

Ethanolamine Activates a Sensor Histidine Kinase Regulating Its Utilization in *Enterococcus faecalis*^{∇†‡}

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***Enterococcus faecalis* is a gram-positive commensal bacterium of the human intestinal tract. Its opportunistic pathogenicity has been enhanced by the acquisition of multiple antibiotic resistances, making the treatment of enterococcal infections an increasingly difficult problem. The extraordinary capacity of this organism to colonize and survive in a wide variety of ecological niches is attributable, at least in part, to signal transduction pathways mediated by two-component systems (TCS). Here, the ability of *E. faecalis* to utilize ethanolamine as the sole carbon source is shown to be dependent upon the RR-HK17 (EF1633-EF1632) TCS. Ethanolamine is an abundant compound in the human intestine, and thus, the ability of bacteria to utilize it as a source of carbon and nitrogen may provide an advantage for survival and colonization. Growth of *E. faecalis* in a synthetic medium with ethanolamine was abolished in the response regulator RR17 mutant strain. Transcription of the response regulator gene was induced by the presence of ethanolamine. Ethanolamine induced a 15-fold increase in the rate of autophosphorylation in vitro of the HK17 sensor histidine kinase, indicating that this is the ligand recognized by the sensor domain of the kinase. These results assign a role to the RR-HK17 TCS as coordinator of the enterococcal response to specific nutritional conditions existing at the site of bacterial invasion, the intestinal tract of an animal host.**

Enterococci are the third most frequently detected nosocomial pathogens in the United States, with *Enterococcus faecalis* accounting for the majority of the clinically relevant isolates (12). *E. faecalis* is a commensal gram-positive nonsporulating bacterium responsible for a wide array of human diseases of various severities, depending on the location of the infection and the host's immune status, including endocarditis, meningitis, and urinary tract infections (34). Infections caused by *E. faecalis* have increased in the past decade due to the acquisition of multiple antibiotic resistances, which has made some enterococci refractory to all antimicrobial regimens (24). This opportunistic pathogen has an extraordinary capacity to grow under hostile conditions and to colonize and survive in a large range of ecological niches (17).

The ability of most bacteria to sense and adapt to changing conditions is frequently mediated through two-component signal transduction systems (TCS). TCS generally consist of a sensory histidine kinase (HK) and a response regulator (RR). The histidine kinase senses the signal and relays the adaptive response through the transfer of a phosphoryl group to the response regulator, which modulates gene expression by acting as a transcriptional regulator (23).

A total of 17 TCS and one orphan response regulator have been identified on the genome of *E. faecalis* V583 (18). Bioin-

formatic analyses on the genomic location of each TCS revealed that the RR-HK17 system is surrounded by genes encoding proteins related to the components of the ethanolamine (EA) utilization pathway in *Salmonella enterica* (27). This large metabolic pathway must have been maintained by selection, yet no condition is known under which *E. faecalis* utilizes EA for growth.

EA is an abundant compound in the human intestinal tract (27, 31) and in processed food (2, 10), and it can be utilized as a source of carbon, nitrogen, and energy under aerobic and/or anaerobic conditions. Korbel et al. (28) reported that three of the most hazardous food-borne pathogens, *Listeria monocytogenes*, *Clostridium perfringens*, and *Salmonella enterica*, carried highly similar EA utilization operons, but EA utilization genes are absent in most other prokaryotes. These authors predicted that the EA utilization pathway is an important genomic determinant of pathogenicity associated with food poisoning. The presence of EA utilization genes could promote anaerobic growth in the host and in processed food and, in parallel, may provide an advantage over natural gut bacteria not possessing this function. Consistent with this notion, *Salmonella enterica* serovar Typhimurium mutant strains unable to grow on EA were found to be attenuated in a mouse model of infection (53).

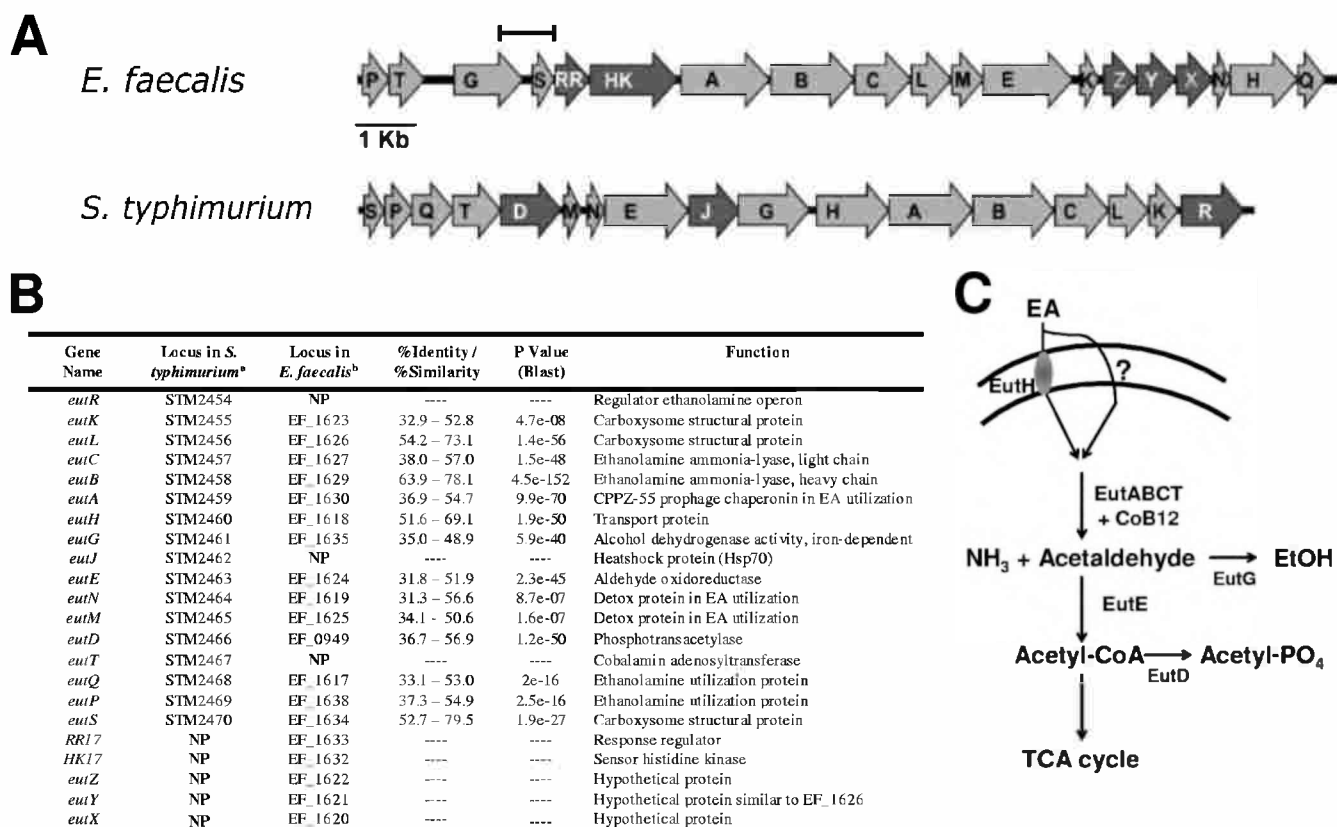
Several investigations have studied the minimal functions and physiological conditions required for growth of *S. enterica* serovar Typhimurium on EA. In general, two sequential reactions convert EA into acetyl coenzyme A (Ac-CoA). First, EA is converted to acetaldehyde and free ammonia by the adenosylcobalamin (CoB12)-dependent EA ammonia-lyase encoded by the *eutBC* genes of the operon (Fig. 1) (13). In the second step, acetaldehyde is oxidized to acetate and activated to Ac-CoA in a step presumably catalyzed by the acetaldehyde dehydrogenase (*EutE*) (53).

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a) *Salmonella typhimurium* LT2 accession number NC_003197.
 b) *E. faecalis* V583 accession number NC_004668.
 NP: Not present

FIG. 1. *eut* operon for EA utilization in *E. faecalis* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). (A) A diagram of the gene organization in the two operons is shown with the darker shade of gray representing genes that are present in one organism but missing in the other. The bar represents the extent of the fragment cloned in the pTCV-RR17p transcriptional fusion plasmid. (B) Table summarizing gene names, annotation numbers in genome databases, the results of ClustalW and BLAST analyses, and function. (C) Schematic representation of the basic EA metabolism in *Salmonella*. EA can enter the cell by diffusion either via EutH or by other, unknown routes (38). EutBC are the two subunits of the cobalamin-dependent EA ammonia-lyase. EutE is an acetaldehyde dehydrogenase. EutG is an alcohol dehydrogenase, and EutD is a phosphotransacetylase (6, 27, 39). Notably, the genes for the tricarboxylic acid cycle (TCA) are absent in the genome of *E. faecalis* V583.

In two additional reactions, acetaldehyde is reduced to ethanol in a reaction catalyzed by EutG with a not-well-defined involvement of EutJ (38). The Ac-CoA is converted to acetyl phosphate in a reaction catalyzed by EutD phosphotransacetylase. Acetyl phosphate is used to conserve energy via substrate level phosphorylation catalyzed by acetate kinase, yielding ATP and acetate. Acetate is excreted into the medium and later recaptured by Ac-CoA synthetase (51). In *S. enterica*, the anaerobic use of EA provides ATP and an electron sink.

The 17-gene operon encoding the requirements for EA utilization in *S. enterica* serovar Typhimurium is induced by the simultaneous presence of EA and cobalamin/vitamin B₁₂ (VitB12) and requires the product of the last gene of the operon, *eutR*. EutR is a transcription factor belonging to the AraC family of DNA-binding proteins (15, 27, 46).

In the present study, we describe a growth condition in which *E. faecalis* utilizes EA as a carbon source under anaerobic growth conditions in the presence of cobalamin (VitB12). We also show that, in contrast to *S. enterica*, EA utilization in *E. faecalis* is dependent upon the RR-HK17 TCS and EA is the

direct effector inducing autophosphorylation of the sensor histidine kinase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. faecalis* strains and plasmids used in this study are listed in Table 1. Strains were cultured in Todd-Hewitt broth. Notably, the V583T strain was used as a control instead of the parental V583 strain in every experiment that involved the use of mutant strains such as RR15, RR17, and RR15-17 (which carry the TetR plasmid p3TET inserted by single crossing-over integration at the deletion site) which were grown in the presence of tetracycline. To our knowledge, the insertion of p3TET in the V583 strain does not cause any detectable phenotype (19). *Escherichia coli* DH5 α and TB1 were used for plasmid constructions and propagation. Strains were cultivated in Luria-Bertani broth. Antibiotics used for selection in *E. coli* and *E. faecalis* were spectinomycin (150 and 750 μ g/ml, respectively), ampicillin (100 μ g/ml), and tetracycline (15 μ g/ml, unless otherwise specified). *Enterococcus* electroporation was carried out as described previously (11).

The minimal medium M9HY is base medium (20 mM HEPES [pH 7.0], 2 g/liter yeast extract [final concentration]) to which individually sterilized M9 salts (47), MgSO₄, and CaCl₂ were added at 1 \times , 2 mM, and 0.1 mM, respectively. Vitamin and amino acid mixes were also added to achieve a 1 \times final concentration from 100 \times stocks. Vitamin and amino acid stocks were prepared essentially as described previously (35). Vitamins at 1 \times concentration were as follows:

TABLE 1. *Enterococcus faecalis* strains and plasmids used in this study

| Strain or plasmid | Relevant information | Reference or source(s) |
|-------------------|---|---|
| Strains | | |
| V583 | <i>E. faecalis</i> | Clinical isolate |
| RR15 | RR15 insertion mutant of V583; Tet ^r | 19 |
| RR17 | RR17 insertion mutant of V583; Tet ^r | 19 |
| RR15-17 | RR15 RR17 double mutant insertion of V583; Tet ^r Cm ^r | This study |
| V583T | Insertion mutant of V583 with p3TET inserted between HK02 and the adjacent open reading frame encoding a putative lipoprotein | 19 |
| V583/pTCV-LacSpec | V583 containing pTCV-LacSpec; Sp ^r | This study |
| V583/pTCV-RR17p | V583 containing pTCV-RR17p; Sp ^r | This study |
| RR17/pTCV-LacSpec | RR17 containing pTCV-LacSpec; Sp ^r Tet ^r | This study |
| RR17/pTCV-RR17p | RR17 containing pTCV-RR17p; Sp ^r Tet ^r | This study |
| Plasmids | | |
| pTCV-Lac | Transcriptional <i>lacZ</i> fusion vector | 3 |
| pML28 | pAT28 with <i>aphA3</i> promoter on a 369-bp EcoRI/BamHI fragment | 20 |
| pML28-RR17 | pML28 derivative containing <i>E. faecalis</i> RR17 coding sequence | This study |
| pTCV-LacSpec | SpcR derivative of pTCV-Lac | This study; Hancock and Perego, unpublished |
| pTCV-RR17p | pTCV-LacSpec derivative containing <i>E. faecalis</i> RR17 promoter; -722 to +122 | This study |

thiamine, 100 µg/ml; biotin, 20 µg/ml; panthothenic acid, 20 µg/ml; nicotinic acid, 2 µg/ml; riboflavin, 2 µg/ml; and folic acid, 0.2 µg/ml. Amino acids at 1× concentration were as follows: arginine, 20 µg/ml; glutamate, 20 µg/ml; glycine, 20 µg/ml; histidine, 20 µg/ml; isoleucine, 20 µg/ml; leucine, 20 µg/ml; methionine, 20 µg/ml; tryptophan, 20 µg/ml; and valine, 20 µg/ml. Growth and survival experiments were done at 37°C. The M9HY medium was supplemented with glucose at 100 mM or EA at 40 or 100 mM final concentration. VitB12 and the coenzyme CoB12 (Sigma-Aldrich) were used as exogenous VitB12 sources. All solutions were prepared separately and sterilized by autoclaving for 15 min. Amino acid mix, vitamin mix, and VitB12 derivative solutions were prepared separately and sterilized by filtration.

For growth under normal oxygen conditions, the M9HY medium was inoculated with *E. faecalis* cells from overnight cultures in M9HY medium with 100 mM glucose. Cells were washed twice in M9HY medium without carbon sources (NCS). Cells were grown at 37°C without agitation and ample air space. This growth condition is referred to as aerobic growth. For the detection of EA utilization, the M9HY medium supplemented with all the necessary components was degassed, inoculated with cells from an overnight culture as described above, and flushed with argon. Argon was flushed in the culture tubes every time a sample was withdrawn. This growth condition is referred to as anaerobic growth throughout this work. Incubations were performed in the dark at 37°C without shaking. Overnight cultures were diluted 1:1,000 (see Fig. 3 and 5), 1:20 (see Fig. 4), or 1:15 (see Fig. 6). The average optical density (OD) at 600 nm of the overnight cultures was ~0.2 to 0.3.

Transcription analysis. The transcriptional fusion vector pTCV-LacSpec was used to monitor transcription of the *eut* operon. The pTCV-LacSpec vector is a derivative of pTCVlac (40) in which the kanamycin resistance marker was replaced by the spectinomycin resistance gene originally from piC333 (52; L. E. Hancock and M. Perego, unpublished data). The region upstream the RR17-encoding gene was amplified by PCR using oligonucleotide primers PduQEco5' and RR17Bam3' (Table 2) and was cloned in vector pTCV-LacSpec using EcoRI and BamHI restriction sites to generate a transcriptional fusion to the promoterless *lacZ* reporter gene (pTCV-RR17p). The 834-bp fragment cloned in pTCV-RR17p included 722 bp upstream the RR17 translational start codon and 112 bp of coding sequence. The sequence of the cloned promoter fragment was confirmed by DNA sequencing analysis.

E. faecalis strains containing pTCV-LacSpec or pTCV-RR17p were grown in M9HY medium in the presence of spectinomycin. Samples were collected at hourly intervals. β-Galactosidase activity was detected by the method described by Poyart and Trieu-Cuot (40), with some modifications. In order to lyse the cells, 10 µl of lysozyme, 10 mg/ml containing 100 U/ml of mutanolysin, were added to 100 µl of sample and incubated at 37°C for 60 min. The β-galactosidase activities were calculated according to Miller (33). Samples were taken and processed in duplicate, and the mean values were used for the calculations.

The RR17-complementing plasmid was constructed in the replicative vector pML28 (20), which is a derivative of pAT28 (55) carrying the *aphA3* promoter. The RR17 coding sequence and approximately 100 bp of the upstream region

were amplified by PCR using oligonucleotide primers RR17Bam25' and RR17SalI3' (Table 2). After digestion by BamHI and SalI, the fragment was cloned in similarly digested pML28.

Expression and purification of RR17-H₆ and HK17-H₆. The RR17 and HK17 coding regions were amplified from *E. faecalis* V583 genomic DNA by using primers EFRR175'NdeI/EFRR173'Xho and EFHK175'NdeI/EFHK173'XhoI, respectively (Table 2), and cloned in pET28a(+)-cut NcoI/XhoI, generating a fusion to six-His codons at the 3' end. The resulting plasmids were transformed in *E. coli* expression strain BL21(DE3)pLys (Novagen). Protein expression was induced at an OD at 600 nm of 0.5 with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and cells were allowed to grow for 4 h at 28°C. Cells were then harvested, washed in buffer B (50 mM Tris-HCl [pH 7.0 for RR17 and pH 8.0 for HK17], 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), resuspended in buffer B with 10 mg/ml lysozyme, incubated 30 min at 4°C, and lysed using sonication. The proteins were then purified by immobilized metal affinity chromatography using the Ni-nitrilotriacetic acid resin (Qiagen, Valencia, CA). The proteins were eluted from the column by a gradient of 30 to 300 mM imidazole in buffer B. Fractions were pooled and dialyzed against 50 mM Tris-HCl (pH 7.0 or pH 8.0), 100 mM NaCl, and 3 mM dithiothreitol. Finally, the proteins were concentrated and stored with 20% glycerol at -20°C.

Protein phosphorylation assay. The phosphorylation assays were carried out at room temperature in the presence of [γ -³²P]ATP (specific activity 6,000 Ci/mmol) and cold ATP (900 µM final concentration) in buffer A (20 mM

TABLE 2. Oligonucleotide primers used in this study

| Primer | Sequence ^a |
|-------------|--|
| EFRR175'Nde |5'-CGTGAAGCATATGGATGGACGAATT GTAATAG-3' |
| EFRR173'Xho |5'-AATCGCTCGAGTTATTCATCATCCAT TACAATC-3' |
| EFHK175'Nde |5'-TGATGAACATATGAAACGATTAGAG CAATTATG-3' |
| EFHK173'Xho |5'-CCTTCTCGAGTCAATGAACAACATC ACTGG-3' |
| RR17Bam25' |5'-GGGAGGATCCAAGCGGTGATTGAAG GTTT-3' |
| RR17SalI3' |5'-TTTAGCTGACTGGTGACATAAATGCT CTAATCG-3' |
| RR17Bam3' |5'-CGCGGATCCAAAACCATCAGCTGC TTCA-3' |
| PduQEco5' |5'-CGCGAATTCAAAATCAAGCAACTCCG TCA-3' |

^a Restriction sites are in boldface type.

HEPES [pH 7.5], 50 mM KCl, 2 mM MgCl₂, 5 mM CaCl₂, 10 mM dithiothreitol, 10 μM bovine serum albumin). The reactions were initiated by the addition of [γ -³²P]ATP, terminated by the addition of a 5% sodium dodecyl sulfate sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% polyacrylamide gels. Proteins and EA concentrations were used as indicated in the figure legends. EA at the concentration used in the assays did not alter the pH of the reaction buffer (data not shown). Gels were dried, and labeled proteins were detected by a PhosphorImager screen (Amersham Molecular Dynamics).

RESULTS

The *E. faecalis* gene cluster for EA utilization (*eut*) contains a TCS. A bioinformatic analysis of the genome context for each of the 17 TCS identified in *E. faecalis* V583 (19) revealed that the RR-HK17 system (EF1633-EF1632 [GenBank NC_004668]) was embedded within genes encoding proteins with strong similarities to members of the EA utilization pathway of *Salmonella enterica* serovar Typhimurium (*eut*) (27) (Fig. 1). The 17 putative *eut* genes of *E. faecalis* were found upstream (*eutP*, *-T*, *-G*, and *-S*) or downstream (*eutA*, *-B*, *-C*, *-L*, *-M*, *-E*, *-K*, *-Z*, *-Y*, *-X*, *-N*, *-H*, and *-Q*) of TCS-17. As shown in Fig. 1, the gene order is not fully conserved between *Enterococcus* and *Salmonella*. Also some genes present in *Salmonella* are missing in *E. faecalis*, and vice versa. Most notably, the *eutR* gene encoding the autoregulator of the *eut* operon in *Salmonella* is missing in *E. faecalis*, which, instead, has the RR-HK17 TCS. Also different from *Salmonella*, the *eutD* gene (EF0949) encoding a phosphotransacetylase is not within this cluster of genes but is located in a different chromosomal region. The only gene whose product is not significantly conserved with the product of an *eut* gene of *Salmonella* is the EF1637 gene. Nevertheless, the EF1637 protein is annotated as a putative cobalamin adenosyltransferase, making it likely to carry out the same function of EutT (STM2467), which is similarly annotated and shares 11% of identical residues.

The genome sequences of *Clostridium perfringens*, *Listeria monocytogenes*, *Listeria innocua*, and *Streptococcus sanguinis* also revealed the presence of a highly conserved TCS upstream of the *eutA* gene. The RR17 and HK17 proteins shared approximately 69% and 47% of identical residues, respectively, with their homologues in all the sequenced *Listeria* species (data not shown).

The 19 genes present in this *E. faecalis* cluster may be transcribed from more than one promoter. Consensus sequences compatible with the -35 and -10 recognition sites of σ^A -containing RNA polymerase are detectable 130 bp upstream of the *eutP* gene (TTGACA-17 bp-TAAGAT) and in the *eutG-eutS* intergenic region (TTGACA-17 bp-TATAAT).

Notably, a B12 riboswitch-like element was found in the *eutT-eutG* intergenic region by the RibEx riboswitch explorer (significance value of $3.16E-10$) (1). This riboswitch expands for approximately 170 bp, starting 40 bp from the stop codon of *eutT* (EF1637) (from nucleotide 1591221 to 1591049, according to Barrick and Breaker [4]). Riboswitches are mRNA control elements that respond to ligand concentration by modulating gene expression via transcription termination or translation mechanisms (59). Similar observations were reported by K. A. Fox, A. Maadani, and D. A. Garsin at the International Conference on Gram-Positive Pathogens in Omaha, NE, in 2006.

Based on GenBank database and BLAST searches, it seems

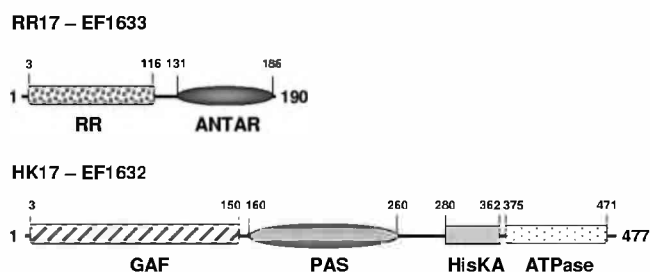


FIG. 2. Schematic representation of the structural and functional domains of the RR17 response regulator (190 amino acids) and the HK17 histidine kinase (477 amino acids). The numbers delineate the approximate initial and terminal amino acid residues for each domain, as deduced from the Pfam and FFAS03 database analyses.

that the *E. faecalis* V583 genome carries all the genes necessary for EA metabolism.

Structural features of the RR-HK17 TCS. Two-component signal transduction proteins often contain structural features, in addition to the conserved response regulatory, histidine kinase (HisKA), and ATP-binding (ATPase) domains, that may provide an indication of specialized function. Searches for the structural features of the RR17 response regulator and the HK17 histidine kinase were conducted in the PSI-BLAST, Pfam, and FFAS03 databases (14, 26). The results are summarized in Fig. 2. RR17 is a member of the family of regulatory proteins with an ANTAR carboxy-terminal domain (49). The ANTAR domain interacts with RNA instead of DNA, and the first ANTAR response regulator, AmiR of *Pseudomonas aeruginosa*, was described as an antiterminator of transcription acting on a *rho*-independent terminator (58). The amino-terminal domain of RR17 is predicted to have a typical response regulator fold, with the aspartate residues at positions 9, 10, and 54 likely to constitute the phosphorylatable active site.

The HK17 histidine kinase is a cytoplasmic protein of 477 amino acids, with the amino-terminal 150 residues recognized as likely to form a GAF domain (probability of <-9.5 by the FFAS03 search program). GAF domains are ubiquitous motifs found in prokaryotic, eukaryotic, and plant proteins. Originally identified as the noncatalytic binding domain of cyclic GMP in cyclic nucleotide phosphodiesterases (9), it is now often found in sensor histidine kinases, among other proteins. GAF domains, however, may interact with alternative ligands, such as heme, in the *Mycobacterium tuberculosis* DevS and DosT histidine kinases (48, 50), photopigments in plant phytochromes (30), or nitric oxide (NO) through a nonheme mononuclear iron center as in the *E. coli* NorR transcription regulator (56). The structure of the GAF domain is very similar to that of the PAS domain, in spite of very limited sequence conservation (22).

A PAS domain is also present in HK17 between the GAF and the HisKA domains. The PAS domain is another ubiquitous structural feature of proteins from bacteria to humans. PAS domains can transduce signals by mediating protein-protein interaction upon sensing signals such as light, oxygen, redox potential, and small ligands (16, 54). These structural features of the HK17 histidine kinase strongly suggest that its activity may be regulated by a ligand such as EA whose met-

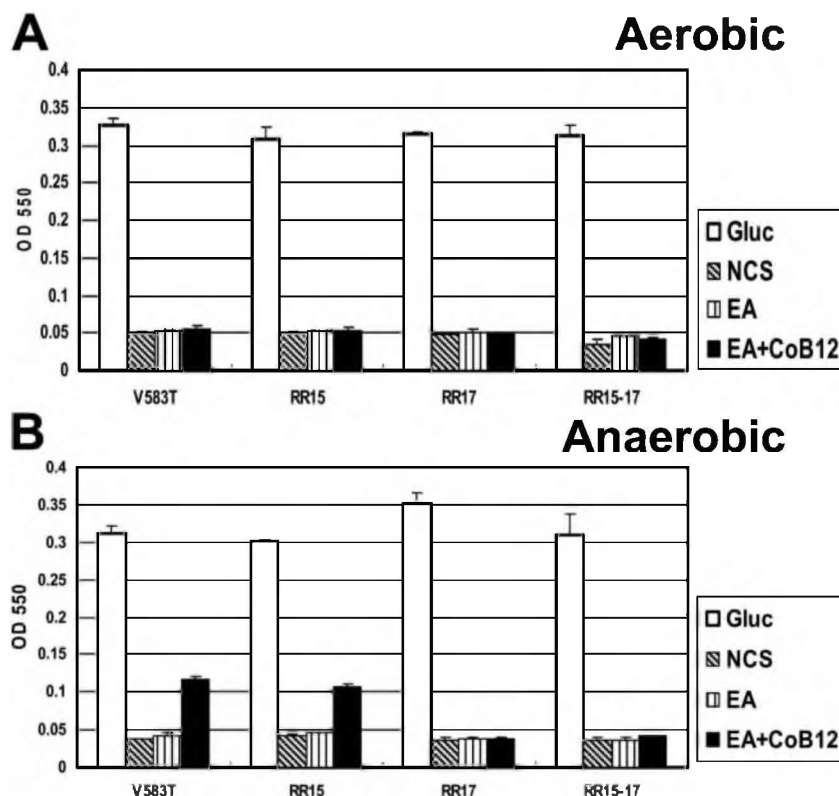


FIG. 3. Growth of *E. faecalis* on EA. ODs of *E. faecalis* cultures after 24 h of growth under aerobic (A) or anaerobic (B) conditions in M9HY medium containing 10 μ g/ml of tetracycline. Gluc, M9HY medium plus 100 mM glucose; NCS, M9HY medium without carbon source; EA, M9HY medium plus 100 mM EA; EA + CoB12, M9HY medium plus 100 mM EA and 40 nM CoB12. The error bars represent standard deviations of three independent cultures.

abolic pathway is encoded in the 19-gene cluster comprising TCS-17.

E. faecalis utilizes EA under anaerobic growth conditions.

The use of EA as a source of carbon and nitrogen could be important, since this compound is a constituent of abundant species of lipid present in the intestinal tract. At present, there are no reports of EA utilization in enterococci, and their complex growth requirement has often restricted the use of synthetic media (35).

In order to determine whether *E. faecalis* could utilize EA as the sole source of carbon, a synthetic medium was devised as a derivative of the M9 minimal salt medium supplemented with HEPES buffer, yeast extract, vitamins, and essential amino acids (see Materials and Methods). The strict *E. faecalis* requirement for essential amino acids did not really allow us to test the utilization of EA as a source of nitrogen. In the absence of carbon sources, the optical density of the initial inoculum in M9HY medium increased by approximately 2 to 3 orders of magnitude after 24 h of incubation. With the addition of glucose at 100 mM, a poor but highly reproducible growth of the control strain V583T in air at 37°C was observed (Fig. 3A). When glucose was replaced by EA, however, no growth was observed above the level reached in the absence of carbon sources. Because the first enzyme in the EA utilization pathway, the EA ammonia-lyase (the product of the *eutBC* genes), is dependent upon VitB12 as cofactor and the *E. faecalis* genome carries genes for VitB12 transport but not for its biosyn-

thesis (43), growth in air was also tested in the presence of EA with either VitB12 or CoB12, but no increase in OD was observed above the level observed for the cultures grown without carbon sources (Fig. 3A and data not shown). Also, no growth stimulation was observed in the presence of VitB12 or CoB12 alone (see Fig. S1 in the supplemental material). The same growth conditions were tested under anaerobic conditions (see Materials and Methods), and the ability of the parental strain to grow poorly but consistently in the presence of VitB12 (data not shown) or CoB12 (Fig. 3B) was observed. This result allowed us to test whether a deletion of the RR17-encoding gene would affect the ability of *E. faecalis* to utilize EA. As shown in Fig. 3B, the RR17 mutant in the presence of EA and CoB12 did not grow to an OD greater than that of the control culture grown without any carbon sources. Notably, in the absence of EA, neither VitB12 nor CoB12 promoted any growth to a level greater than that of the control culture grown without carbon sources (data not shown; see Fig. S1 in the supplemental material).

The observation that most of the genes from EF1617 (*eutQ*) to EF1638 (*eutP*) were highly repressed by the Fsr system in a microarray study (5) prompted us to test whether a deletion of the response regulator FsrA-encoding gene (RR15, or EF1822) affected EA utilization. As shown in Fig. 3B, the RR15 single mutant strain grew as the parental strain under anaerobic conditions and did not overcome the requirement for the RR17 protein in the double mutant strain, thus

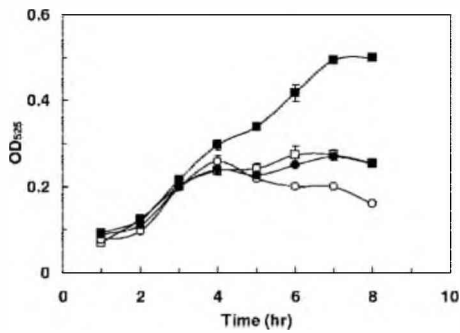


FIG. 4. Growth curves of *E. faecalis* parental strain V583T and RR17 mutant strain on EA as the sole carbon source in M9HY medium containing 10 µg/ml of tetracycline. EA was provided at 100 mM and CoB12 at 40 nM. Filled and open symbols represent growth under anaerobic and aerobic conditions, respectively. Squares represent *E. faecalis* V583T, while circles indicate the RR17 mutant. The error bars represent standard deviations calculated from two independent cultures. OD₅₂₅, OD at 525 nm.

ruling out a significant role for FsrA in regulating the *eut* genes under these growth conditions.

A time course of growth of the parental strain, V583T, and the RR17 mutant (Fig. 4) confirmed that EA with CoB12 allowed *E. faecalis* to grow, albeit poorly, only under anaerobic conditions, and the RR17 response regulator was required for this effect.

Notably, the supernatant of the parental V583T strain grown under anaerobic conditions in the presence of EA and CoB12 acquired a yellow-brown color after more than 24 h of incubation. The color was less intense when the cells were grown with EA and VitB12 (data not shown). The formation of this pigment was dependent upon the presence of bacteria, EA, CoB12, and anaerobiosis. This pigment was soluble in phenol (pH 8.0) and, in an absorption spectrum, showed a maximum in the UV region at an OD of ~265 nm (see Fig. S2 in the supplemental material). The interference of the yellow-brown pigment to the cell OD at 550 nm was minor and did not affect the conclusion that EA and CoB12 promoted bacterial growth

under the assay conditions tested (see Fig. S1 in the supplemental material).

In order to ensure that the increase in OD observed upon incubation in the presence of EA and VitB12 or CoB12 corresponded to an increase in CFU levels (CFU/ml), the V583T strain grown in M9HY medium for 24, 48, or 72 h was plated on Todd-Hewitt agar plates. The results of the colony count shown in Fig. 5 indicated that a small (10-fold) increase in the number of CFU/ml was consistently observed in the presence of EA and either VitB12 or CoB12 after 24 h of incubation, thus confirming the ability of *E. faecalis* to grow with these supplements.

Nevertheless, prolonged incubation (48 and 72 h) resulted in a reproducible, drastic loss of viability, as measured by CFU/ml, particularly in the cultures containing CoB12 (Fig. 5), suggesting that a product of EA utilization may be toxic to the cells.

A promoter upstream of RR17 is induced by EA-CoB12 in anaerobiosis. The 17-gene operon for EA utilization in *S. enterica* serovar Typhimurium is transcribed from a main promoter, located upstream of *eutS*, and from a minor promoter, adjacent to the *eutR* gene that provides a low constitutive level of EutR regulator sufficient to initiate induction of the main promoter.

In order to test whether in *E. faecalis*, as in *Salmonella*, the regulator of *eut* expression was immediately preceded by a promoter, plasmid pTCV-RR17p was constructed by fusing a fragment ranging from position -722 to +112, relative to the RR17 start codon, to the promoterless *lacZ* reporter gene in pTCV-LacSpec. This construct and the vector itself were transformed by electroporation into the V583T parental strain, and the β-galactosidase activities were analyzed in M9HY medium under anaerobic growth conditions. As shown in Fig. 6A, induction of transcription was observed in the parental strain grown in the presence of EA and CoB12, but when cells were grown in the presence of only EA, or CoB12 or VitB12, the level of transcription was not significantly different from the level detected in the parental strain V583T carrying the vector pTCV-LacSpec alone (Fig. 6B). Also, no induction of tran-

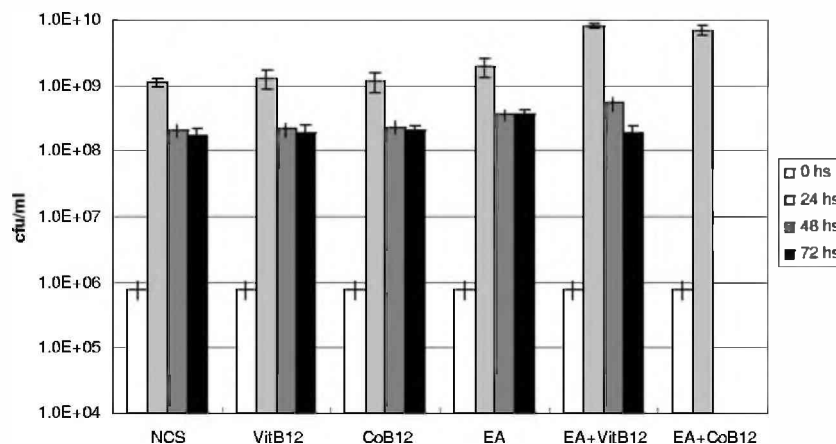


FIG. 5. Growth and survival of *E. faecalis* V583 under anaerobic conditions utilizing EA as a carbon source. CFU per ml was measured after 0, 24, 48, and 72 h. NCS, M9HY medium without carbon source; EA, 100 mM EA; CoB12, 40 nM CoB12; VitB12, 40 nM VitB12. The 48-h and 72-h time points of the EA plus CoB12 culture conditions are missing because counts were below the minimal experimental limit set for the experiment. The error bars denote standard deviations calculated from three independent cultures.

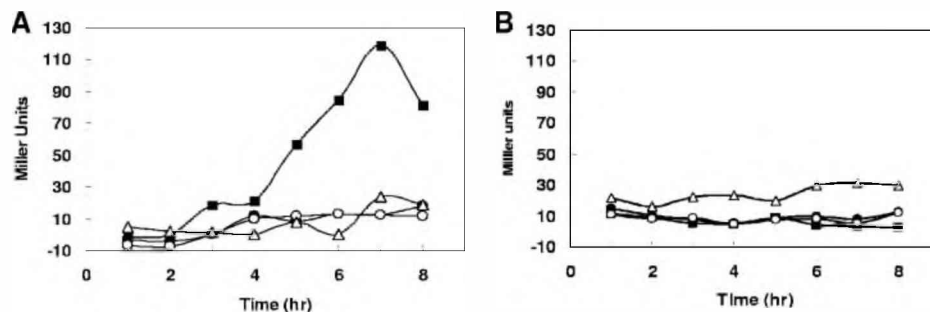


FIG. 6. Transcription analysis of the RR17-encoding gene. Time course of β -galactosidase activity was taken from a culture of the parental strain, V583, carrying the pTCV-RR17p transcriptional *lacZ* fusion construct (A) or the pTCV-LacSpec vector (B). Cells were grown in M9HY medium containing 750 μ g/ml spectinomycin and supplemented with the following: 100 mM EA plus 40 mM CoB12 (■); 100 mM EA (●); NCS plus 40 nM CoB12 (○); and 100 mM glucose plus 40 nM CoB12 (△).

scription was observed when cells were grown in the presence of EA, CoB12, and glucose at 100 mM (data not shown), suggesting that carbon catabolite repression may regulate this promoter, as previously shown in *Salmonella* for the main promoter of the *eut* operon (46).

The inability of the RR17 mutant strain to grow under these assay conditions did not allow us to test the role of the RR17 response regulator on the transcription generated by this *lacZ* fusion construct.

These results, together with the observation that RR17 is required for *E. faecalis* growth in the presence of EA, indicate that the RR-HK17 TCS is likely expressed from an autoregulated promoter in response to EA and VitB12 but subjected to repression by a carbon catabolite repression mechanism. This promoter is likely located in the *eutG-eutS* intergenic region where putative -10 (TATAAT) and -35 (TTGACA) consensus sequences separated by 17 bp and compatible with recognition by RNA polymerase containing a σ^A -type sigma factor are located approximately 150 bp upstream of the *eutS* translational start site.

Autophosphorylation of HK17 is stimulated by EA. The EutR protein of *S. enterica* serovar Typhimurium induces the expression of the *eut* operon in response to EA and VitB12, but binding of either or both effectors has not been demonstrated experimentally.

In order to test whether EA could induce the autophosphorylation of the HK17 sensor histidine kinase, an *in vitro* assay was carried out with the protein purified from an overexpressing *E. coli* strain (see Materials and Methods). As shown in Fig. 7A, the presence of an equimolar concentration of EA stimulated the enzyme autophosphorylation activity by approximately 10-fold. With a 2:1 stoichiometry of ligand versus enzyme, the autophosphorylation activity essentially reached a plateau. No stimulation of autokinase activity was observed when VitB12 or CoB12 was added to the reaction (see Fig. S3 in the supplemental material), and the presence of CoB12 did not affect the stimulation brought about by EA (data not shown). A time course analysis of HK17 autophosphorylation in the presence and absence of EA revealed an approximately 15-fold increase in the rate of the reaction in the presence of a saturating concentration of effector (Fig. 7B).

In the presence of purified RR17 protein, the autophosphorylated HK17 sensor kinase was able to efficiently transfer

phosphoryl groups to its mated response regulator, thus confirming the functionality of the TCS in sensing EA and responding to its presence by activating the transcription factor (see Fig. S4 in the supplemental material).

DISCUSSION

For the lifestyle of *E. faecalis* in the digestive tract of its host, it may be important to be able to use EA as a source of carbon and nitrogen. EA is, in the form of phosphatidylethanolamine, an essential component in the membranes of both prokaryotic and eukaryotic organisms (42), and it is a constituent of an abundant class of lipids available to gut inhabitants as part of

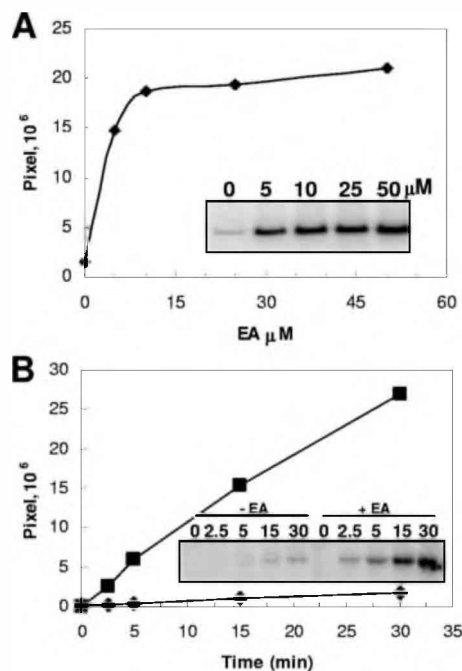


FIG. 7. EA stimulated HK17 autophosphorylation activity. (A) Concentration-dependent activation of the autophosphorylation activity of the HK17 histidine kinase (5 μ M) was measured after 30 min of incubation in the presence of EA concentrations shown on the x axis. (B) Time course of HK17 (5 μ M) autophosphorylation in the presence (■) or absence (◆) of EA (25 μ M).

the host's dietary intake (2, 10). Therefore, it is not surprising that EA utilization occurs in enterobacteria (such as *Escherichia coli* and *Salmonella* species) and that *S. enterica* serovar Typhimurium mutants defective in EA utilization were shown to be slightly attenuated in a mouse model of infection (53).

Here, we reported the identification of the *E. faecalis* gene cluster encoding the components for EA utilization and provided evidence for EA as the direct effector of a TCS required for this metabolic function. The RR-HK17 TCS was found to be embedded within genes encoding proteins with strong similarity to the EA utilization components of *S. enterica* serovar Typhimurium, and the addition of EA in an in vitro assay induced the autophosphorylation rate of the HK17 histidine approximately 15-fold from a low but measurable basal level. Autophosphorylation activity of HK17 reached a plateau at a 2:1 molar ratio between effector and kinase, supporting a physiological significance for EA as inducer of this histidine kinase.

The HK17 sensor histidine kinase is one of the few TCS kinases whose molecular environmental effector is now known. Furthermore, EA is only one of two small molecule ligands, along with toluene for the TodS histidine kinase of *Pseudomonas putida*, shown to be directly responsible for activation of autophosphorylation activity of a full-length, cytoplasmic kinase in vitro (29). The possibility that the GAF and/or the PAS domains in HK17 are the target for EA binding resulting in kinase activation is currently being investigated.

Phosphorylated HK17 specifically transferred its phosphoryl groups to the response regulator RR17 but no phosphotransfer was observed to the FsrA response regulator (RR15) used as a control (data not shown). Phosphorylation of RR17 by HK17 distinguishes this response regulator from AmiR, the first response regulator identified with an ANTAR output domain. In fact, AmiR lacks the residues essential for phosphotransfer, and its activation occurs via ligand disruption of its sequestering complex with the AmiC sensor protein (36).

Phosphorylation of RR17 by the EA-activated HK17 kinase provides the functional link between ligand detection and transcription activation for bacterial adaptation to growth in the presence of EA as the sole source of carbon.

The presence of EA in the medium as the sole carbon source induced transcriptional activity within a fragment located 722 bp upstream of the RR17 translational start site, suggesting the presence of an autoregulated promoter, because this induction was not observed in the absence of EA and growth of *E. faecalis* on EA required RR17. The inability of the RR17 mutant strain to grow in the medium containing only EA and CoB12 did not allow us to make a direct correlation between RR17 and its requirement for transcriptional induction, but the indirect evidence strongly supports this hypothesis. This promoter is likely located in the *eutG-eutS* intergenic region and must be required for the transcription of the genes downstream of the RR17-encoding gene, because expression of the RR17 response regulator from the constitutive *aphA3* promoter in the multicopy plasmid pML28 (20) failed to complement the RR17 deletion and restore growth on EA in this mutant strain (data not shown). Nevertheless, the presence of an additional promoter(s) downstream of the RR17-encoding gene cannot be ruled out at this time. An additional promoter for the transcription of *eut* genes may also be present upstream of the *eutP* gene (EF1638) as suggested by the presence of

putative -10 and -35 recognition sequences for σ^A -containing RNA polymerase, although read-through from an upstream putative ABC transport operon (EF1641-1639) could occur. Also, in the *eutT-eutG* intergenic region that contains the putative B12 riboswitch, the presence of a promoter cannot be excluded.

The presence of a putative B12 riboswitch, together with the VitB12 cofactor requirement for the activity of the EA ammonia lysase, indicates that a complex regulatory mechanism must exist for the expression of the EA utilization pathway. An additional B12 element has been identified by Barrick and Breaker (4) at position 1595486 to 1595326 on the *E. faecalis* V583 genome corresponding to the region upstream of EF1641, the first gene of a three-gene operon annotated as "iron compound ABC transporter," whose transcription could read through to the *eutP* and *eutT* genes. The product of the EF1641 gene shares 24% identical residues (BLAST E value, $3e-08$) with BtuF of *S. enterica* serovar Typhimurium, the substrate-binding protein that, with the BtuC permease and the BtuD ATPase, constitutes the ABC transporter for cobalamin (25, 57).

Bacterial riboswitches are regulatory elements located in the 5' untranslated regions of mRNAs and induce the formation of a terminator hairpin that prematurely terminates transcription or the formation of a structure at the ribosome binding site that blocks ribosome binding. Most riboswitches block the production of biosynthetic enzymes or transporters in the presence of the ligand that is the final product of the regulated pathway. Other riboswitches activate the expression of a salvage or degradation pathway when their effector molecule is present in excess (4).

The locations of the putative riboswitches identified upstream of *eutG* and EF1641 are both suggestive of a transcription termination regulatory mechanism. This implies that the expression of the *eut* gene cluster in the presence of EA as the sole carbon source, which occurs only in the concomitant presence of EA and cobalamin, requires a mechanism counteracting the transcription termination effect brought about by the B12 riboswitch. The transcription antitermination role must be played by the ANTAR-containing RR17 response regulator upon activation by EA of the mated HK17 histidine kinase. Detailed characterization of this possible new mechanism of riboswitch regulation of gene expression through a TCS mechanism is ongoing in order to define what seems like a complex pathway for EA utilization in *E. faecalis*.

Curiously, the *eutD* gene encoding a phosphotransacetylase (Pta), located within the *eut* operon in *S. enterica* serovar Typhimurium (Fig. 1) (6), is likely to correspond to the EF0949 monocistronic gene located outside the *E. faecalis* *eut* gene cluster and possibly encoding the only Pta in this organism. EutD is responsible for the conversion of Ac-CoA to acetyl- PO_4 (6). In *Salmonella*, the Ac-CoA that is not converted in acetyl- PO_4 by EutD enters the tricarboxylic acid cycle, but this cycle is absent in *E. faecalis* (37). This suggests that the *eutD* gene may be located outside the *eut* operon in order to be regulated differently and/or not exclusively by the presence of EA and thus avoid the accumulation of Ac-CoA.

The regulation of *eut* gene expression by a TCS and the genome organization of this pathway is not unique to *E. faecalis* but is also found in *S. sanguinis*, *Listeria* species, *Clostrid-*

ium species, and *Paenibacillus larvae*; in these organisms, the highly conserved TCS is also located upstream of the gene annotated *eutA*. Because the sources of EA in the environment are largely man-made (e.g., toothpaste, mouthwash, and anti-freeze), while it is naturally abundant in the human gut (27, 31) and in processed food (2, 10) and its utilization is dependent upon VitB12, it is possible that this large gene cluster may have been selected in these bacterial pathogens by exposure to the host environment. Interestingly, McBride et al. have searched for specific sets of genes in order to identify genes common to maximally diverse strains of *E. faecalis*, in order to define a core genome, and they found that TCS-17 was present in all strains tested (32).

In *Enterococcus*, EA degradation has not yet been studied, and the role of the *eut* genes within the context of enterococcal metabolism is still unknown. It was previously reported that salmonellae can use EA as a sole source of carbon and nitrogen under aerobic and anaerobic conditions (41, 44, 45). Of the 17 genes in the *Salmonella eut* operon, only 6 (*eutA*, *-B*, *-C*, *-D*, *-E* and *-R*) are required under standard conditions (37°C, pH 7.0). Five of the extra genes (*eutM*, *-N*, *-L*, *-K*, and *-G*) become necessary under conditions that favor loss of the volatile intermediate acetaldehyde, which escapes as a gas during growth on EA.

Acetaldehyde is highly toxic to the cells, and we cannot exclude the possibility that the limited growth of *E. faecalis* in the M9HY medium supplemented with EA and CoB12 is due to the accumulation of this compound (39). The alternative possibility that the OD would not increase because of the lack of an electron acceptor in the medium was tested by growing the cells in the supplemented M9HY medium under anaerobic conditions in the presence of potassium nitrate (41), but no improvement in growth was detected (data not shown).

The anaerobic growth of *E. faecalis* in the M9HY medium supplemented with EA and CoB12 is accompanied by the production of a yellow-brown pigment which is indicative of active metabolism and is likely the result of bacterium-dependent alteration of the corrin nucleus of VitB12 (8). This pigment strongly resembles the pigments derived from VitB12 by anaerobic growth of *Pseudomonas rubescens* or aerobic growth of *Aerobacter aerogenes* that have been characterized in the past (7, 21). It cannot be ruled out that it is the accumulation of this pigment that limited bacterial growth and reduced cell viability after prolonged incubation, as its production or characterization in *E. faecalis*, to our knowledge, has never been reported.

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