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Cloning, Sequencing, and Expression of the Pantothenate Kinase (*coaA*) Gene of *Escherichia coli*

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Pantothenate kinase catalyzes the rate-controlling step in coenzyme A (CoA) biosynthesis. The structural gene (*coaA*) located at 90 min of the *Escherichia coli* chromosome was cloned and sequenced. The *coaA* gene was transcribed in the opposite direction to the flanking genes *birA* and *thrU* and produced a single 1.1-kb transcript. Translation of the *coaA* gene produced two protein products (36.4 and 35.4 kDa) that differed by eight amino acids at the amino terminus. The poor homology of the *coaA* promoter region to consensus *E. coli* promoter sequences and the low frequency of optimal codon usage (0.565) were consistent with the low abundance of pantothenate kinase. Strains containing multiple copies of the *coaA* gene possessed 76-fold-higher specific activity of pantothenate kinase; however, there was only a 2.7-fold increase in the steady-state level of CoA. These data corroborate the conclusion that regulation of pantothenate kinase activity by feedback inhibition is the critical factor controlling the intracellular CoA concentration.

Coenzyme A (CoA) and CoA thioesters are essential cofactors in numerous biosynthetic and energy-yielding metabolic pathways, and they function as regulators of several key reactions of intermediary metabolism such as pyruvate dehydrogenase and phosphoenolpyruvate carboxylase (for reviews, see references 1 and 6). CoA also donates the 4′-phosphopantetheine moiety to acyl carrier protein, an essential cofactor in the biosynthesis of fatty acids (30). Metabolic labeling experiments and direct mass measurements show that the cellular concentrations of CoA and its thioesters fluctuate depending on the carbon source and growth state of *Escherichia coli* (14, 16, 33). Regulation at the pantothenate kinase step is the most important determinant of the CoA biosynthetic rate. Pantothenate kinase activity is inhibited by CoA and less effectively by CoA thioesters in vitro (33). Feedback inhibition is also effective in vivo, since only a fraction of the total catalytic capacity of pantothenate kinase is used, leading to the copious excretion of pantothenate into the medium (12, 33). Pantothenate kinase is an essential protein in the CoA biosynthetic pathway, and a pantothenate kinase mutant [*coaA15*(Ts)] exhibits a temperature-sensitive growth phenotype (34). The *coaA* gene is located at kb 3532 on the *E. coli* physical map and is allelic with *rts* (27), a previously uncharacterized locus identified by a mutant strain with a temperature-sensitive growth phenotype (7). The crucial role for pantothenate kinase in controlling the intracellular CoA content is corroborated by the significantly elevated intracellular CoA concentrations found in pantothenate kinase mutants [*coaA16*(Fr)] refractory to feedback inhibition by CoA (32). A second, less-stringent point of regulation is at the 4′-phosphopantetheine adenylyltransferase step. Regulation at this step is thought to control the recycling of 4′-phosphopantetheine arising from the degradation of CoA either by acyl carrier protein prosthetic-group turnover (14) or direct cleavage of CoA by a phosphodiesterase (32).

To test the hypothesis that the intracellular CoA concentration is regulated primarily by allosteric control of pantothenate kinase activity, we have cloned and sequenced the pantothenate kinase structural gene (*coaA*) and examined the impact of pantothenate kinase overexpression on the size of the CoA pool.

MATERIALS AND METHODS

Materials. Sources of supplies were Amersham Corp. for L-[^{35}S]methionine (specific activity, 1,112 Ci/mmol) and ACS scintillation solution; Amicon for the Centricon-30; Bio-Rad, Inc., for Bradford dye-binding protein assay solution; Anatech Inc., for 250-μm Silica Gel H plates; Applied Biosystems for ProBlott membrane; Bio 101 for Circleprep and GeneClean II kits; Du Pont-New England Nuclear for D-[1-^{14}C]pantothenate (specific activity, 54.5 Ci/mol), [α-^{35}S]dATP (specific activity, 1,200 Ci/mmol), [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol), and [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol); Pharmacia P-L Biochemicals for Blue Sepharose CL-6B, ATP, and CoA; Promega for the *E. coli* S30 transcription-translation kit, restriction endonucleases, exonuclease III, T4 DNA ligase, T4 polynucleotide kinase, S1 nuclease, and Klenow fragment of DNA polymerase I; Research Organics, Inc., for dithiothreitol; U.S. Biochemical Corp. for the DNA sequencing kit; and Whatman, Inc., for DE81 filter circles. Oligonucleotides were synthesized with an Applied Biosystems model 381A synthesizer and purified with oligonucleotide purification cartridges as recommended by the manufacturer. All other materials were reagent grade or better.

Bacterial strains and growth conditions. The bacterial strains used in this work were derivatives of *E. coli* K-12 (Table 1). Rich medium was L broth (21), and minimal medium consisted of medium E salts (35) supplemented with glucose (0.4%), thiamine (0.001%), and required amino acids (0.01%). Antibiotic concentrations were 50 μg/ml for ampicillin and kanamycin, 15 μg/ml for tetracycline, and 200 μg/ml for rifampin. Cell number was monitored during growth by using a Klett-Summerson colorimeter with a blue filter. The colorimeter was calibrated with strain SJ16 by determining the number of viable bacteria in the range of colorimeter readings encountered.

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
DH1	*recA1 endA1 relA1 supE44 gyrA96 thi-1 hsdR17* λ^-	CGSC[a]
DV73	*metB1 relA1 spoT1 gyrA216 coaA15 srl*::Tn*10 recA1*	D. Vallari
SJ16	*panD2 metB1 relA1 spoT1 gyrA216 zad-220*::Tn*10* λ^- λ^r F^-	12
SJ51	*panD2 metB1 relA1 spoT1 gyrA216* λ^- λ^r F^-	17
UB1005	*metB1 relA1 spoT1 gyrA216* λ^- λ^r F^-	12

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

Pantothenate kinase assay. Enzyme preparation and assays were performed as described previously (33) by using a logarithmically growing culture harvested at a density of 6.3 × 10^8 cells per ml. The pantothenate kinase specific activities in cell lysates prepared from overnight cultures were consistently lower than those in lysates prepared from logarithmically growing cells. Protein concentrations were measured by the method of Bradford (4) with bovine serum albumin as a standard.

DNA sequencing. The sequentially deleted pWS7 plasmid derivatives (Fig. 1) made by the method of Henikoff (8) and two M13 recombinant phages carrying 0.5- and 1-kb segments were sequenced by using a set of synthetic primers. The nucleotide sequence was determined by the dideoxynucleotide chain termination method (25) with [α-^{35}S]dATP on double- or single-stranded DNA substrates. Regions obscured by G-C compressions were clarified by using a labeling mixture substituting dITP for dGTP.

RNA analysis. Total RNAs from strains UB1005 and DV73/pWS6 were isolated as described by Aiba et al. (2). To determine the transcriptional start sites, a synthetic oligonucleotide (18-mer, 5′-CGTCATTAACGTTTGCTC-3′) complementary to nucleotides 493 to 510 was end labeled with [γ-^{32}P]ATP with polynucleotide kinase (24) and used for primer extension. The labeled probe was annealed with 100 μg of RNA, and cDNA was generated with avian myeloblastosis virus reverse transcriptase (23). Primer extension and sequencing samples were loaded side by side on an 8% polyacrylamide sequencing gel.

The size of the *coaA* transcript was determined by Northern (RNA) blot hybridization as described by Thomas (29). Total RNA was isolated from strain UB1005, and 30 μg was fractionated on a 1.1% formaldehyde agarose gel. The *Nco*I-*Ssp*I fragment (429 to 1,345 bp in the nucleotide sequence) of plasmid pWS7-13-2 was used to prepare a randomly primed DNA probe.

Expression and purification of pantothenate kinase. Cultures of strain DH1/pWS7-13-2/pGP1-2 were labeled with [^{35}S]methionine in vivo following treatment of the cells with rifampin and induction of the T7 RNA polymerase encoded by pGP1-2 as described by Tabor (28). Also, an *E. coli* S30-coupled transcription-translation system was used to express the protein(s) encoded by pWS7-13-2 in vitro (Promega). Proteins from each procedure were separated by 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis as described by Laemmli (20), and ^{35}S-labeled proteins were visualized by autoradiography.

Pantothenate kinase protein(s) was also expressed (28) on a larger scale in strain UB1005/pWS7-13-2/pGP1-2 for subsequent partial purification and determination of the amino-terminal sequence. The cell extract was prepared and fractionated with ammonium sulfate as described previously (33). The kinase preparation was affinity purified as described by Vallari (31). Briefly, it was loaded onto a Blue Sepharose CL-6B column that was washed with 50 mM Tris-HCl, pH 7.5. Specifically bound proteins were eluted with 0.25 mM CoA–50 mM Tris-HCl (pH 7.5), concentrated by using a Centricon-30 membrane, and separated by SDS-polyacrylamide gel electrophoresis. The proteins in the gel were electroblotted onto a ProBlott membrane; the discrete, Coomassie-stained pantothenate kinase bands were excised; and the amino-terminal amino acid sequence was determined by the St. Jude Molecular Resources Center using an Applied Biosystems model 470A Sequenator.

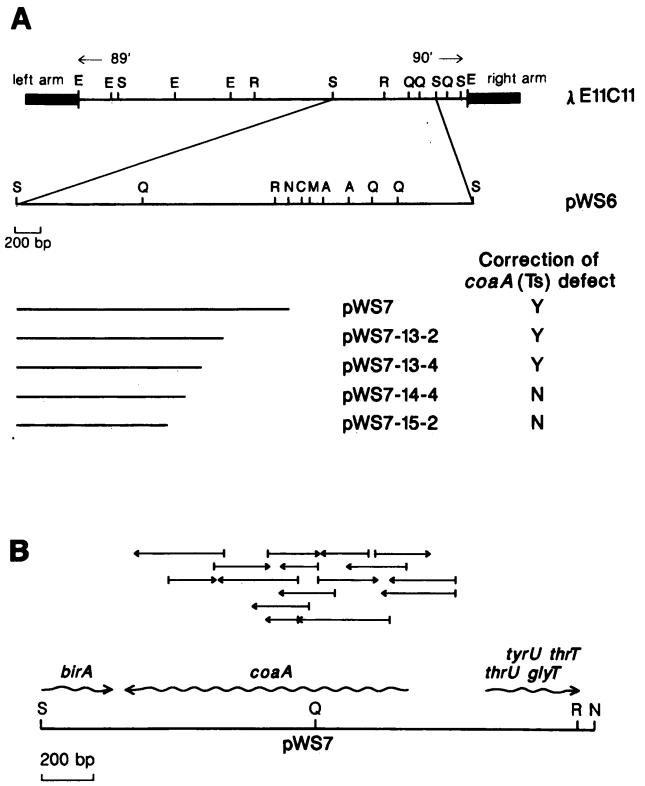

FIG. 1. Localization of the *coaA* gene and sequencing strategy. (A) The 3.5-kb *Pst*I fragment of λE11C11, carrying *coaA* (27) and other genes at 90 min on the *E. coli* chromosome (19), was inserted into the *Pst*I site of plasmid pBS(+) (Stratagene). The 2.1-kb *Pst*I-*Hin*cII fragment from plasmid pWS6 was ligated into *Pst*I-*Hin*cII-digested pBS(+), resulting in plasmid pWS7. Unidirectional deletions of plasmid pWS7 were made to pinpoint the location of *coaA* by complementation of the temperature-sensitive growth phenotype of strain DV73 [*coaA15*(Ts)]. (B) The direction and extent of sequencing of all the unidirectionally deleted plasmids and recombinant M13mp19 phages covering the 1.2-kb fragment containing the *coaA* gene are indicated by arrows. Neighboring genes and directions of transcription are indicated by wavy lines. Abbreviations for restriction endonucleases: E, *Eco*RI; S, *Pst*I; Q, *Kpn*I; R, *Eco*RV; N, *Hin*cII; C, *Cla*I; M, *Sma*I; A, *Acc*I.

Measurement of pantothenate-derived metabolites. CoA-depleted (12) strain SJ16 (*panD*) and SJ16/pWS7-13-2 cells were inoculated into minimal medium containing 60 μM [1-^{14}C]pantothenate and grown at 37°C to a density of 6.3 × 10^8 cells per ml. The pantothenate-derived metabolites were

quantitated by thin-layer chromatography as described by Jackowski and Rock (12).

The rate of pantothenate phosphorylation in vivo was measured in CoA-depleted cells at a density of 5×10^8 cells per ml. [1-^{14}C]pantothenate (60 μM) was added to 1 ml of cultures incubated at 37°C. At different time intervals, a 200-μl aliquot was removed, and the cells were lysed by a freeze-thaw lysis method (26). Aliquots (20 μl) of the cell extracts were deposited on DE81 filter disks and washed (33) to determine the incorporation of extracellular pantothenate into phosphorylated products.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of *coaA* is M90071.

RESULTS AND DISCUSSION

Cloning and sequencing the *coaA* gene. We previously showed that the *coaA* gene is allelic with *rts* and is located at kb 3532 (λE11C11; 18) on the *E. coli* physical map (27). On the basis of the restriction enzyme map of λE11C11, pWS6 that complemented the *coaA*(Ts) growth phenotype of strain DV73 was prepared (Fig. 1A). To localize one end of the *coaA* gene and to generate a set of overlapping deletions for DNA sequencing, the chromosomal insert in plasmid pWS7 was unidirectionally deleted by using exonuclease III. The resulting pWS7 derivatives were tested for complementation of the *coaA*(Ts) mutation, and one end of the sequence required for expression of functional pantothenate kinase was located between the termini of the recombinant inserts of plasmids pWS7-13-4 and pWS7-14-4 (Fig. 1A).

The set of sequentially deleted plasmids and M13mp19 subclones were sequenced by using the strategy outlined in Fig. 1B. A restriction map of pWS6 compared with the Kohara restriction map disclosed an additional *Kpn*I site (19), which indicated a total of two *Kpn*I sites within this *Pst*I-*Pst*I fragment. DNA sequence analysis of the *coaA* fragment confirmed the presence of the additional *Kpn*I restriction site. Placements of the *Eco*RI, *Eco*RV, and *Pst*I sites (Fig. 1A) were in generally good agreement with the data accompanying the Kohara library miniset. Further analysis of the nucleotide sequence revealed the presence of a single open reading frame corresponding to the location of the *coaA* gene (Fig. 2). There were four potential ATG initiation sites located at nucleotides 460, 475, 481, and 505. The 5′ end of the *coaA* DNA sequence overlapped with the published sequences for the *thrU-thyU* tRNA gene cluster (3), and the 3′ end overlapped with the carboxyl terminus and transcriptional terminator for the *birA* gene (9) (Fig. 2). The *coaA* gene is transcribed in the opposite direction to the flanking genes at 90 min (18, 27). Between the convergently transcribed *coaA* and *birA* genes is a shared sequence with the characteristic of a factor-independent transcription terminator (22). The transcriptional start sites were determined by primer extension (Fig. 3). One major and three minor bands were consistently observed with RNA derived from strain UB1005 or strain DV73/pWS6. Densitometric analysis showed that the major start site (nucleotide 400; Fig. 2 and 3) comprised 75% of the total transcripts. The size of the *coaA* transcript was determined by Northern blotting (Fig. 4). One major 1.1-kb band was detected, leading to the conclusion that *coaA* was the only gene expressed from the promoter located upstream of nucleotide 400. The putative −10 (TACCCT) and −35 (TTATTA) regions exhibited poor homology to the consensus sequences recognized by the σ^{70} RNA polymerase subunit for *E. coli* promoters (−10,

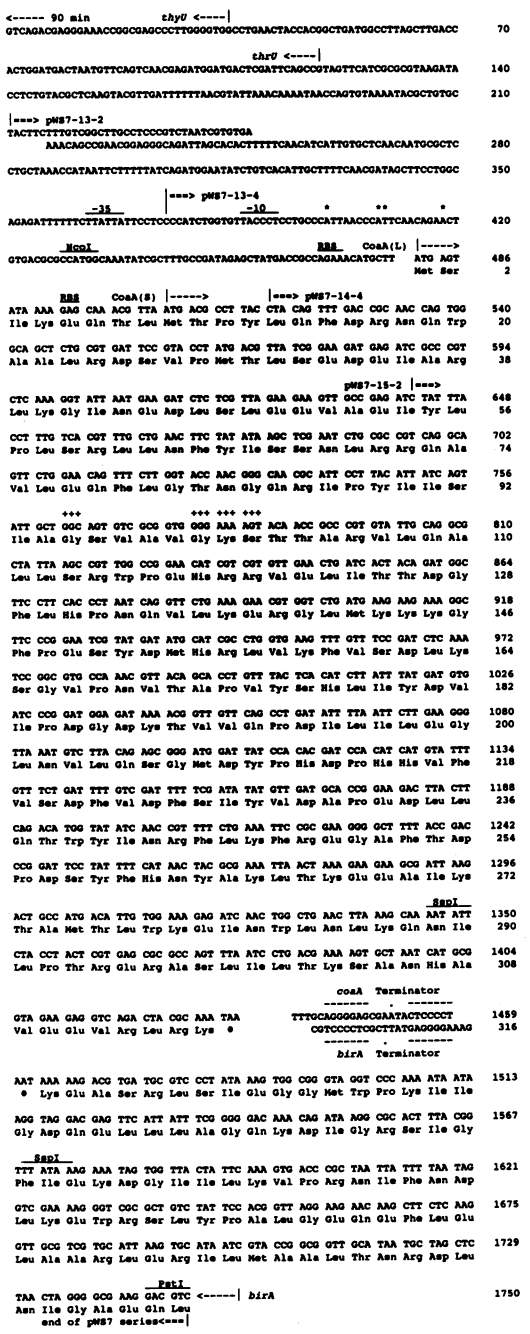

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *coaA* gene. DNA sequence of *coaA* gene and its flanking genes (*thrU*, *thyU*, and C terminus of *birA*) are shown. The direction (<---) to the 90-min region of the *E. coli* linkage map is shown at the top of the sequence. The nucleotide sequences upstream of nt 210 and downstream of nt 1411 overlapped the nucleotide sequences of *thrU*, *thyU*, and *birA*. The nucleotides from 217 to 248 and from 1435 to 1453 are shown as a double strand. Abbreviations: *, transcriptional start sites determined from primer extension; +++, amino acids representing the consensus sequence for ATP binding; RBS, two putative ribosome-binding sites used for the translation of CoaA(L) and CoaA(S); |--->, amino-terminal residues and directions of translation of CoaA(L) and CoaA(S); | ===>, start sites for the unidirectional pWS7 deletions; <===| , end of the pWS7 insert deletion series; and <---| , transcriptional directions of neighboring genes.

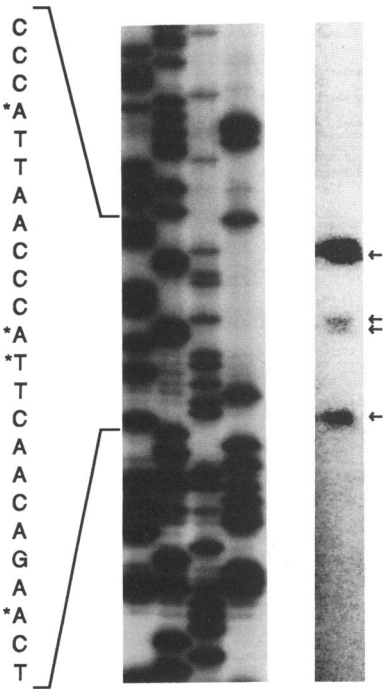

FIG. 3. Identification of the transcriptional start sites for the *coaA* gene. RNA (100 μg) from strain DV73/pWS6 was annealed with a 5'-end-labeled oligonucleotide probe, and cDNA was prepared with reverse transcriptase as described in Materials and Methods. The same oligonucleotide was used to sequence the pWS7 plasmid. The two samples were loaded side by side on an 8% DNA-sequencing polyacrylamide gel. *, four transcriptional start sites corresponding to four bands (←) detected on gel.

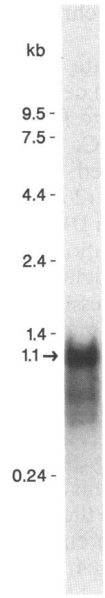

FIG. 4. Determination of the size of the *coaA* gene transcript. RNA from strain UB1005 was fractionated on a 1.1% formaldehyde gel and transferred to a nitrocellulose membrane that was hybridized with a ^{32}P-labeled randomly primed probe prepared from the *Nco*I-*Ssp*I fragment of the *coaA* gene.

TAtAaT, and −35, TTGACaT). These data suggest that the promoter may not be very active, contributing to the low relative abundance of pantothenate kinase. Another possibility is that the promoter regions bind a different σ subunit. The promoter does have −10 and −35 regions that could be related to heat shock promoters (σ^{32}); however, we have not observed an increase in the level of *coaA* transcripts following a temperature shift from 30 to 42°C for 5 min (unpublished observations). The primer extension data did not rule out any of the four potential translational start sites, and all of the potential start sites were preceded by sequences with homology to the consensus ribosome-binding sites. The frequency of optimal codon usage was calculated by the method of Ikemura (10) to be 0.565. This value is characteristic of rare proteins and is similar to the codon usage of the pantothenate permease (*panF*) gene (11). The poor homology to the consensus promoter sequence and the suboptimal codon usage are consistent with the low abundance of pantothenate kinase (33).

Expression of pantothenate kinase. A T7 expression system employing strain DH1/pWS7-13-2/pGP1-2 was used to selectively label the protein products of the *coaA* gene and to overproduce the gene products for conventional protein purification. Two protein products with apparent molecular masses of 38 and 36 kDa on SDS-polyacrylamide gel electrophoresis were expressed (Fig. 5). To determine the relationship between the two protein products, pantothenate kinase was purified by dye-ligand affinity chromatography from the overproducing strain (see Materials and Methods). The partially purified pantothenate kinase preparation exhibited two bands on a Coomassie-stained gel that corresponded to the two protein bands that were selectively labeled with the T7 expression system (Fig. 5). These purified proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to ProBlott membranes, and the amino-terminal amino acid sequence of each band was determined. The actual amino acid sequence was identical to the deduced amino acid sequence (Fig. 2). The higher-molecular-mass protein [CoaA(L)] began with the sequence SIKEQTLMTPYL..., yielding a protein with a molecular mass of 36.4 kDa (pI 6.35) that had the initiator methionine removed by posttranslational processing. The lower-molecular-mass protein [CoaA(S)] began with the sequence MTPYLQFDR..., demonstrating that this protein has a molecular mass of 35.4 kDa (pI 6.34) and was eight amino acids shorter than its larger counterpart. To determine whether the two pantothenate kinase proteins were independent translation products or were related by posttranslational processing, we performed a pulse-chase experiment (Fig. 5). The products of the *coaA* gene were selectively labeled with [^{35}S]methionine for 5 min and chased by the addition of nonradioactive methionine to the culture. During the 30-min chase, there was no detectable conversion of the higher-molecular-mass gene product to the lower-molecular-mass form (Fig. 5). Furthermore, two proteins of the same molecular masses were produced in a coupled transcription-translation system containing five protease inhibitors (sodium *meta*-bisulfate, aprotinin, leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride; data not shown). These data confirm that only two of the four potential translational start sites in the *coaA* transcript were active. The significance, if any, of the production of two translation products is unknown.

The deduced amino acid sequence contained an A-type ATP-binding consensus sequence, GXXXXGKS(T) (5), located between residues 95 and 102 (Fig. 2). An amino acid

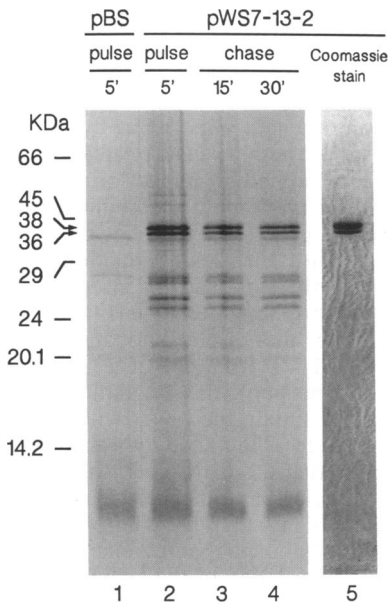

FIG. 5. Identification of the protein products of the *coaA* gene. T7 RNA polymerase directed labeling of pantothenate kinase proteins. Selective [^{35}S]methionine labeling of plasmid-coded proteins and purification of pantothenate kinase were carried out as described in Materials and Methods, and the resulting protein preparations were fractionated by SDS-polyacrylamide gel electrophoresis. Lane 1, autoradiograph of control strain DH1/pBS/pGP1-2 selectively labeled by using the T7 system with [^{35}S]methionine for 5 min; lane 2, autoradiograph of the products synthesized by strain DH1/pWS7-13-2/pGP1-2; lane 3, protein products after a 15-min chase with cold methionine; lane 4, protein products after a 30-min chase with cold methionine; lane 5, affinity-purified pantothenate kinase stained with Coomassie brilliant blue.

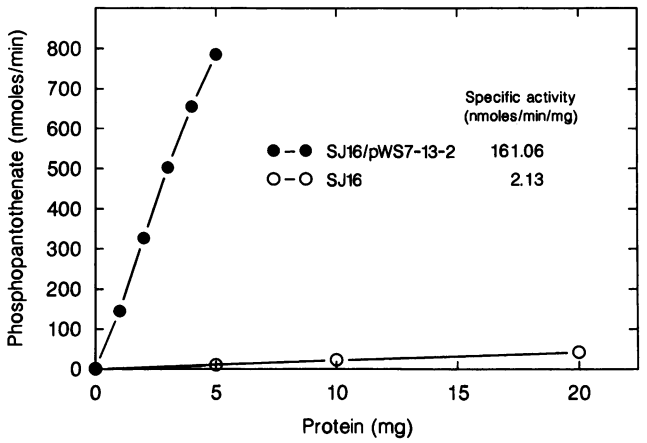

FIG. 6. Overproduction of pantothenate kinase. Samples of ammonium sulfate-purified dialyzed cell extracts from logarithmic cultures were assayed for pantothenate kinase activity for 5 min at 42°C. The specific activity of pantothenate kinase was determined from the slope of the protein curve.

homology search was performed on the GenBank and unique EMBL data bases by using Intelligenetics software (release 5.37, version 64). No other proteins with significant overall homology to pantothenate kinase were found in the data bases.

Physiological consequences of pantothenate kinase overproduction. Cell extracts from strains harboring the pWS7-13-2 plasmid possessed significantly higher specific activities of pantothenate kinase than did wild-type strains and the *coaA* mutant (Fig. 6). The extracts were fractionated with ammonium sulfate and exhaustively dialyzed. The specific activity of pantothenate kinase in extracts from strain SJ16/pWS7-13-2 was 76-fold higher than the specific activity of strain SJ16 (Fig. 6).

To ascertain whether the higher specific activity of pantothenate kinase resulted in elevated steady-state levels of CoA, strains SJ16 and SJ16/pWS7-13-2 were equilibrium labeled with [^{14}C]pantothenate, and the concentrations of intracellular and extracellular pantothenate-derived metabolites were measured (Fig. 7). The cells were provided with excess (60 μM) extracellular pantothenate, a level 7.5 times higher than the concentration of extracellular CoA precursor that is required to maximize the CoA pool in strain SJ16 (12). The 76-fold increase in pantothenate kinase specific activity in strain SJ16/pWS7-13-2 (161 nmol/min/mg; Fig. 6) resulted in a more modest 2.7-fold increase in the intracellular CoA concentration (Fig. 7). The level of extracellular 4-phosphopantetheine also increased in strain SJ16/pWS7-13-2, indicating that the 4′-phosphopantetheine adenylyltransferase could not utilize the amount of 4′-phosphopantetheine arising from biosynthesis from pantothenate, CoA degradation (32), or acyl carrier protein prosthetic-group turnover (15). The intracellular 4′-phosphopantetheine pool remained small (12 pmol/10^8 cells; Fig. 7) in cells with or without the *coaA* clone, suggesting that the pool was regulated. Cells were grown on extracellular pantothenate concentrations of 10 to 60 μM, and the metabolite levels, including those of CoA, acyl carrier protein, and phosphopantetheine, did not change significantly (data not shown). Therefore, the intracellular level of CoA was elevated but still efficiently regulated in the presence of a large excess of pantothenate kinase protein.

Our results support the conclusion that feedback regulation of pantothenate kinase activity by the components of the CoA pool is the major determinant of the CoA biosyn-

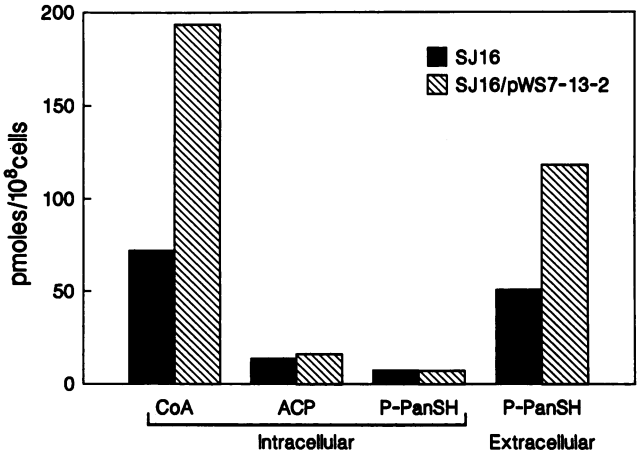

FIG. 7. Effect of pantothenate kinase overexpression on the intracellular CoA level. Strains SJ16 and SJ16/pWS7-13-2 were labeled with 60 μM pantothenate during the logarithmic phase of growth. At a density of 6.3 × 10^8 cells per ml, cells were separated from the medium, and pantothenate-derived metabolites were extracted and quantitated by scintillation counting following separation by thin-layer chromatography as described in Materials and Methods. Abbreviations: ACP, acyl carrier protein; P-PanSH, 4′-phosphopantetheine.

thetic rate. The intracellular pantothenate kinase activity can be drastically reduced without affecting CoA biosynthesis. *coaA15*(Ts) mutants possess only 20% of wild-type kinase activity at 30°C, yet the cells grow normally at this temperature (34). The importance of feedback regulation is corroborated by analysis of the *coaA16*(Fr) allele (32). Pantothenate kinase specific activity in these mutants is only 29% of the specific activity of kinase in its wild-type counterpart; however, the enzyme is considerably less sensitive to inhibition by CoA. These *coaA16*(Fr) mutants each have a 3.2-fold increase in CoA content, illustrating that a low level of pantothenate kinase activity that is refractory to feedback inhibition has as much impact on the biosynthetic rate as a 76-fold increase in specific activity.

The rates of [^{14}C]pantothenate phosphorylation in vivo were very similar in strains SJ16/pWS7-13-2, which overproduced pantothenate kinase, and strain SJ16 (Table 2), which suggests either that intracellular pantothenate phosphorylation was down-regulated or that the supply of pantothenate substrate was limiting. To test the latter hypothesis, a multicopy plasmid (pSJ2; Amp^r Tet^s; 11) carrying the *panF* gene encoding pantothenate permease was transformed into strain SJ51 (Tet^s derivative of strain SJ16; 17) together with a second multicopy plasmid (pWS11-3; Amp^s Tet^r) carrying the *coaA* gene. Plasmid pWS11-3 was constructed by transferring the *Pst*I-*Eco*RI (in multiple cloning site) fragment of pWS7-13-2 into *Eco*RI-*Pst*I-digested pBR322, resulting in a plasmid encoding resistance to tetracycline rather than ampicillin. The pantothenate kinase specific activities were determined in cell lysates prepared from overnight cultures of strain SJ51 (1.24 nmol/min/mg of protein) and of the isogenic strain harboring the pBR322-derived *coaA* plasmid pWS11-3 (43.00 nmol/min/mg of protein) to ensure that the enzyme was significantly overexpressed in vitro at a level comparable to the overexpression observed in overnight cultures of strains with the pBS-derived *coaA* plasmid pWS7-13-2 (43.75 nmol/min/mg; 24). Plasmid pSJ8 (11), which carried a nonfunctional *panF* gene, was substituted for pSJ2 in some experiments as a technical control and was derived from pBR322, as were pSJ2 and pWS11-3. Pantothenate phosphorylation was then measured in vivo with CoA-depleted cells (13) as a function of time in the presence of 60 μM [^{14}C]pantothenate and was linear for 60 min. The phosphorylation rate increased to 14.84 pmol/min/10^8 cells in the presence of a functional multicopy *panF* clone and a multicopy *coaA* clone (Table 2), indicating that the supply of pantothenate was in fact limiting when the intracellular CoA level was minimal. However, when cells with maximal intracellular CoA levels were assayed, the rate of pantothenate phosphorylation was reduced to the rate observed in wild-type cells carrying single copies of both the *coaA* and *panF* genes. This indicates that the rate of pantothenate phosphorylation in strain SJ16/pWS7-13-2 was not limited by the rate of pantothenate import but by the modulation of pantothenate kinase activity via CoA feedback inhibition. In fact, the *panF* clone had no impact on the size of the total pool of pantothenate-derived metabolites in cells overexpressing the *coaA* gene (data not shown).

TABLE 2. Rate of pantothenate phosphorylation in vivo

Strain	CoA level (pmol/10^8 cells)	Multicopy clone		Rate of phosphorylation (pmol/min/10^8 cells)
		coaA	*panF*	
SJ16	<5[a]	−	−	2.81
SJ16/pWS7-13-2	<5	+	−	4.66
SJ51	<5	−	−	3.44
SJ51/pWS11-3/pSJ8	<5	+	−	3.47
SJ51/pWS11-3/pSJ2	<5	+	+	14.84
SJ51	156	−	−	4.76
SJ51/pWS11-3/pSJ8	243	+	−	4.66
SJ51/pWS11-3/pSJ2	267	+	+	6.82

[a] See reference 16.

Concomitant with the downregulation of pantothenate phosphorylation, there is also an increase in the level of extracellular 4′-phosphopantetheine (14). The adenylyltransferase regulates the flux of phosphopantetheine through the pathway, and judging from the size of the extracellular phosphopantetheine pool, this regulation is significant but not sufficient to maintain the lower wild-type CoA concentration. If regulation at the adenylyltransferase step did not contribute to limiting the CoA level, the amount of CoA in strains overproducing pantothenate kinase activity would be at most threefold higher than the wild-type level. The size of the intracellular 4′-phosphopantetheine pool is strictly limited (Fig. 7) by modulation of the adenylyltransferase activity, efflux of excess phosphopantetheine from the cell, or possibly both mechanisms. The efflux could occur by passive diffusion, but this mechanism is unlikely, considering the charged phosphate moiety of phosphopantetheine. Another possible efflux mechanism could be mediated by a carrier molecule or related to a coupled metabolic function involving passage through the inner membrane, the peptidoglycan, and the outer membrane of *E. coli.*

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