JOURNAL OF BACTERIOLOGY, Oct. 2000, p. 5799–5806 Vol. 182, No. 20
0021-9193/00/$04.00+0

Characterization of the *ccpA* Gene of *Enterococcus faecalis*: Identification of Starvation-Inducible Proteins Regulated by CcpA

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Received 30 May 2000/Accepted 26 July 2000

Inactivation of *ccpA* in *Enterococcus faecalis* leads to reduction of the growth rate, derepression of the *galKETR* operon in the presence of a mixture of glucose and galactose, and reduction of transcription of *ldh* in the presence of glucose. Moreover, the *E. faecalis ccpA* gene fully complements a *Bacillus subtilis ccpA* mutant, arguing for similar functions of these two homologous proteins. Protein comparison on two-dimensional gels from the wild-type cells and the *ccpA* mutant cells revealed a pleiotropic effect of the mutation on gene expression. The HPr protein of the carbohydrate-phosphotransferase system was identified by microsequencing, and a modification of its phosphorylation state was observed between the wild-type and the mutant strains. Moreover, at least 16 polypeptides are overexpressed in the mutant, and 6 are repressed. Interestingly, 13 of the 16 polypeptides whose synthesis is enhanced in the mutant were also identified as glucose starvation proteins. The N-terminal amino acid sequences of four of them match sequences deduced from genes coding for L-serine dehydratase, dihydroxyacetone kinase (two genes), and a protein of unknown function from *Deinococcus radiodurans*.

In their natural surroundings, microorganisms are usually subjected to environmental fluctuations, i.e., in the composition and abundance of carbon and energy sources. Bacteria have a high adaptability potential against these modifications. In many cases, the presence of a rapidly metabolizable carbon source leads to the reduction of expression of genes involved in the metabolism of other carbon substrates. This regulation by preferential nutrients has been named catabolite repression (CR). Conversely, carbon starvation leads to the entry of cells into stationary phase. Some bacteria, like *Bacillus* species, form endospores to survive nutrient-poor conditions. However, this morphological differentiation is not encountered in the vast majority of microorganisms. Nevertheless, nondifferentiating bacteria exhibit a variety of alterations in genetic regulation and physiological changes that ensure survival during periods of prolonged starvation and resistance to multiple environmental stresses (12, 15, 22, 23). In *Escherichia coli*, two classes of genes encoding starvation proteins have been defined: *cst* genes, subjected to activation by the cyclic AMP-cyclic AMP receptor protein complex, and *pex* genes, independent of catabolite repression (31). Carbon starvation (Cst) proteins are involved in escape from starvation, whereas postexponential (Pex) proteins are implicated in cross protection against exogenous stresses (37). Many of these Pex proteins are known to be regulated by the transcriptional factor σ^S (16, 26). In *Bacillus subtilis* and numerous other gram-positive bacteria, the transcriptional factor σ^B is involved in the stationary-phase response. In these microorganisms, the distinction between Pex and Cst is not yet established. It has been shown that CR in low-G+C-content gram-positive bacteria is mediated via a negative regulatory mechanism (38) involving at least three components: a *trans*-acting factor called catabolite control protein A (CcpA), *cis*-acting sequences termed catabolite responsive elements (*cre*s), and the HPr protein of the phosphoenolpyruvate-sugar-phosphotransferase system (PTS). CcpA is a DNA binding protein that belongs to the LacI/GalR family of transcriptional regulators (41) and was first identified in *B. subtilis* as a gene responsible for the catabolite repression of *amyE*, encoding α-amylase (18). Its action is mediated via binding to *cre* sequences, located within or near the promoter of the targeted genes. Weickert and Chambliss (42) proposed a consensus sequence for this 14-bp region of dyad symmetry on the basis of point-mutationa1 analysis in the *amyE* promoter region: TG(T/A)NANCGNTN(T/A)CA. The specific binding of CcpA to *cre*s requires an additional factor, the HPr protein of the PTS. In addition to the phosphorylation site at histidine 15 implicated in the sugar transport process, HPr of gram-positive bacteria can be phosphorylated at the serine 46 residue by an ATP-dependent HPr kinase (7). HPr(Ser-P), but not free HPr, can bind to CcpA in vitro, and this interaction is stimulated by high concentrations of fructose-1,6-bisphosphate (FBP), one of the intermediates of the glycolytic pathway (6). Repression of targeted genes results from the fixation of CcpA with its cofactors to *cre*s, which blocks transcription initiation by RNA polymerase (19). *cre*s confer not only repression of genes but also glucose-mediated transcriptional activation of the acetate kinase gene (*ackA*) in *B. subtilis* (14) and the *las* operon, encoding pyruvate kinase, phosphofructokinase, and lactate dehydrogenase, in *Lactococcus lactis* (29). The involvement of CcpA in catabolite repression has also been established in other low-G+C-content gram-positive bacteria, including *Bacillus megaterium*, *Staphylococcus xylosus*, *Lactobacillus casei*, and *L. lactis* (9, 20, 29, 33).

Enterococcus faecalis is a nonsporulant low-G+C-content gram-positive bacterium which is able to develop a multiresistant state when deprived of glucose (12). This multiresistance is correlated with the synthesis of at least 42 glucose starvation proteins (Glsp) (13). In a previous paper, we reported the cloning and sequencing of an *E. faecalis* gene homologous to

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ccpA of *B. subtilis* (27). In this report, we analyze its role in CR and, using a two-dimensional (2-D) gel electrophoresis approach, we attempt to distinguish Pex and Cst proteins among the glucose starvation proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Cultures of *E. faecalis* JH2-2 (21, 43) were grown at 37°C without shaking in 20-ml glass tubes containing 10 ml of semisynthetic medium (for the composition, see Bacto Folic AOAC Medium [Difco, Detroit, Mich.]) supplemented with various carbon sources. *E. coli* XL1Blue (Stratagene, La Jolla, Calif.) was used as a plasmid host and was cultivated under vigorous agitation at 37°C in 2TY medium (32) with ampicillin (100 μg/ml) as required. *B. subtilis* QB7144 [*trpC2 amyE*::(pA *ynaJ'-lacZ*$^+$ *cat*)] and QB7147 [*trpC2 ccpA*::Tn*917 spc amyE*::(pA *ynaJ'-lacZ*$^+$ *cat*)] (11) were used for complementation experiments and were cultivated in CSK medium (11) at 37°C under vigorous agitation.

Analysis of mRNA transcription by Northern blotting. Total RNA of *E. faecalis* was isolated by using the RNeasy Midi Kit (Qiagen, Inc., Valencia, Calif.). After DNase treatment, samples were precipitated and the amount of RNA was determined by spectrophotometry. Northern blots of exactly 10 μg of electrophoresed RNA were prepared by using Hybond N+ membranes and standard procedures (36). For quantification of the relative intensities of the hybridizing bands in the Northern blots, rRNA bands observed after ethidium bromide staining of gels were used as an internal standard for each sample. For this purpose, the stained 23S and 16S rRNA bands were scanned and quantified by densitometry with OptiQuant image analysis software (Packard Instrument Company, Canberra, Australia). The sizes of transcripts were estimated by comparing the band mobilities of standards in an RNA ladder (0.56 to 9.4 kb) (Amersham International, Little Chalfont, United Kingdom). Oligonucleotide primers were used in PCRs to generate specific fragments of genes: *ldh*, 5′-GGAATGGTACACATGACTGC-3′ and 5′-CGTCAGGATTATTTTTCACC-3′; *pfk*, 5′-GCATTGGTATTTTAACCAGC-3′ and 5′-TCACCATGTGAAAAGTTCAA-3′; *galK*, 5′-TTGGTGAGAAAGGGACAGCC-3′ and 5′-GCAGGATAAAAATCAGCAGC-3′; *gls27*, 5′-AATAATGCACTAGATGCTGC-3′ and 5′-TAAAAGACATTCAAACATGG-3′; and *gls17*, 5′-GAAGAATTTATCGATAAAGC-3′ and 5′-GGCCATCGCTGAAGCACTGC-3′. These PCR fragments were then used to generate specific probes by PCR, using 200 pmol of the reverse primers; 2 μM of dGTP, dCTP, and dTTP; 1.5 mM $MgCl_2$; 1 μl of purified PCR product; 1× PCR buffer (Amersham); 5 U of *Taq* DNA polymerase (Amersham); and 20 μCi of [α^{32}P]dATP (Amersham Pharmacia Biotech). Reactions were run for 10 cycles. Prehybridization and hybridization of membrane-bound RNA with single-stranded DNA probes were performed at 60°C with gentle agitation.

Mapping the transcriptional start sites. Primer CCPA3 (5′-TAGATACATTTGCCTCTCTAGC-3′), complementary to nucleotides +33 to +53 of *ccpA*, was labeled with 10 U of polynucleotide kinase (Roche Molecular Biochemicals) and 2 μCi of [γ^{32}P]ATP (Amersham International; 10 mCi/ml) and then mixed with 10 μg of total RNA in 14 μl of the reverse transcriptase buffer containing 40 U of RNase inhibitor (Roche Molecular Biochemicals). After the mixture was heated at 65°C for 5 min, annealing was obtained by a slow decrease of the temperature to 25°C. The extension reaction was then performed in a 20-μl final volume with 50 U of avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) and 0.5 mM deoxynucleoside triphosphates at 42°C for 1 h. After heat denaturation, 2-μl samples were loaded onto a 6% polyacrylamide-urea sequencing gel for electrophoresis, together with a sequencing reaction performed with the same primer (T7 sequencing kit; Pharmacia Biotech), and the bands were detected after exposure to a storage phosphor screen (Packard Instrument Company).

General molecular methods. Restriction endonucleases, alkaline phosphatase, and ligase were obtained from Roche Molecular Biochemicals and Amersham International and used according to the furnished instructions. PCR was carried out in a reaction volume of 25 μl with 100 ng of chromosomal DNA of *E. faecalis* using Ready To Go PCR beads (Pharmacia Biotech). PCR products were purified with the QIAquick kit (Qiagen). DNA and amino acid sequences were analyzed using the Mac Vector (Kodak Scientific Imaging Systems) program, and database searches were performed with the BLAST program (1). Other standard techniques were carried out as described by Sambrook et al. (36). Competent *B. subtilis* cells were used for transformation (2). *E. faecalis* and *E. coli* were transformed by electroporation with a Gene-pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.).

Construction of the *ccpA* insertional mutant. To construct an insertional mutant with a disruption in the *E. faecalis ccpA* gene, a 440-bp internal *E. faecalis ccpA* fragment was amplified from chromosomal DNA with primers CCPA1 (5′-GTGTTGTCCATCGGTAATCC-3′) and CCPA1rev (5′-GCAGAATTCGTTGCTTCTGTGTAATC-3′) and, after being polished with *Pfu* polymerase (Stratagene), ligated with the insertional vector pUCB300 (10) previously digested with *Sma*I. The resulting plasmid, pCCPA1, obtained after transformation of *E. coli* XL1Blue, was used to transform competent cells of *E. faecalis* JH2-2. Erythromycin-resistant colonies were selected on agar plates containing 15 μg of erythromycin per ml. Integrations were verified by PCR and Southern blot analysis, and the disappearance of CcpA was confirmed by Western blotting with antibodies raised against CcpA from *B. megaterium* (25).

Western blot analysis. *E. faecalis* JH2-2 and CL14 strains were grown to an optical density at 600 nm (OD_{600}) of 0.4 in 10 ml of semisynthetic medium supplemented with 0.15% glucose. Crude extracts were prepared by vortexing the pellets in 500 μl of extraction buffer (Tris [pH 7], 50 mM; EDTA, 2 mM; β-mercaptoethanol, 0.74% [vol/vol]) with glass beads (0.1- to 0.25-mm diameter) and subsequent removal of cell debris by centrifugation. The proteins of cell extracts were separated by nondenaturing polyacrylamide gel electrophoresis on a 14% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) by electroblotting (MilliBlot-Graphite electroblotter; Millipore). HPr was detected with a rabbit polyclonal antiserum raised against HPr of *Staphylococcus carnosus*. HPr antibodies were visualized by using the ECL Western blot analysis system (Amersham).

Complementation of a *B. subtilis ccpA* mutant. To express *ccpA* from *E. faecalis* in *B. subtilis*, the gene was amplified by PCR with primers CCPA2for (5′-GGACAAGATCTTATTTATAGGAGGAGAACATGG-3′) and CCPA2rev (5′-CAATGCATGCCGGACTGATTTACTTAATCAAC-3′). These primers changed the ribosome-binding site of *ccpA* to a more appropriate sequence for *Bacillus* and introduced *Bgl*II and *Sph*I sites that were used to clone the gene under the control of the *xynCB* promoter in pHT*xyn*, resulting in pCCPA2. Plasmid pHT*xyn* was obtained by introducing a 1.5-kbp *Eco*RI/*Hin*dIII fragment from pHM12 (H. Putzer, unpublished data) containing the *xynCB* promoter and the regulator *xynR* in plasmid pHT315 (3), a shuttle vector with 15 copies/chromosome in *B. subtilis*, digested by the same restriction enzymes.

Two-dimensional protein gel electrophoresis. Cells were cultured in semisynthetic medium supplemented with 0.15% glucose. Culture aliquots of 5 ml were pulse labeled between OD_{600}s of 0.2 and 0.4 with 250 μCi of [^{35}S]methionine and [^{35}S]cysteine protein-labeling mix (New England Nuclear Co.; 1,000 Ci/mmol). Protein extraction and 2-D electrophoresis were performed as previously described (13). The dried gels were exposed to a storage phosphor screen (Packard Instrument Company) for 48 h, and the intensity of synthesis of proteins was determined by the quantification of the corresponding spot using the OptiQuant image analysis software. For the preparative electrophoresis, 50 ml of bacterial culture was used. After separation, the gel was transferred onto a polyvinylidene difluoride membrane (Immobilon-P) by electroblotting (MilliBlot-Graphite electroblotter) according to the manufacturer's instructions. After Coomassie blue staining of the membrane, the interesting spots were cut off and proteins were sequenced by the Institut für Biochemie (University of Vienna, Austria). Preliminary sequence data were obtained from The Institute for Genomic Research (TIGR) (website at http://www.tigr.org).

RESULTS

Transcriptional analysis of the *E. faecalis ccpA* gene. Northern blot analysis revealed a unique transcript of approximately 1.2 kb, indicating that the *E. faecalis ccpA* gene was expressed as a monocistronic mRNA (data not shown). A potential *rho*-independent terminator structure, with a $\Delta G°$ value of −25.6 kcal/mol, was identified downstream of *ccpA*. Primer extension analysis performed on total RNA extracted from cells grown in glucose-supplemented semisynthetic medium and harvested in mid-exponential growth phase suggested that the transcriptional initiation site was a guanine (G) located 139 bp upstream of the *ccpA* open reading frame (ORF) translational initiation codon (Fig. 1). No obvious sequences corresponding to a −10/−35 hexanucleotide pair was identified at the correct position upstream from this transcriptional initiation site. Another putative promoter deduced from the sequence has been previously described 48 bp upstream of the translational start site (27), but it did not seem to be active, at least under the culture conditions used for primer extension.

Physiological impact of *ccpA* mutation in *E. faecalis*. To determine the function of CcpA in *E. faecalis*, the chromosomal *ccpA* gene was disrupted by integration of a nonreplicative vector carrying an internal *ccpA*. The mutant strain obtained was designated CL14. The fermentative pattern of 50 carbohydrates by the API 50-CH (Biomerieux) series was identical for both wild-type and mutant strains. To determine phenotypic alteration(s) in the mutant, the growth of cultures was monitored in semisynthetic medium containing glucose, galactose, mannitol, mannose, sucrose, fructose, or lactose as a carbon source. The doubling times of CL14 were clearly affected (Table 1). They were higher than that of the wild type

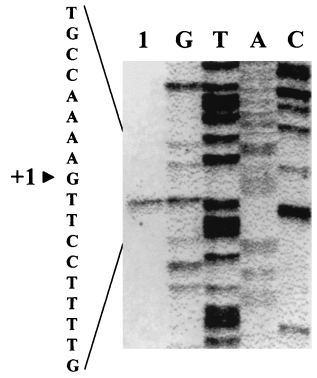

FIG. 1. Determination of the 5′ end of the *ccpA* transcript by primer extension. A DNA-sequencing preparation was run in parallel using the same primer. The arrowhead corresponds to the point within the sequence representing the apparent 5′ end.

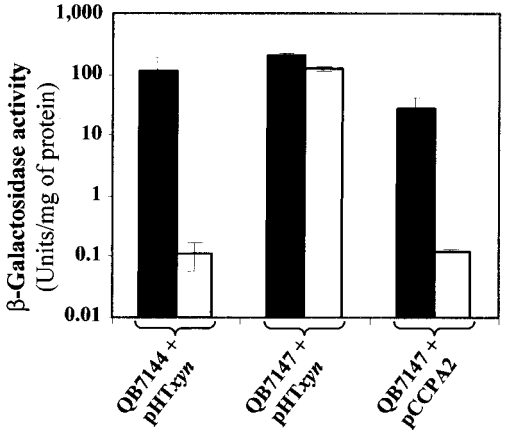

FIG. 2. β-Galactosidase activities of *B. subtilis* strains QB7144 and QB7147 containing different plasmids. The specific activities of β-galactosidase were determined in extracts prepared from exponentially growing cells (OD_{600} = 0.5). The mean values of three independent experiments are presented. Cells were grown in CSK medium supplemented with 0.2% xylose (solid bars) or with 0.2% xylose and 1% glucose (open bars). The method of Miller (32) was used for the determination of β-galactosidase activity.

on the seven carbohydrates tested. For instance, in semisynthetic medium supplemented with 0.15% glucose or mannose, the *ccpA* mutation led to a 45% increase in the doubling time.

Complementation of a *B. subtilis ccpA* mutant. The similarities between *ccpA* of *Bacillus* species and *E. faecalis* prompted us to test the complementation of a *B. subtilis ccpA* mutant with the *E. faecalis* gene. For this purpose, we used two strains of *B. subtilis*: QB7144 and QB7147 (11). The QB7147 strain carries a fusion of the *xynB* promoter with the *lacZ* gene (*ynaJ′-lacZ*) as well as a *ccpA* mutation. In this strain, the expression of *lacZ* is induced by xylose and is not repressed by glucose, while in strain QB7144, which contains a wild-type *ccpA* gene, addition of glucose induces a strong repression of the β-galactosidase gene. Cloning the *E. faecalis ccpA* gene downstream of the *B. subtilis xynCB* promoter in a replicative plasmid and changing its ribosome-binding site to adapt it to its new host (resulting in plasmid pCCPA2) permitted the expression of *E. faecalis* CcpA in *B. subtilis*. With this construct, the glucose-specific repression of the *ynaJ′-lacZ* fusion was restored (Fig. 2).

CcpA-mediated transcriptional regulation of galactose utilization genes. Using the *E. faecalis* genome sequence provided by TIGR, the potential *galK* gene of *E. faecalis* was identified as part of an operon comprising three other genes: *galETR*. The deduced amino acid products of these genes are 76% homologous to galactokinase of *Streptococcus thermophilus*, 80% homologous to UDP-galactose 4-epimerase of *L. lactis*, 71% homologous to galactose-1-*P*-uridyl transferase of *Streptococcus mutans*, and 56% homologous to the galactose operon repressor of *S. thermophilus*. A potential *rho*-independent terminator was identified downstream of the *galR* gene, and a potential *cre* box (TGTA**C**ACGTTTTCA) with only one mismatch (boldface) with the consensus *cre* sequence, was localized 94 bp upstream of the putative translational start codon of the first gene, *galK*.

TABLE 1. Doubling times of *E. faecalis* strains JH2-2 and CL14 with different carbon sources[a]

Semisynthetic medium + sugar (0.15%)	Doubling time (min)	
	Wild type	*ccpA* mutant
Glucose	44.6 ± 4.2	64.6 ± 5.9
Lactose	52.7 ± 2.8	68.1 ± 8.8
Fructose	55.1 ± 1.1	71.3 ± 1.7
Galactose	83.6 ± 9.2	98.4 ± 1.2
Sucrose	54.6 ± 0.8	66.7 ± 1.3
Mannitol	66.2 ± 0.7	85.5 ± 11.7
Mannose	42.0 ± 0.3	64.6 ± 1.9

[a] Results presented correspond to the mean value of three experiments, and standard deviations are indicated.

Northern blots of total RNA extracted from strains JH2-2 and CL14 grown in semisynthetic medium supplemented with 0.15% glucose, 0.15% glucose plus 0.15% galactose, or 0.15% galactose were performed with a *galK*-specific probe (Fig. 3A). No or weak bands were detected when the two strains were grown on glucose (Fig. 3A, lanes 1 and 4), while a strong signal corresponding to a 4.8-kb transcript was detected, suggesting transcriptional regulation, when cultures were performed in the presence of galactose (Fig. 3A, lanes 3 and 6). The size of this transcript corresponded to that expected for the putative *galKETR* operon. Analysis of total RNA extracted from strains cultured on a mixture of glucose and galactose revealed a partial derepression of the *galK* transcription in strain CL14 compared to that in the wild-type strain (Fig. 3A, lanes 2 and 5). Repression factors, corresponding to the ratio between the amount of transcript under nonrepressive and repressive conditions, were about 4.5 and 17.5 for the *ccpA* mutant and the wild-type strains, respectively.

Effects of CcpA on regulation of transcription of glycolysis enzymes. Recently, Luesink et al. (29) reported that the *las* operon of *L. lactis*, encoding the glycolytic enzymes lactate dehydrogenase, pyruvate kinase, and phosphofructokinase, was transcriptionally activated by CcpA in the presence of glucose. In order to determine whether such regulation was effective in *E. faecalis*, we first searched for the corresponding genes in the partially determined genome sequence at the TIGR database. Three genes whose deduced amino acid sequences share 88% homology with that of *L. casei* lactate dehydrogenase (*ldh*), 80% homology with that of *Bacillus stearothermophilus* phosphofructokinase (*pfk*), and 81% homology with that of *Bacillus licheniformis* pyruvate kinase (*pyk*) were identified. While in *L. lactis* the three genes form an operon, the organization of genes found in *E. faecalis* was different: *pfk* and *pyk* seemed to form an operon, whereas *ldh*

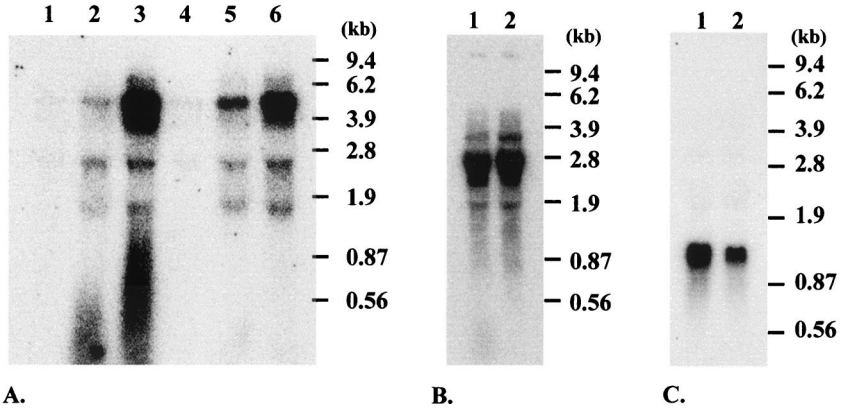

FIG. 3. (A) Northern blot analysis of the expression of the *galK* gene in the *E. faecalis* strains JH2-2 (lanes 1, 2, and 3) and CL14 (lanes 4, 5, and 6) grown with 0.15% glucose (lanes 1 and 4), 0.15% glucose plus 0.15% galactose (lanes 2 and 5), or 0.15% galactose (lanes 3 and 6). (B) Northern blot analysis of the expression of the *pfk* gene in *E. faecalis* strains JH2-2 (lane 1) and CL14 (lane 2) grown with glucose. (C) Northern blot analysis of the expression of the *ldh* gene in *E. faecalis* strains JH2-2 (lane 1) and CL14 (lane 2) grown with glucose.

was monocistronic. A potential *cre*-box (TGAAAACTGTAT CA), with one mismatch (boldface) with the consensus sequence, was identified 114 bp upstream of the ATG start codon of the *ldh* gene, whereas no sequence matching this consensus was identified near the putative promoter region of the *pfk-pyk* operon.

Northern blot analyses performed with total RNA extracted from exponentially growing cells in semisynthetic medium supplemented with glucose and hybridized with a *pfk*-specific probe (Fig. 3B) showed no significant differences in the amounts of transcript between the wild-type and the *ccpA* mutant strains. The size of the transcript corresponds to that expected for the *pfk-pyk* operon. Similar results were obtained when a *pyk*-specific probe was used (data not shown). Northern blotting carried out with an *ldh*-specific probe showed one unique transcript of 1.3 kb (Fig. 3C), corresponding to the expected size for *ldh*. The amount of transcript was 2.2-fold lower in the *ccpA* mutant than in the wild-type strain, suggesting a role for CcpA in a weak activation of *ldh* transcription.

Pleiotropic effect on protein synthesis and influence on phosphorylation state of HPr of the *ccpA* mutation. To determine whether CcpA would affect the synthesis of other *E. faecalis* proteins, a 2-D polyacrylamide gel electrophoresis approach was used. Several differences were observed in the 2-D protein patterns of strains CL14 and JH2-2 when cells were harvested in mid-growth phase (Fig. 4). Indeed, the synthesis of at least 16 polypeptides is obviously enhanced in the *ccpA* mutant, whereas 6 are repressed. Interestingly, most of the polypeptides with enhanced synthesis had already been identified in a previous work as glucose starvation-inducible proteins (Glsp) in *E. faecalis* (13).

In addition to these variations in the amount of protein synthesis, the case of the HPr protein seems special. This protein was identified on the 2-D gels by microsequencing and by cross reaction with antibodies raised against *S. carnosus* HPr. Two spots corresponded to this protein; these are likely due to nonphosphorylated and Ser(46)-phosphorylated forms of HPr, the His15~P being heat unstable (30). The amounts of total HPr in the two strains did not show significant differences but displayed variations in the phosphorylation states. CL14 shows a twofold amplification of the HPr(Ser-P) form, whereas the unphosphorylated form of HPr decreased by the same factor. This modification of the phosphorylation state was verified by Western blotting of native proteins (Fig. 5). This analysis confirmed a larger amount of HPr(Ser-P) but also revealed that the level of the double-phosphorylated form of this protein in particular is increased in the *ccpA* mutant. On the other hand, unphosphorylated HPr and, more significantly, the HPr(His~P) fraction showed reduced levels (Fig. 5).

Analysis of some CcpA-dependent proteins. Among the 16 polypeptides with enhanced synthesis in the *ccpA* mutant strain, the N-terminal parts of 4 of them were determined by microsequencing as follows: for Gls10, MKKIINEP; for Gls17, YLXIEEFI; for Gls27, MELTVKDI, and for Gls40, MKA DILLV. The corresponding genes were found in the genome sequence provided by TIGR, and adjacent regions were analyzed. The results of sequence analyses indicated that *gls40*, *gls27*, and *gls10* are part of an operon of four genes, which would terminate at a potential *rho*-independent terminator. ORF1 shares 38% identity with glycerol dehydrogenase from *E. coli*, Gls10 and Gls27 share 42 and 40% identity with putative dihydroxyacetone kinase from *E. coli*, and Gls40 shares 35% identity with a protein of unknown function from *Deinococcus radiodurans*. Upstream of this operon, three potential *cre*s with two mismatches (boldface) in comparison to the consensus sequence were identified. The first (TATCAACGATG TTA) is located 437 nucleotides upstream of the potential translational start site of *orf1*, and the two degenerations conserved the symmetry. The two others are located 36 (TGAA AGCGTTTTAT) and 70 (AGAAAACGATACCA) nucleotides upstream of this translational start site.

Analysis of adjacent regions of *gls17* indicates that this gene is part of an operon of four genes. ORF1 shares 42% identity with the regulatory protein PfoR from *Clostridium perfringens*, and ORF2 and Gls17 share 39 and 51% identity with probable L-serine dehydratase beta and alpha chains from *B. subtilis*, respectively. Finally, ORF4 is 60% identical with seryl-tRNA synthetase from *B. subtilis*. A perfect *cre* box (TGAAAACGT TATCA) was identified 1 nucleotide after the translational start site of *orf1*.

The obvious induction of these proteins in the *ccpA* mutant strain and at the onset of glucose starvation of strain JH2-2 has been verified at the transcriptional level. Northern blot analyses were performed with total RNA extracted from growing cells of strain CL14 and growing and starved cells of strain JH2-2 (Fig. 6). The results of hybridization with a *gls27*-specific probe showed one band, the size of which corresponds to that of the entire operon (Fig. 6A). This approximately 4.2-kb tran-

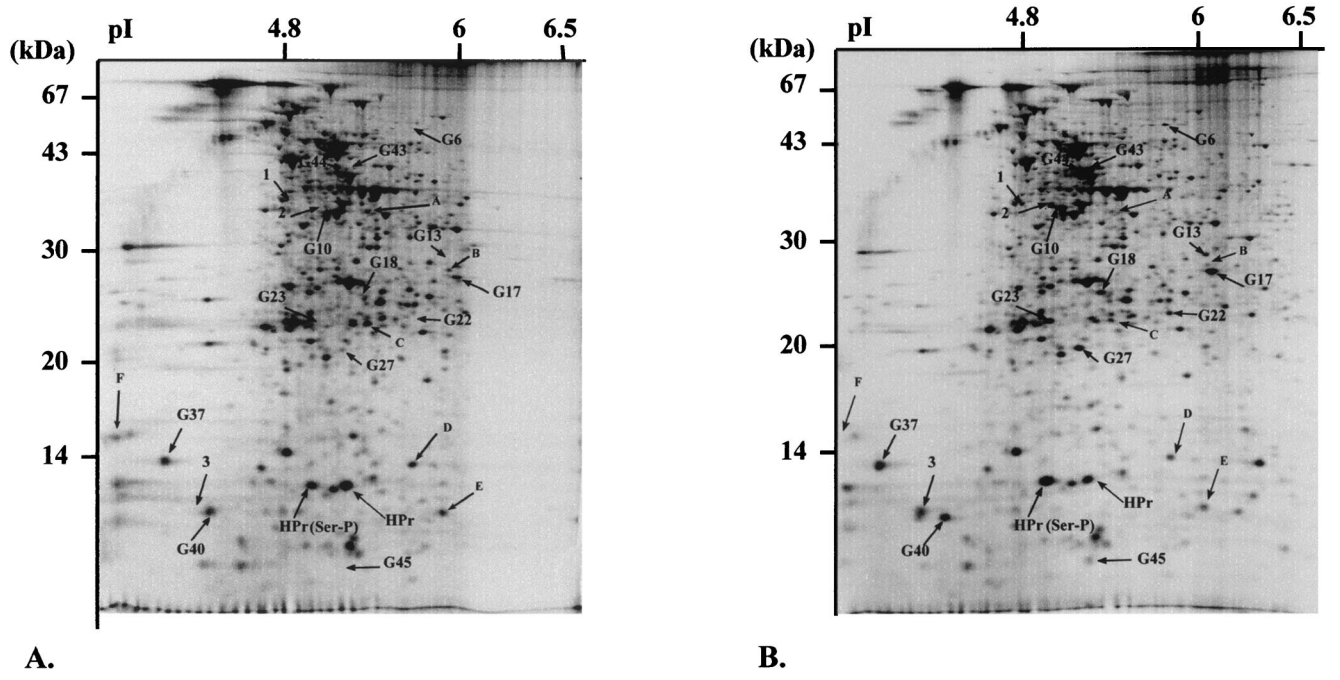

FIG. 4. The 2-D electrophoresis protein pattern of *E. faecalis* strains JH2-2 (A) and CL14 (B) grown on glucose and harvested in the exponential growth phase. The arrows indicate proteins showing modified expression in the *ccpA* mutant strain. The spots indicated by a G and a number and those indicated only by numbers are polypeptides under negative control by CcpA, whereas proteins indicated by letters are under positive control by CcpA. The majority of the G proteins have been identified in a previous study (13) as inducible upon glucose starvation (Gls proteins). Three additional Gls proteins (G43, G44, and G45) have been identified since that time (unpublished results). The proteins indicated by numbers are specific to the *ccpA* mutant. N-terminal microsequencing of proteins isolated from two spots showed that they correspond to the HPr protein of *E. faecalis*. The more acidic spot seems to be the form of this protein phosphorylated on serine 46. For more details, see the text.

script showed a 4.5-fold increase in the *ccpA* mutant strain and at the onset of glucose starvation compared to the level in growing cells of JH2-2. The results of hybridization with a *gls17*-specific probe are shown in Fig. 6B. A transcript of approximately 4.9 kb was detected, which corresponds to the size of the putative operon. This transcript was strongly induced (7.4-fold) in the mutant cells during growth phase compared to the JH2-2 strain. Moreover, the mRNA level was 2.2-fold higher at the onset of glucose starvation compared to that in growing cells of JH2-2.

DISCUSSION

In this communication, we report the characterization and functional analysis of a *ccpA* homologue from *E. faecalis*. Our transcriptional analyses indicated that transcription is monocistronic and takes place from a promoter located 139 bp upstream from the *ccpA* reading frame. In the next step, we tried to determine the putative regulatory role of CcpA in *E. faecalis* and its implication in carbon metabolism. Analysis of *galK* transcription in a *ccpA* mutant strain of *E. faecalis* indicated that transcription of the corresponding operon is partially derepressed in the presence of a mixture of glucose and galactose (Fig. 3A). This phenomenon could be correlated with the presence of a putative *cre* sequence in the promoter region of the *galKETR* operon. Similarly, in *L. lactis*, disruption of the *ccpA* gene did not result in a complete derepression of *gal* operon transcription (29), suggesting that either the induction of the *gal* transcription is reduced by the disruption of the *ccpA* gene or an additional system of glucose repression might be active. Processes such as inducer exclusion and inducer expulsion, which have been demonstrated in *E. faecalis* (44), or other control mechanisms involved in the regulation of the *gal* operon may also account for the observed residual glucose repression in the *E. faecalis ccpA* mutant. The *E. faecalis ccpA* gene could also restore glucose repression of a *ynaJ′-lacZ* fusion in a *B. subtilis ccpA* mutant, showing that the sequence conservation of *ccpA* between *E. faecalis* and *B. subtilis* was paralleled by similar functions in these microorganisms. These data clearly demonstrate the implication of CcpA in CR of *E. faecalis*.

Inactivation of the *E. faecalis ccpA* gene also resulted in a reduction of the growth rate on different sugars, a phenomenon generally observed for *ccpA* mutants of other bacteria (4, 9, 19). This suggests that, in addition to its role in CR, CcpA could also be involved in other regulatory processes. Indeed, CcpA is responsible for glucose-mediated transcriptional activation of *alsS*, *ackA*, and some glycolytic enzymes in *B. subtilis* (14, 17, 39). Moreover, Luesink et al. (29) reported the transcriptional activation of the *las* operon by CcpA in the presence of glucose in *L. lactis*. Our observations indicated that the organization of these genes in *E. faecalis* was different. Genes encoding pyruvate kinase and phosphofructokinase form an operon whose transcription seemed independent of CcpA, whereas the gene encoding lactate dehydrogenase is monocistronic and its transcription is 2.2-fold reduced in the *ccpA* mutant strain (Fig. 3). In *B. subtilis*, *pfk* and *pyk* are also CcpA independent (39). However, in that microorganism, an activation of the *gap* gene and the *pgk* operon by glucose, which seems to be dependent on CcpA, has been reported (39). In *E. faecalis*, Northern blot experiments showed that transcription of the operon comprising *ygaP*, *gap*, *pgk*, and *tpi* seems independent of CcpA (data not shown). This result is further strengthened by the absence of a *cre*-like sequence in the

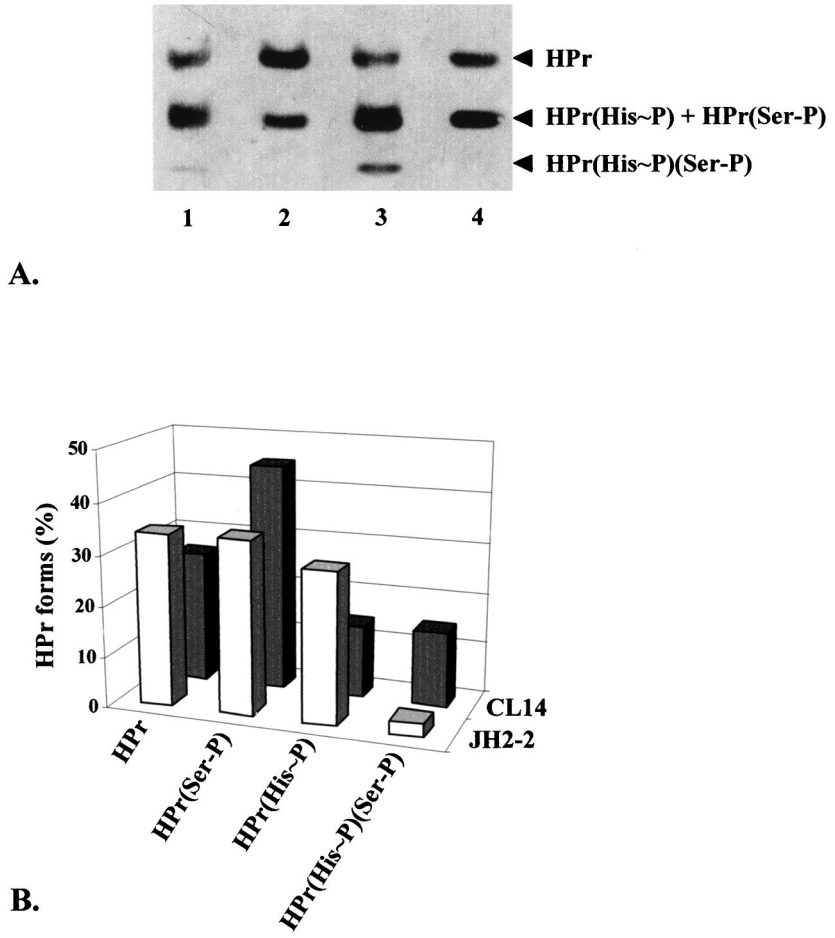

FIG. 5. (A) Western blot analysis of HPr of *E. faecalis* strains JH2-2 (lanes 1 and 2) and CL14 (lanes 3 and 4). The extracts in lanes 1 and 3 correspond to native proteins, and the extracts in lanes 2 and 4 were boiled for 5 min before loading, leading to the dephosphorylation of histidine. The positions of the different forms of HPr are indicated by arrowheads. (B) Percentages of the different HPr forms in *E. faecalis* wild-type and *ccpA* mutant strains were deduced from the results of the Western blot analysis after scanning and densitometry analysis by OptiQuant image analysis Software.

promoter region of this operon. A potential *cre* was identified in the promoter region of *ldh* in *E. faecalis* and could be implicated in the CcpA-mediated activation of this gene. This result suggests that CcpA in *E. faecalis* could also act as a transcriptional activator, as in *B. subtilis* and *L. lactis*, which is further supported by the 2-D gel analysis indicating that six proteins showed reduced expression in the *ccpA* mutant. However alternative explanations, such as indirect effects on transcription or changes in RNA stability, cannot be excluded. The probable lower glycolytic capacity of the *E. faecalis ccpA* mutant, due to the lack of activation of at least *ldh* in the presence of glucose, might be one of several factors explaining the growth deficiency. Among these are the unbalanced expression of catabolic enzymes that might be a burden to the cells, the lack of ammonium assimilation, as demonstrated for the *B. subtilis ccpA* mutant, and an accumulation of glycolytic intermediates that cannot be excreted (17, 40).

In order to identify other proteins that may belong to the CcpA regulon, we used a 2-D electrophoresis approach. A comparison of the protein pattern of the wild-type and *ccpA* mutant cells harvested in mid-exponential growth phase revealed that several proteins were affected by the *ccpA* mutation. Among them, a variation in the phosphorylation states of HPr was observed. One might hypothesize that this phenomenon is correlated with the observed smaller amount of *ldh* transcript in the *ccpA* mutant, which could provoke higher levels of glycolytic intermediates, such as FBP, required for activation of the HPr kinase of *B. subtilis* (34). Such results were obtained for the HPr kinase of *E. faecalis* (5), but a recent study indicated that for highly purified recombinant *E. faecalis* HPr kinase, FBP could also be omitted in vitro (24), suggesting that its implication is not so clear as for *B. subtilis*.

In addition to its effects on HPr, the *ccpA* mutation leads to an obviously enhanced synthesis of at least 16 polypeptides. This number certainly does not reflect the totality of proteins that belong to the CcpA regulon. Indeed, genes and operons required for the utilization of specific carbon sources are in most cases subjected to CR and to substrate induction; thus, even in a *ccpA* mutant, they will be expressed only if the carbon sources are present in the medium (for a review, see reference 35). In this way, the 16 polypeptides with enhanced synthesis in a *ccpA* mutant genetic background probably do not need any inducer or corresponding inducers are already present in the culture medium.

Four of the polypeptides with enhanced synthesis in the *E. faecalis ccpA* mutant were microsequenced, and the corresponding genes were found in the unfinished genome sequence of *E. faecalis*. One of the microsequences obtained corre-

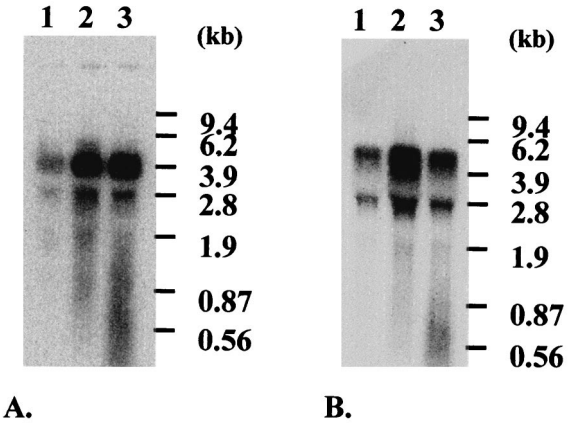

FIG. 6. Northern blot analysis of the *E. faecalis gls27* and *gls17* genes. Total RNA was isolated from strains JH2-2 (lanes 1 and 3) and CL14 (lane 2) exponentially grown on glucose (lanes 1 and 2) or at the onset of glucose starvation (lane 3). Hybond N+ membranes were hybridized with a *gls27*-specific probe (A) or a *gls17*-specific probe (B).

sponds to a gene whose product shares high identity with the putative L-serine dehydratase alpha subunit of *B. subtilis*. L-Serine dehydratase (or L-serine deaminase) catalyzes the conversion of L-serine to pyruvate, the first step of the degradative pathway of this amino acid.

The other microsequences obtained correspond to genes that were part of an operon encoding glycerol dehydrogenase, an ORF coding for a putative protein of unknown function, and two polypeptides corresponding to putative dihydroxyacetone kinases. As in other bacteria, this result suggests that glycerol dissimilation in *E. faecalis* can be achieved by two biochemical pathways. Following uptake via the glycerol facilitator, glycerol may be first phosphorylated by glycerol kinase and subsequently oxidized to dihydroxyacetone phosphate by a flavin-linked glycerol-3-phosphate dehydrogenase. Corresponding enzymatic activities have been identified in *E. faecalis* (8). Alternatively, glycerol is first oxidized by an NAD-linked glycerol dehydrogenase to dihydroxyacetone (DHA) and subsequently phosphorylated to DHA-phosphate by an ATP-dependent DHA kinase (28). The roles of these activities in the starvation stress response remain to be analyzed.

ACKNOWLEDGMENTS

This work was partly supported by financial aid from the Agence de l'Eau Seine-Normandie. C. Leboeuf is the recipient of an award from the Ministère de la Recherche et de l'Enseignement Supérieur of France.

We thank I. Martin-Verstraete for kindly providing us strains QB7144 and QB7147, H. Putzer for plasmid pHM12, E. Küster and W. Hillen for CcpA antibodies, and W. Hengstenberg for HPr antibodies. The expert advice of C. Karmazyn-Campelli for the *B. subtilis* experimentations were greatly appreciated.

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