity *in vivo* (Fig. 4B), and we found that the spontaneously arising mutants synthesized levels of saturated and unsaturated fatty acids comparable to those of the wild-type strain. However, consistent with their lower ENR activities and poorer growth, the strains carrying the plasmid-borne mutated segments incorporated less [^{14}C]acetate into fatty acids than the wild-type strain (Fig. 4B), and no increase in ENR activity or [^{14}C]acetate incorporation was seen when the plasmid carried the wild-type genome segment. Hence, the increased ENR activity due to mutations within the *fabK* RBS sequence was sufficient to restore growth and fatty acid synthesis to the Δ*fabI* strain.

The spontaneous mutations increase *fabK* translation. Although the location of the nucleotide changes in the mutant strains argued in favor of increased translation rather than increased transcription, we measured the mRNA levels of *fabK* in the mutant and wild-type strains. No significant change in mRNA levels was observed (Fig. 5A), and thus the primary effect of the

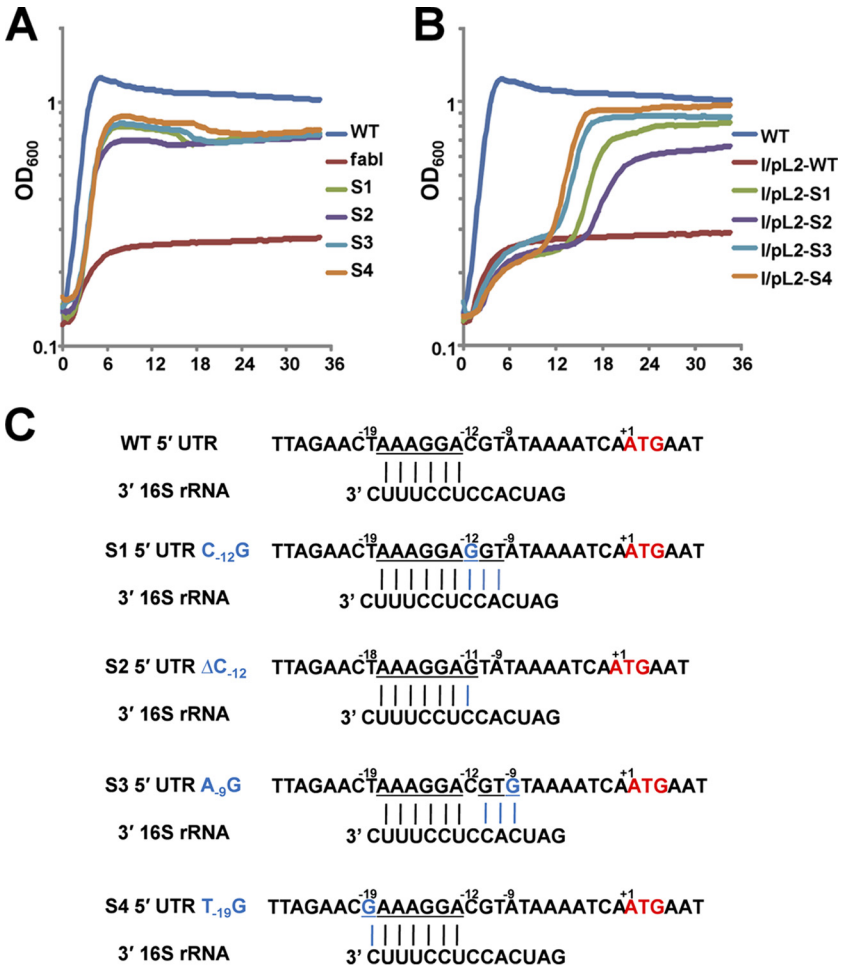

FIG 2 Growth and sequences of the spontaneously arising mutant strains and complementation of the Δ*fabI* strain by plasmid-borne fragments that included the *fabK* mutant or wild-type alleles. (A) Growth of the four spontaneously arising mutant Δ*fabI* strains (S1 to S4) on GM17 medium in the absence of oleate supplementation. (B) Complementation of the Δ*fabI* strain FAZL1 by plasmids (Table 1) carrying a mutant *fabK* allele plus about 300 bp of upstream and downstream sequence. Each growth assay was carried out in triplicate in GM17 medium at 37°C, and the averages are shown. Strain FAZL1 in panel A and the strain carrying wild-type *fabK* in panel B were used as negative controls. Panels A and B have the same color coding for the mutant *fabK* alleles. (C) Alignments of 5′ untranslated regions of the wild-type gene *fabK* or of the four spontaneously arising mutant strains with the 3′ end of the *E. faecalis* 16S rRNA. The *fabK* genes of wild-type strain FAZL1 and the four spontaneous mutants plus about 300 bp upstream and downstream were sequenced. Each point mutation expanded the *fabK* ribosome binding site. The underlined letters indicate bases capable of Watson-Crick base pairing with the 16S rRNA. +1 indicates the A of the initiation ATG codon.

mutations was indeed at the translational level. We constructed *fabK-lacZ* translational fusions that contained the *acpP-fabK* intergenic 136 bp plus the first 35 bp of the *fabK* coding sequence under the control of *Lactococcus lactis* promoter P32 and then measured *fabK* translation based on the β-galactosidase activity. The β-galactosidase levels in cultures expressing fusions with the spontaneous *fabK* mutant sequences were 4- to 7-fold higher than that of the fusion plasmid carrying the wild-type genomic segment (Fig. 5B), whereas the *lacZ* mRNA levels were essentially the same for the wild-type and mutant constructs (Fig. 5A). Hence, the spontaneous mutations overcome the normally weak translational initiation of *fabK*. Note that the 136-bp intergenic DNA fragment chosen for construction of the translational fusions was based on two considerations. The observed three intergenic amplicons from RT-PCR analysis (Fig. 1C) showed that at least the first four genes of the *fab* cluster comprise a transcription unit and hence constitute an operon. We also performed RNA ligase-mediated RACE, an improved version of 5′-RACE for amplification of the 5′ end of the cDNA, and we failed to amplify a PCR product (data not shown). Therefore, as in Fig. 1D, we were unable to detect a promoter between *acpP* and *fabK*.

Effects of site-directed and random mutagenesis of the RBS sequence on *fabK* expression and cell growth. The increased complementarity of the *fabK* RBS to the 3′ end of the 16S rRNA (Fig. 2C) seemed very likely to be responsible for the increased ENR activity of the spontaneous mutants. If so, then substitution of the mutant base with another base should result in loss of growth of the *fabI* null strain in the absence of oleic acid. Therefore, the mutated base was replaced with other nucleotides and, as expected, this resulted in loss of the abilities of plasmids carrying these constructs to complement growth of the Δ*fabI* strain (Fig. 6A) and to increase *fabK* translation (Fig. 6B).

We also randomly mutagenized the wild-type *fabK* coding sequence plus the upstream 275 bp by using error-prone PCR and

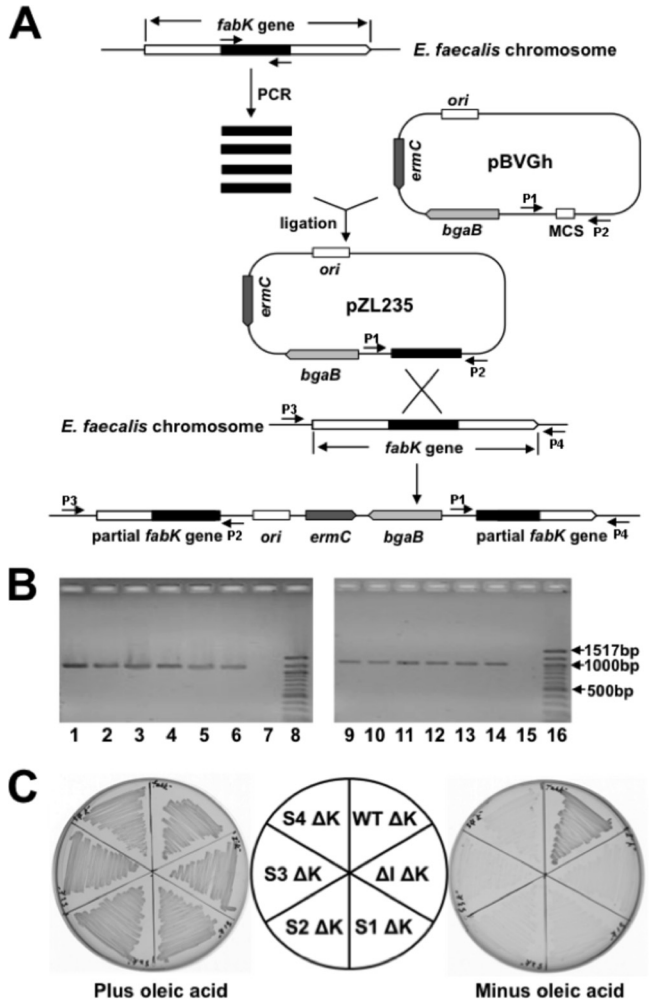

FIG 3 Construction, characterization, and growth of *fabK* disruption mutant strains. (A) Strategy for isolation of erythromycin-marked chromosomal *E. faecalis fabK* disruption mutant strains via plasmid insertion. P1, P2, P3, and P4 were the PCR primers used for characterization of the *fabK* disruption mutant strains. (B) Characterization of the *fabK* disruption strains by PCR analyses. Lanes 1 to 6, PCR products amplified from lane 1, the *fabK* disruption strain (FAZL6); lane 2, the *fabK* disruption Δ*fabI* double mutant strain (FAZL7); lanes 3 to 6, the S1 to S4 *fabK* strains in which *fabK* was disrupted (strains FAZL8 to -11, respectively). Genomic DNA preparations were used as templates with primer sets pBVGh For (P1) and *fabK* check Down (P4). Lanes 9 to 14 are the PCR products amplified using the same template DNAs as for lanes 1 to 6 but with primers pBVGh Rev (P2) and *fabK* check Up (P3). Lanes 7 and 15 are the PCR products formed from the genomic DNA of the wild-type strain FA2-2. Lanes 8 and 16 are molecular size standards. (C) Growth of *E. faecalis* mutant strains on GM17 medium plus 5 μg/ml of erythromycin at 42°C with (left) or without (right) oleic acid. Cell growth proceeded at 42°C for 36 h. ΔK, the disrupted *fabK*; WT, *E. faecalis* wild-type strain; ΔI, the *E. faecalis* Δ*fabI* gene deletion strain; S1, S2, S3, and S4, the spontaneously arising suppressors the of *E. faecalis fabI* deletion. The normal growth in the absence of fatty acid supplementation of the disrupted *fabK* derivative of the wild-type strain indicated that the disruption cassette is not polar on the downstream fatty acid synthesis genes.

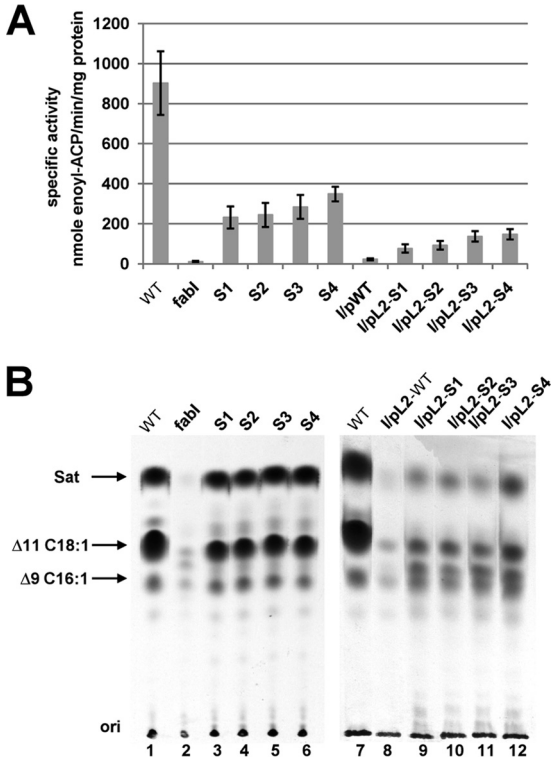

FIG 4 The spontaneous mutations increased expression of the FabK ENR. (A) ENR activities of the wild-type FA2-2, spontaneous mutants, and the complementing plasmid-bearing strains. The 100-μl ENR reaction mixtures contained 0.1 M sodium phosphate buffer (pH 7.0), 0.15 mM NADH, and 2 μg of cell extract protein, and NADH-dependent ENR activity was assayed. The reactions were initiated by addition of 100 μM *trans*-2-decenoyl-ACP. The data are from three independent experiments and are expressed as means ± standard deviations. (B) Incorporation of [1-^{14}C]acetate into the membrane phospholipids of the wild-type strain FA2-2, the spontaneous mutant strains, and the Δ*fabI* strain FAZL1 transformed with the wild-type *fabK* gene or the mutant alleles. The strains used here are the same with those in Fig. 2. The fatty acid methyl esters were prepared and analyzed by argentation thin-layer chromatography as described in Materials and Methods. Designations: Sat, saturated fatty acids; Δ9C16:1, palmitoleic (*cis*-9-hexadecenoic) acid; Δ11C18:1, *cis*-vaccenic (*cis*-11-octadecenoic) acid.

isolated plasmids that suppressed the *fabI* phenotype. A total of 13 colonies were isolated, and their plasmids were sequenced. Two isolates had the A-9G mutation found in the spontaneous mutant BHK262. Another 11 isolates had the A-9G or C-12G mutations, found in the spontaneously isolated strains, and also contained phenotypically silent coding sequence mutations that were generally conservative residue substitutions (Table 3). The reisolation of the mutations found in the spontaneously arising strains confirmed their abilities to increase the efficiency of mRNA-ribosome pairing.

DISCUSSION

Selection for growth of the *E. faecalis* Δ*fabI* strain in the absence of fatty acid supplementation gave four spontaneous mutant strains. The mutations in these strains were four different single-base changes (three single-base substitutions and one single-base deletion) in the ribosome binding region of *fabK*, which encodes a fully functional but physiologically cryptic ENR. The discrete nature of our mutations is strikingly different from previously reported spontaneous translation-activating mutants, in which large DNA fragments carrying RBS sequences have been inserted or deleted (20–22).Translation begins with the association between a 30S ribosomal subunit and the mRNA. Translational efficiency is in part determined by the RBS interaction, i.e., the base

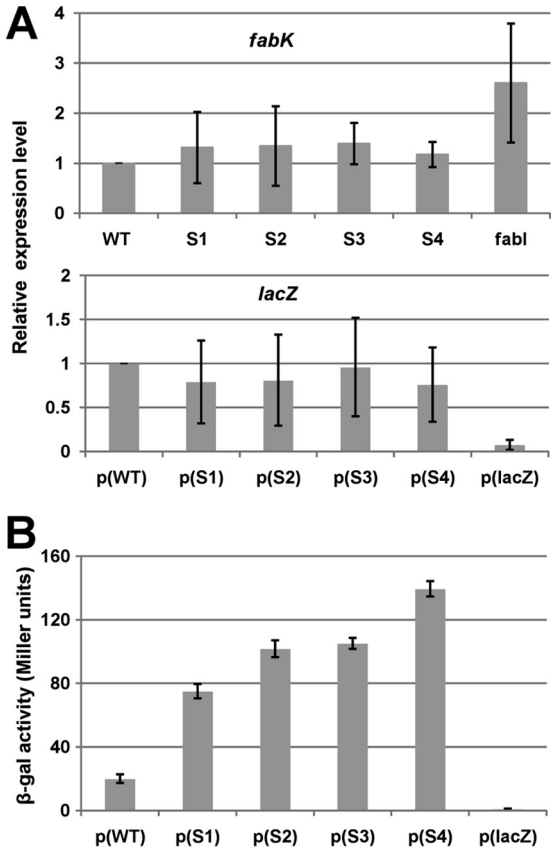

FIG 5 Effects of the spontaneous mutations on *fabK* transcription and translation. (A) qRT-PCR analyses of the effects on *fabK* and *lacZ* transcription. Transcription of *fabK* was analyzed in the wild-type strain and the spontaneous mutant strains, whereas *lacZ* transcription was analyzed for the wild-type strains carrying the translational fusion plasmid. Cells were grown in GM17 medium to mid-log phase, and RNA was isolated as described in Materials and Methods. The qRT-PCR data were from no less than four independent tests and are expressed as means ± standard deviations. The p(lacZ) designation denotes the promoterless *′lacZ* vector. (B) Effects of the spontaneous mutations on β-galactosidase expression from a plasmid carrying a P32::*fabK*::*lacZ* translational fusion. The wild-type strain FA2-2 carrying the fusion plasmids was grown in GM17 medium to mid-log phase, and β-galactosidase activities were measured from more than three independent experiments. The error bars indicate standard deviations. p(WT), p(S1), p(S2), p(S3), p(S4), and p(lacZ) indicate fusion plasmids pBHK323, pBHK324, pBHK325, pBHK326, pBHK327, and pBHK322 carrying the −136 to +35 fragments of wild-type *fabK*, one of the four spontaneous *fabK* mutants, or the empty *′lacZ* vector, respectively.

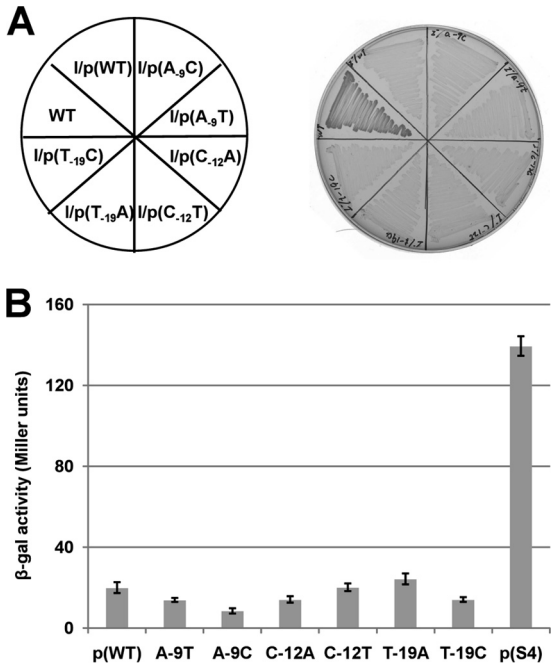

FIG 6 16S rRNA-mRNA complementarity is required for growth of the spontaneous mutants. (A) Growth was tested on GM17 plates incubated for 36 h at 37°C. In three of the spontaneous mutants, the unique nucleotide of the mutation was changed to two other nucleotides. Each site-directed *fabK* gene was cloned into the vector pTRKL2 and then transformed to the Δ*fabI* strain, FAZL1. (B) Effects of the nucleotide changes on β-galactosidase expression from a plasmid carrying a P32::*fabK*::*lacZ* translational fusion. The wild-type strain FA2-2 carrying the fusion plasmids was grown in GM17 medium to mid-log phase, and β-galactosidase activities were measured from more than three independent experiments. The error bars indicate standard deviations. The designations p(WT), A-9T, A-9C, C-12A, C-12T, T-19A, T-19C, and p(S4) indicate the fusion plasmids pBHK323, pBHK334, pBHK335, pBHK336, pBHK337, pBHK338, pBHK339, and pBHK327, respectively. The p(WT) and p(S4) plasmids were used as negative and positive controls, respectively.

pairing of the 3′ end of 16S rRNA to a stretch of complementary nucleotides in the messenger, located upstream of the initiation codon (23, 24). Indeed, as a result of the C-12 single-base change of mutant strain BHK260, the substitution with G extended the pairing potential with the 3′ end of the 16S rRNA by 3 bp (Fig. 2C) and increased *fabK* expression (Fig. 5B). Moreover, site-directed mutagenesis, in which A or T was substituted for C-12, resulted in decreased *fabK* translation (Fig. 6B). The other three single mutations appeared to have similar effects on base pairing and translation. Moreover, mutagenesis by error-prone PCR produced two of the same mutations that were isolated spontaneously (Table 3). Previous studies suggested that Gram-positive ribosomes require a stronger interaction between 16S rRNA and mRNA than Gram-negative ribosomes in order to initiate translation (21, 25). Our findings are also in agreement with a proposed scheme for the movement of mRNA at different stages of translation, in which a lengthening of the RBS duplex takes place in order to contact ribosomal protein S2 following translation initiation (26). The increased extent of complementarity between the RBS sequence and the 16S rRNA 3′ end (Fig. 2C) resulting from the mutations

TABLE 3 Results of error-prone PCR mutagenesis of *fabK* and selection for complementation of the Δ*fabI* strain FAZL1

Isolate	FabK mutation(s)
Ran2[a]	A-9G
Ran9	A-9G, L9 (CUU to CUC)
Ran16	A-9G, D78G, L154I, I163V
Ran26	A-9G, I285V
Ran12	C-12G, A185V, I220V, I261V
Ran13[a]	C-12G, V84I, K124R, L148 (UUA to CUA)
Ran15	C-12G, M281L
Ran17	C-12G, K250 Stop (AAA to UAA)

[a] Note that in Ran2 and Ran13, two synonymous mutations occurred for residues L9 and L148, respectively. In Ran17, a nonsense mutation truncated the protein at residue K250, suggesting that the last 69 FabK residues are not essential for ENR activity.

aids the mRNA-16S rRNA interaction and perhaps also aids the lengthening process.

The broad question is why all *E. faecalis* genomes encode a fully functional FabK when the enzyme activity is both redundant and is not expressed at physiologically useful levels (at least under laboratory conditions). There seems no selection for retention of this silent gene, because FabI can fully support *E. faecalis* fatty acid synthesis (18). Given this situation, the *fabK* sequence would be expected to first degenerate to encode inactive proteins and finally be lost (as may have been the case in the related bacterium *Enterococcus faecium*). Instead, the *fabK* sequence has been retained intact and still encodes an active ENR. The only straightforward explanation for this puzzling situation is that FabK expression must be activated by physiological conditions that are not duplicated in the laboratory. However, our data argue that the RBS-16S rRNA interaction is hardwired and, if that is the case, how can FabK expression be activated? Increased transcription from a cryptic promoter is a possible means, although since *fabK* is in an operon, increased expression of downstream genes would be expected, and in other bacteria overproduction of some of the encoded proteins is toxic. A common means of translational control is sequestration of the RBS by base pairing to an mRNA sequence located upstream of the RBS. The sequestration is released upon synthesis of a small RNA that base pairs with the inhibitory sequence and thereby releases the RBS for rRNA interaction and translational initiation. Another possibility is activation of a translational enhancer sequence, such as those studied in *E. coli* (26–29). *E. coli* enhancers are pyrimidine-rich sequences located upstream of the RBS that interact with ribosomal protein S1, and their effects can be augmented by tracts of adenosine bases between the RBS and enhancer (28). To our knowledge, no translational enhancer sequences have been reported in *Firmicutes* bacteria, but this is not surprising, considering the dearth of data on translational initiation in these bacteria. However, even in the *E. coli* system the details of enhancer function remain unclear, because the S1 protein is not present in the extant mRNA/ribosome crystal structures. If an *E. faecalis* translational enhancer were present upstream of *fabK*, then it could be sequestered by base pairing with a complementary sequence in the mRNA (RNA folding programs predict a high degree of base pairing within the *acpP-fabK* intergenic region transcript). The putative sequestration by base pairing could be disrupted by a small RNA (or perhaps a protein) that binds the complement of the enhancer sequence and thereby frees the enhancer to bind ribosomal protein S1.

However, it should be noted that our mutational data argue against the above scenarios, because no mutations, either spontaneous or those obtained by error-prone PCR, mapped in the region upstream of the RBS. The PCR mutagenesis produced 15,000 clones, and those that survived the selection for both increased *fabK* translation and preservation of FabK enzymatic activity all contained two of the RBS-extending mutations we had isolated spontaneously. Moreover, the fact that several of the error-prone PCR mutants had multiple base changes within the coding sequence despite the fact that FabK function was required for their isolation indicates that the mutagenesis was robust.

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