JOURNAL OF BACTERIOLOGY, Nov. 1993, p. 7290–7300
0021-9193/93/227290-11$02.00/0

Vol. 175, No. 22

dcp Gene of *Escherichia coli*: Cloning, Sequencing, Transcript Mapping, and Characterization of the Gene Product

BERNHARD HENRICH,* SUSANNE BECKER, ULRICH SCHROEDER, AND ROLAND PLAPP

Fachbereich Biologie, Abteilung Mikrobiologie, Universität Kaiserslautern, Postfach 3049, D-67653 Kaiserslautern, Germany

Received 1 June 1993/Accepted 31 August 1993

Dipeptidyl carboxypeptidase is a C-terminal exopeptidase of *Escherichia coli*. We have isolated the respective gene, *dcp*, from a low-copy-number plasmid library by its ability to complement a *dcp* mutation preventing the utilization of the unique substrate *N*-benzoyl-L-glycyl-L-histidyl-L-leucine. Sequence analysis of a 2.9-kb DNA fragment revealed an open reading frame of 2,043 nucleotides which was assigned to the *dcp* gene by N-terminal amino acid sequencing and electrophoretic molecular mass determination of the purified *dcp* product. Transcript mapping by primer extension and S1 protection experiments verified the physiological significance of potential initiation and termination signals for *dcp* transcription and allowed the identification of a single species of monocistronic *dcp* mRNA. The codon usage pattern and the effects of elevated gene copy number indicated a relatively low level of *dcp* expression. The predicted amino acid sequence of dipeptidyl carboxypeptidase, containing a potential zinc-binding site, is highly homologous (78.8%) to the corresponding enzyme from *Salmonella typhimurium*. It also displays significant homology to the products of the *S. typhimurium opdA* and the *E. coli prlC* genes and to some metalloproteases from rats and *Saccharomyces cerevisiae*. No potential export signals could be inferred from the amino acid sequence. Dipeptidyl carboxypeptidase was enriched 80-fold from crude extracts of *E. coli* and used to investigate some of its biochemical and biophysical properties.

Escherichia coli has a series of peptidolytic activities to meet the requirement for intracellular protein breakdown. Peptidases are involved not only in the utilization of peptides supplied in the medium (52) but also in the turnover of cytoplasmic proteins during cell growth as well as under conditions of nutritional starvation (39). The analysis of mutant strains of *E. coli* and of the closely related *Salmonella typhimurium* led to the detection of a series of exopeptidases with partly overlapping substrate specificities (reference 37 and references therein). These include at least six aminopeptidases (PepA, -B, -N, -P, iminopeptidase, and methionine aminopeptidase), one aminotripeptidase (PepT), three dipeptidases (PepD, -E, and -Q), and one oligopeptidase (OpdA). Some of them (PepA, -D, -N, -P, and OpdA) have been characterized on the molecular level by isolation and sequencing of the respective genes (2, 10, 22, 25, 27, 55, 64), but no significant similarities between these genes or their products were detected. In addition, a C-terminal exopeptidase, dipeptidyl carboxypeptidase (Dcp), has been described for *E. coli*. It cleaves dipeptides off the C termini of various peptides and proteins, the smallest substrates being N-blocked tripeptides and unblocked tetrapeptides (62, 63). Dcp from *E. coli* in its catalytic properties closely resembles mammalian angiotensin I-converting enzyme (ACE), which is involved in the regulation of blood pressure (16, 53). The *E. coli* enzyme, therefore, has been used in the evaluation of drugs designed to ameliorate human hypertensive disease.

We recently localized the *dcp* gene to the 34-min position between 1,642 and 1,645 kb on the physical map of the *E. coli* chromosome (4). In this communication, we report the cloning, sequencing, and transcript mapping of the *E. coli dcp* gene. To supplement the data of Yaron et al. (63), we also analyzed some biochemical and biophysical properties of purified Dcp. Results are discussed in relation to the recently sequenced *dcp* gene of *S. typhimurium* (24).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and growth conditions. *E. coli* K-12 strains used in this study are listed in Table 1. To obtain strain UK191, the *hisG*::Tn*10* marker of NK5526 was transduced to strain K93 by use of phage P1*vir* (51). Phages M13mp18 and M13mp19 (41) and plasmid pBR322 (5) were from Pharmacia, and phage P1*vir* (40) was from H. Schmieger (University of Munich). The vector pLG339 (56) was provided by N. G. Stoker (University of Leicester, Leicester, United Kingdom). The use of the positive selection vector pUH84 (26) to establish a plasmid library of the *E. coli* genome has been described previously (31). To construct plasmid pUS39, the 3.7-kb *Sal*I-*Eco*RI fragment of pBS10 (see Fig. 1) was ligated with *Sal*I and *Eco*RI-digested pBR322. Media used were Luria-Bertani medium (40) and Davis minimal medium (14) which was supplemented with synthetic peptides at final concentrations of 50 μg/ml. When appropriate, antibiotics were added to media at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml in the case of plasmid-encoded resistance or 30 μg/ml in the case of Tn*10*-encoded resistance.

Materials. Restriction endonucleases and nucleic acid-modifying enzymes were from Boehringer Mannheim, United States Biochemical, New England Biolabs, or Pharmacia and were used according to the instructions of the suppliers. *N*-Acetyl-L-alanyl-L-alanyl-L-alanine (Ac-Ala_3), *N*-benzoyl-L-glycyl-L-histidyl-L-leucine (Hip-His-Leu), and *p*-nitrobenzyloxycarbonyl-L-glycyl-(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinyl-L-glycine [*p*-NO_2-Z-Gly-(S-NBD)-Cys-Gly] were obtained from Bachem. The peptide *p*-nitrobenzyloxycarbonyl-L-glycyl-L-phenylalanyl-L-glycine (*p*-NO_2-Z-Gly-Phe-Gly) was synthesized by conventional peptide chemistry methods. Radiochemicals (α-^{35}S-dATP, 600 or 1,240 Ci/mmol; L-[^{35}S]methionine, 1,000 Ci/mmol; ^{14}C-protein molecular weight mark-

TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Source or reference
e23716	Wild type	American Type Culture Collection
K79	Hfr *thi-1 relA1 dcp-1*	16
K93	Hfr *thi-1 relA1 dcp-2*	16
UK191	K93 *hisG*::Tn*10*	This work
MM294	F⁻ *endA1 hsdR17 supE44 thi-1*	38
ER1562	MM294 *mcrA1272*::Tn*10 hsdR2 mcrB1*	New England Biolabs
NK5526	*hisG*::Tn*10* IN(*rrnD-rrnE*)*1*	N. Kleckner[a]

[a] Harvard University, Cambridge, Mass.

ers, 10 to 50 μCi/mg of protein) were from Amersham. L-Amino acid oxidase, RNase A, and a digoxigenin DNA labelling and detection kit were obtained from Boehringer Mannheim. Protease inhibitors and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-tetrazolium chloride (INT) were from Sigma, and phenazine methosulfate and ampholines were from Serva. The purification of peptidase D from *E. coli* UK61(pJK13) (30) will be published elsewhere (unpublished data).

Recombinant DNA and sequencing techniques. Preparation and analysis of plasmid and phage DNA and transformation of *E. coli* were carried out by standard procedures (48). DNA fragments for cloning were isolated from agarose or polyacrylamide gels by use of an HSB-Elutor (Biometra). To sequence the 2.9-kb *Kpn*I fragment of plasmid pBS10 (see Fig. 1), each of two subfragments (a 1.8-kb *Kpn*I-*Acc*I fragment and a 1.3-kb *Hin*cII-*Kpn*I fragment) was inserted into M13mp18 and M13mp19 in both orientations. With single-stranded templates and a T7 sequencing kit (Pharmacia), dideoxy sequencing (49) of overlapping regions was primed with synthetic oligonucleotides, synthesized on a Gene Assembler Plus apparatus (Pharmacia).

Detection of Dcp in native polyacrylamide gels. To prepare cell extracts, cells from a 1-liter culture, grown in Luria-Bertani medium to an optical density at 600 nm of about 1.3, were harvested by centrifugation, washed with 30 mM Tris hydrochloride (pH 7.5), and resuspended in 4 ml of the same buffer. After disruption of the bacteria by ultrasonication, the suspension was centrifuged at 220,000 × *g* for 90 min at 4°C, and the protein concentration in the supernatant was determined by the Coomassie blue method (54). About 50 to 200 μg of total protein was applied to prerun 6% polyacrylamide rod gels (15) and electrophoresed at 3 mA per tube and 4°C. To visualize Dcp activity, a coupled assay including purified peptidase D (24) was used. The gels were removed from the glass tubes after electrophoresis and embedded in 3 ml of molten 1% agar (45°C), containing 25 mM Tris hydrochloride (pH 8.0), 5 mM $CoCl_2$, 2.5 mg of Hip-His-Leu, 0.2 μg of purified *E. coli* peptidase D, 0.17 U of L-amino acid oxidase, 0.75 mg of INT, and 0.15 mg of phenazine methosulfate. During the following incubation at 37°C, Hip-His-Leu was specifically cleaved by the action of Dcp to give *N*-benzoyl-L-glycine (hippuric acid) and L-histidyl-L-leucine, which in turn was hydrolyzed by peptidase D. Oxidative desamination of the liberated amino acids by L-amino acid oxidase, according to the method of Sugiura et al. (58), finally resulted in the formation of red formazan at the sites of Dcp activity.

In vitro expression of *dcp*. Plasmid pBS15 DNA (see Fig. 1) was highly purified by two successive CsCl density gradient centrifugations, and 2.5 μg was used as template to direct protein synthesis in a coupled transcription-translation system derived from *E. coli* (Amersham). Of the resulting samples, aliquots (1/7 volume) were analyzed on discontinuous sodium dodecyl sulfate–12% polyacrylamide gels (34).

Construction and screening of a low-copy-number library. Chromosomal DNA of *E. coli* wild type, prepared by the method of Wilson (61), was partially digested with *Sau*3A, and fragments were fractionated by centrifugation through a 10 to 40% sucrose gradient (rotor SW27, 26,000 rpm, 20°C, 24 h). Fragments in the size range of 5 to 20 kb were ligated with the *Bam*HI-linearized and dephosphorylated low-copy-number vector pLG339. To screen for hybrid plasmids containing the *dcp* gene, the *dcp hisG*::Tn*10* strain UK191 was transformed with the ligation mixture by electroporation (18). About 9,000 of the clones obtained on Luria-Bertani plates supplemented with kanamycin (selection for pLG339) and tetracycline (selection for *hisG*::Tn*10*) were pooled, washed with 0.9% NaCl, and plated on minimal agar containing Hip-His-Leu as sole histine source.

Isolation of RNA. Total RNA from *E. coli* was prepared by the hot-phenol-extraction method (1) as modified by Gerendasy and Ito (23). RNA was stored in the presence of 75% ethanol and quantified as described previously (25). Aliquots were checked on 1% agarose gels before use.

Primer extension analysis. To identify the 5′ end of *dcp*-specific transcripts by primer extension, we used two synthetic oligonucleotides (primer 1, 37-mer; primer 2, 26-mer; see Fig. 3) in parallel reactions. Annealing was done in volumes of 10 μl of H_2O, containing 30 μg of RNA and 20 pmol of primer, by heating at 90°C for 2 min and subsequent cooling to 30°C within 30 min. To obtain labelled extension products, annealing reaction mixtures were combined with 22.5 U of avian myeloblastosis virus reverse transcriptase (United States Biochemical) in 20-μl reaction volumes, containing 50 mM Tris hydrochloride (pH 8.3), 8 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 0.15 μM (each) dCTP, dGTP, and dTTP, and 15.1 pmol (18.75 μCi) of α-^{35}S-dATP. After a 7-min incubation at 42°C, labelled cDNA was extended by addition of 18 μl of 50 mM NaCl containing 0.4 mM (each) all four deoxyribonucleoside triphosphates and further incubation at 42°C for 60 min. To stop the reactions, 3.3 μl of 0.2 M EDTA (pH 8.0) containing 3 ng of RNase A (DNase-free) per μl was added and followed by incubation at 37°C for 30 min. The samples were mixed with 160 μl of 2.5 M ammonium acetate (pH 7.0) and 5 μg of carrier *Saccharomyces cerevisiae* tRNA, extracted with 200 μl of phenol-$CHCl_3$-isoamyl alcohol (25:24:1), precipitated with ethanol, and dissolved in 9 μl of formamide dye (48). Aliquots (1 to 3 μl), after being heated at 95°C for 3 min, were applied to 6% polyacrylamide–urea sequencing gels.

S1 nuclease mapping. Two uniformly labelled single-stranded probes (nucleotides 499 to 773 and 2507 to 2862; see Fig. 3), overlapping the putative 5′ and 3′ endpoints of *dcp* transcription, were synthesized by a modification of the extension procedure of Burke (8). Single-stranded M13mp18 DNA, carrying the 1,845-bp *Kpn*I-*Acc*I fragment of pBS10 inserted into the respective restriction sites of the polylinker region, was annealed with primer 1 (see Fig. 3), and single-stranded M13mp19 DNA, carrying the 1,295-bp *Hin*cII-*Kpn*I fragment of pBS10, was annealed with primer 3. Annealing reaction mixtures containing 0.5 pmol of template DNA, 5 pmol of primer, 15 mM Tris hydrochloride (pH 7.5), 15 mM $MgCl_2$, 75 mM NaCl, and 1.5 mM dithiothreitol in a total volume of 20 μl were heated at 85°C for 5 min and then slowly cooled to 37°C within 30 min. After addition of 2 μl of 100 mM dithiothreitol, 50 pmol (62.5 μCi) of α-^{35}S-dATP, and 1 μl of a mixture of dCTP-dGTP-dTTP (each at 20 mM), primers were extended with 5 U of DNA polymerase (Klenow fragment) for 30 min at room temperature in total volumes of 30 μl. The reaction

mixtures were supplemented with 1 μl of 20 mM dATP and chased for a further 20 min at room temperature. Double-stranded DNA regions were subsequently digested with restriction enzymes cleaving at appropriate distances upstream of the respective primer hybridization sites (*Nsi*I in the case of the 5′-end-specific probe, *Pst*I in the case of the 3′-end-specific probe; see Fig. 3). The samples were applied to 6% polyacrylamide–urea sequencing gels from which the single-stranded probes were isolated by standard methods. This procedure yielded about 10^7 cpm of labelled probe. The ends of *dcp* mRNA were mapped by S1 nuclease protection analysis essentially as described previously (25). The probes (5×10^5 cpm) each were hybridized with total RNA (130 μg) for 15 h at 30°C and then digested with 140 U of S1 nuclease for 50 min at 37°C. After the final precipitation step, samples were dissolved in 9 μl of formamide dye, and portions (2 to 3 μl) were electrophoresed in 6% polyacrylamide–urea sequencing gels. Northern (RNA) blot analysis of RNA was performed as described elsewhere (25).

Assay of Dcp activity. Hydrolysis of the substrates *p*-NO_2-Z-Gly-Phe-Gly and *p*-NO_2-Z-Gly-(S-NBD)-Cys-Gly by Dcp was monitored essentially as described by Persson and coworkers (43, 44). Dcp was routinely assayed with Hip-His-Leu as a substrate by a modification of the method described by Cushman and Cheung (12). The respective samples (10 to 100 μl) were incubated in the presence of 1 mM Hip-His-Leu and 30 mM Tris hydrochloride (pH 7.5) for 30 min at 37°C in total volumes of 200 μl. To stop the reaction, 100 μl of 1 N HCl was added, and hippuric acid, liberated by the action of Dcp, was extracted from the aqueous phase with 600 μl of ethyl acetate. After evaporation of the solvent at 120°C, remaining material was dissolved in 400 μl of H_2O. The concentration of hippuric acid was determined by measuring the A_{228} of the solution.

Purification of Dcp. Strain UK191(pUS39) was used to prepare cell extract by the same procedure as that applied for the analysis of Dcp in native gels (see above). Extract from 42 g (wet weight) of cells was fractionated on an anion-exchange column (Q-Sepharose Fast Flow, Pharmacia) with a linear gradient from 0 to 1 M NaCl in 30 mM Tris hydrochloride (pH 7.5) at 4°C and a flow rate of 120 ml/h. The most active fractions were pooled and again applied to a Q-Sepharose column which then was eluted with a 0 to 0.4 M NaCl gradient. Active fractions were concentrated by ultrafiltration and further purified by gel permeation through Fraktogel TSK HW-55(s) (Merck) at 4°C and a flow rate of 60 ml/h. As deduced from the elution profiles of various marker proteins, Dcp eluted from this column with an apparent molecular mass of 80 kDa. A pool of the active fractions was subjected to preparative isoelectric focusing in an LKB type 8100-1 column for 47 h exactly as recommended by the supplier. The pH gradient, generated by use of ampholines (Serva) in the pH range of 4 to 6, was stabilized by a 5 to 50% sucrose density gradient. The pH of the most active fraction was 5.2. Proteins contained in this fraction were separated on a sodium dodecyl sulfate–8% polyacrylamide gel and subsequently blotted to a glass fiber membrane (Glassybond, Biometra) by the method of Eckerskorn et al. (19). As estimated after Coomassie blue staining of the membrane, Dcp constituted about 20% of the total protein in the final preparation. The Dcp band was excised from the membrane and forwarded to Beckman Inc. (Munich, Germany) for commercial sequencing of the amino terminus.

Computer analysis. Nucleotide and amino acid sequence data were processed by use of the Microgenie (Beckman), Husar (Geniusnet), and PC/Gene (Intelligenetics) programs.

Nucleotide sequence accession number. The GenBank accession number assigned to the nucleotide sequence reported in this paper is X57947.

RESULTS

Phenotypic detection of *dcp*. *E. coli dcp* mutants K79 and K93 were previously characterized by their failure to cleave Ac-Ala_3 (16). As the use of this substrate as sole nitrogen source results in poor cell growth, we chose another N-blocked tripeptide, Hip-His-Leu, for the detection of Dcp activity. The unique specificity of Dcp for Hip-His-Leu was demonstrated by two pieces of evidence. (i) Derivatives of *dcp* mutants, carrying a *hisG*::Tn*10* insertion, were not able to utilize Hip-His-Leu as the sole histidine source. (ii) Cell extracts of *E. coli* K-12 wild type, after electrophoretic separation in native polyacrylamide gels and activity staining by use of a coupled PepD–L-amino acid oxidase test, yielded a single band of Hip-His-Leu cleaving activity. This band was absent from the protein pattern of *dcp* mutants.

Identification of the *dcp* gene in a plasmid library. Initially, a gene bank of the *E. coli* chromosome, constructed in the high-copy-number positive selection vector pUH84, was screened, but no recombinant plasmids conferring the ability to utilize Hip-His-Leu on a *dcp hisG*::Tn*10* mutant were detected. Subsequent screening of a low-copy-number plasmid library, constructed with the vector pLG339, allowed the isolation of two plasmids (pBS10 and pBS20) that complemented the *dcp* mutation and contained inserts of 5 and 13 kb, respectively. Some common restriction fragments were present in both inserts, indicating that the same genetic locus (*dcp*) had been cloned in both plasmids. From restriction and deletion analysis of plasmid pBS10, the *dcp*-complementing activity could be localized to the 2.6-kb *Bgl*I-*Kpn*I fragment (Fig. 1). In a coupled in vitro transcription-translation system, the recombinant plasmid pBS15 directed the synthesis of a 78-kDa product in addition to the proteins encoded by the vector pLG339 (Fig. 2).

Nucleotide sequence analysis of the *dcp* gene. The nucleotide sequence of both strands of a 2,899-bp *Kpn*I fragment (Fig. 1) was determined. Analysis of the sequence revealed a complete open reading frame extending from an ATG codon at nucleotide 677 to a TAA stop codon at position 2720, which is sufficient to encode a protein of 681 amino acids (Fig. 3). The deduced molecular mass (77.5 kDa) of this protein agreed well with the 80- and 78-kDa values determined by gel filtration (see Materials and Methods) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2), respectively. However, there is a second ATG codon, located only 6 bp downstream from the first one, which was also a likely candidate for the initiation of *dcp* translation. To resolve this ambiguity, the sequence of the first nine amino acid residues of the purified *dcp* gene product was determined. It was in complete agreement with the amino acid sequence obtained by translation initiation at the first ATG codon of the open reading frame, with the exception that the N-terminal methionine was absent from the mature protein. As in other *E. coli* genes (57), a putative ribosome binding site was present 5 bp upstream of the ATG start codon. It has complementarity with 6 of 8 nucleotides of the 3′-OH terminus of *E. coli* 16S rRNA (50).

Transcription of the *dcp* gene. In a Northern blot experiment (not shown) with RNA from strain MM294(pBS15), both a *Pvu*II fragment, corresponding to nucleotides 1255 to 2788, and an *Nsi*I-*Eco*RV fragment (nucleotides 501 to 993) (Fig. 1 and 3) hybridized to a single species of *dcp*-specific transcript

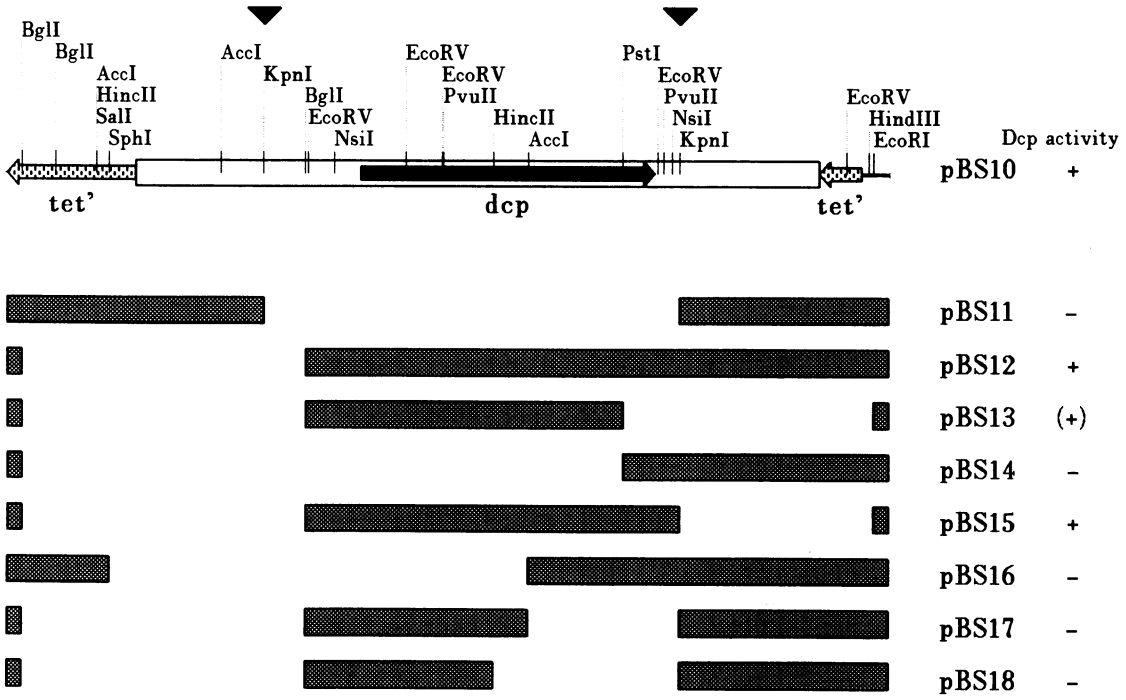

FIG. 1. Restriction and deletion analysis of the recombinant plasmid pBS10. Plasmid sections encompassing the regions of inserted chromosomal DNA are shown. The 5-kb insert of pBS10 (open box) is flanked by fragments of the tetracycline resistance gene (tet'). The extension and orientation of the *dcp* coding region are given by the solid arrow. Some pertinent restriction sites are shown. Arrowheads point to the ends of the 2.9-kb *Kpn*I fragment that has been sequenced (Fig. 3). Shaded bars below the map of pBS10 represent sequences retained in the deletion products pBS11 to pBS18. Dcp activities, as indicated to the right, reflect the ability of transformants of strain UK191 harboring the respective plasmids to grow on agar plates containing Hip-His-Leu as the sole source of histidine. (Colonies were visible after 2 days of incubation at 37°C in the case of pBS10, pBS12, and pBS15 and after 3.5 days in the case of pBS13.)

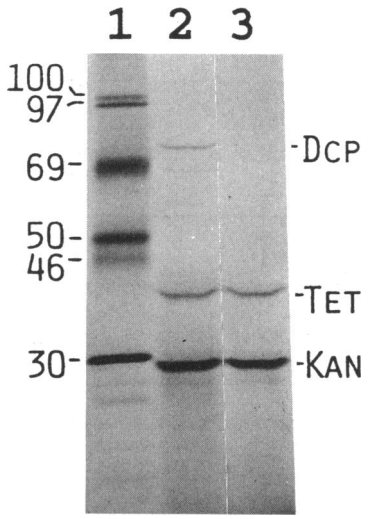

FIG. 2. Electrophoretic identification of in vitro-synthesized *dcp* product. ^{35}S-labelled proteins, encoded by plasmid pBS15, were synthesized with a prokaryotic DNA-directed translation system and separated on a sodium dodecyl sulfate–12% polyacrylamide gel (lane 2). Proteins encoded by the vector pLG339 are the tetracycline resistance protein (TET) and aminoglycoside phosphotransferase (KAN) (lane 3). The sizes of protein standards are given in kilodaltons (lane 1).

of about 2.3 kb, whereas an *Eco*RV-*Nsi*I fragment (nucleotides 316 to 500) did not generate any visible signal. This indicated that transcription is initiated downstream from the *Nsi*I site at position 498. The estimated length of *dcp* mRNA correlates well with the presence of possible transcription initiation and termination signals (46) in the *dcp* 5′- and 3′-flanking regions. The ATG initiation codon is preceded by potential sigma^{70} promoter elements at distances of 29 (−10 region) and 52 (−35 region) nucleotides, respectively. The spacing between these elements is 17 nucleotides. Downstream of the TAA stop codon, at a distance of only 5 nucleotides, a G+C-rich sequence with the potential to form a stem-loop structure (ΔG = −36.4 kcal/mol) can be identified (see Fig. 5B). This structure, however, lacks the run of consecutive T residues, typical of rho-independent transcription terminators (46). To verify the physiological significance of these potential transcription signals, we performed primer extension and S1 nuclease protection experiments. Extension products which were synthesized after annealing of *dcp* mRNA with an oligonucleotide, complementary to nucleotides 809 to 834 (primer 2, Fig. 3), were terminated at position 653 or 655 (Fig. 4A). A different primer (primer 1, Fig. 3), synthesized to confirm this result, was extended to exactly the same positions (not shown). For high-resolution S1 nuclease mapping of the 5′ and 3′ ends of *dcp* transcripts, we used single-stranded probes, generated by the extension of primer 1 (5′ end) and primer 3 (3′ end), respectively. RNA transcribed from the *dcp* gene was found to protect 119 ± 3 nucleotides of the 5′-end-specific probe and 262 ± 3 nucleotides of the 3′-end-specific probe from S1 nuclease digestion. Thus, S1 mapping localized the

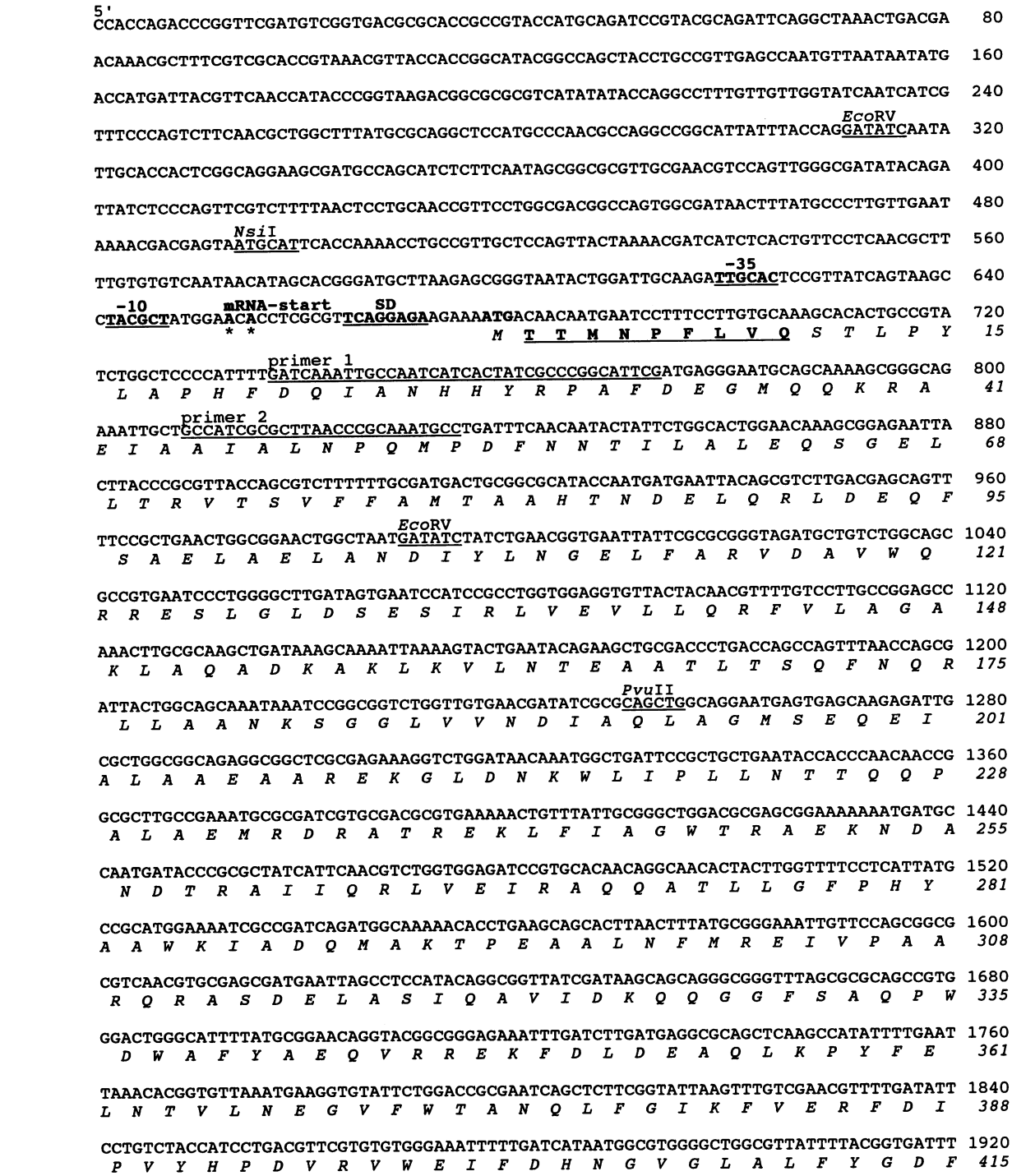

FIG. 3. Nucleotide sequence of the noncoding strand of the *dcp* gene and of its flanking regions. Numbering starts at the last residue of the left *Kpn*I recognition site shown in Fig. 1. Start points and endpoints (mRNA-start, mRNA-end) of *dcp* mRNA, as detected by primer extension and S1 nuclease analysis, are marked by asterisks. Potential −35 and −10 regions, a possible Shine-Dalgarno sequence (SD), and an inverted repeat (IR) capable of forming a stem-loop structure are underlined. Sequences complementary to synthetic oligonucleotides (primer 1, primer 2, and primer 3) which were used in transcript mapping are also underlined. The positions of some relevant restriction sites are indicated. The deduced amino acid sequence of Dcp is given below the nucleotide sequence. The sequence of the underlined residues at the amino terminus was confirmed by partial sequencing of purified Dcp. A potential zinc-binding site (positions 470 to 474) (29) within the active center of the enzyme is underlined. It contains two zinc-binding histidine residues (marked by asterisks) and a catalytically active glutamic acid residue (in boldface).

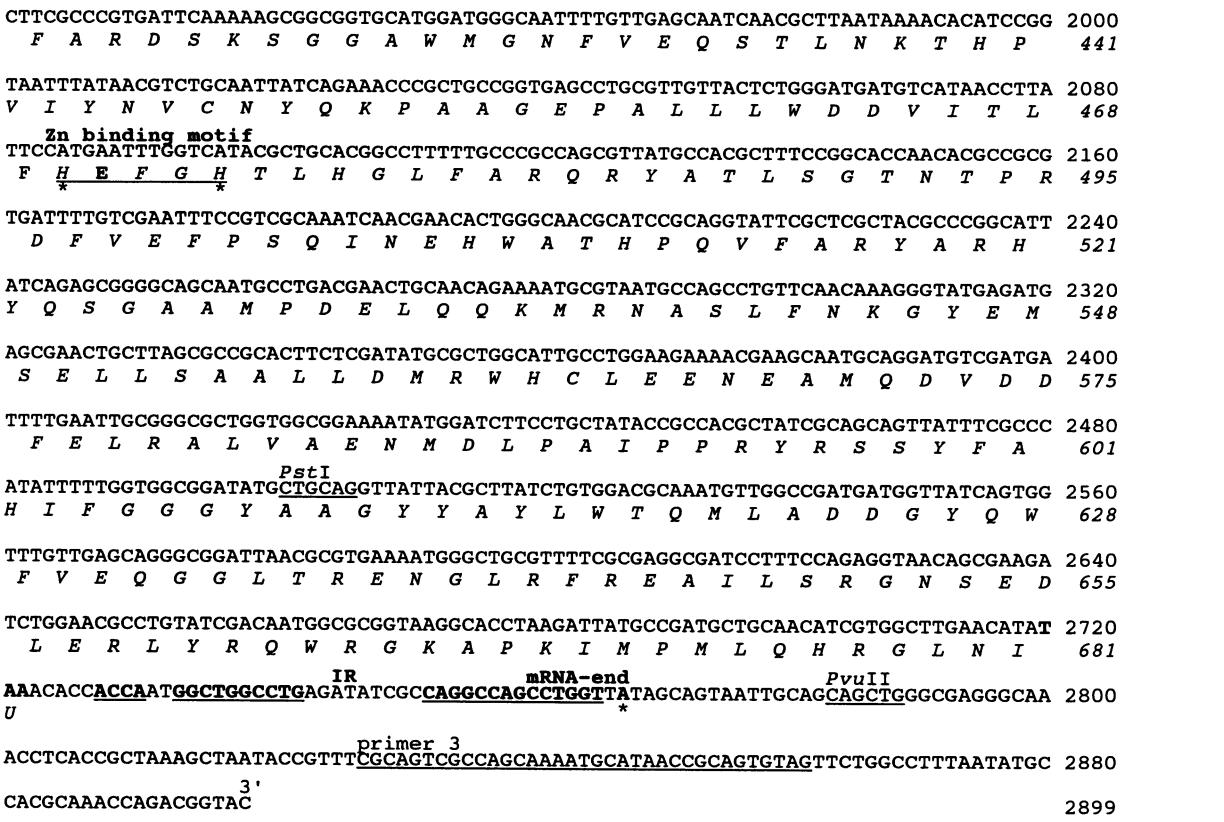

FIG. 3—*Continued.*

initiation site of *dcp* transcription to position 655 (Fig. 4B), which is in excellent agreement with the results from primer extension. The 3′ endpoint of *dcp* RNA could be assigned to position 2768 (Fig. 5A), which, in accordance with typical *E. coli* terminators (46), follows the center of the dyad symmetry of the predicted stem-loop structure (Fig. 5B) at a distance of 22 nucleotides. Since primer extension and S1 mapping experiments with RNA from strain ER1562 carrying only the unmodified vector pLG339 produced the same (although weaker) signals as experiments with RNA from strains harboring the *dcp* plasmids pBS10, pBS12, or pBS15 (Fig. 4 and 5A), artifacts, which occasionally occur with elevated gene copy numbers, can be excluded. It therefore appears that the *dcp* coding region represents an individual transcription unit which is not associated with other genes in an operon.

Codon usage. To estimate the efficiency of *dcp* translation, we calculated the frequencies of rare and optimal codon usage. A set of eight rare codons were reported to occur at an average of 4% in the reading frames of 25 nonregulatory *E. coli* genes (33). In the *dcp* coding frame, these rare codons, however, make up 9.5% of the total triplets. Optimal codons, as defined by the codon preference list of Ikemura (28), on the other hand, occur in the *dcp* gene at a frequency of 0.57. Frequencies of optimal codon usage in the range of 0.5 to 0.6 seem to be typical of proteins which are present in less than 100 copies per cell (28).

Amino acid sequence analysis of Dcp. The deduced primary sequence of Dcp shows a preponderance of leucine and alanine, which often occur in stretches of two to four residues and which constitute 23.2% of the protein. However, no matches to a consensus leucine zipper sequence, characteristic of some DNA-binding proteins (35), could be detected. The sequence contains a Zn^{2+}-binding motif (His-Glu-X-X-His) (Fig. 3) which is common to the active centers of various thermolysin-type zinc-dependent metallopeptidases (29).

Computer-assisted comparison of Dcp with protein sequence data banks uncovered extensive homologies to the recently published sequences of the *S. typhimurium* peptidases Dcp (78.8% identity within 676 residues) and OpdA (31.7% identity within 678 residues) (10, 24) and to OpdA of *E. coli* (31.8% identity within 676 residues), which is the product of the *prlC* gene (11). In addition, we identified two eukaryotic proteins which, according to their normalized alignment scores (17), also display a significant degree of similarity to Dcp of *E. coli*. These are the rat metalloendopeptidase EP 24.15 (45), previously shown to be homologous to Dcp of *S. typhimurium* (24), and a hypothetical zinc metalloproteinase of *S. cerevisiae* (6). In Fig. 6A, a multiple alignment of *E. coli* Dcp with the amino acid sequences of these enzymes is presented. Apart from the highly conserved potential zinc-binding site (motif 2, Fig. 6A), the C-terminal halves of the six proteins share two additional regions (motifs 1 and 3) of remarkably high (>40%) identity. On the basis of sequence similarity analysis (21), we calculated the degrees of relationship between these proteins. The resulting dendrogram (Fig. 6B) shows that, of the six sequences compared, the *prlC* and *opdA* products of *E. coli* and *S. typhimurium* displayed the highest degree of amino acid identity (94.5%).

Earlier growth and cell fractionation studies with *dcp* mutants of *E. coli* suggested that about 10% of the Dcp activity is located in the periplasmic space (16). This led us to inspect the *dcp* product for the presence of typical export signals. Cleavage

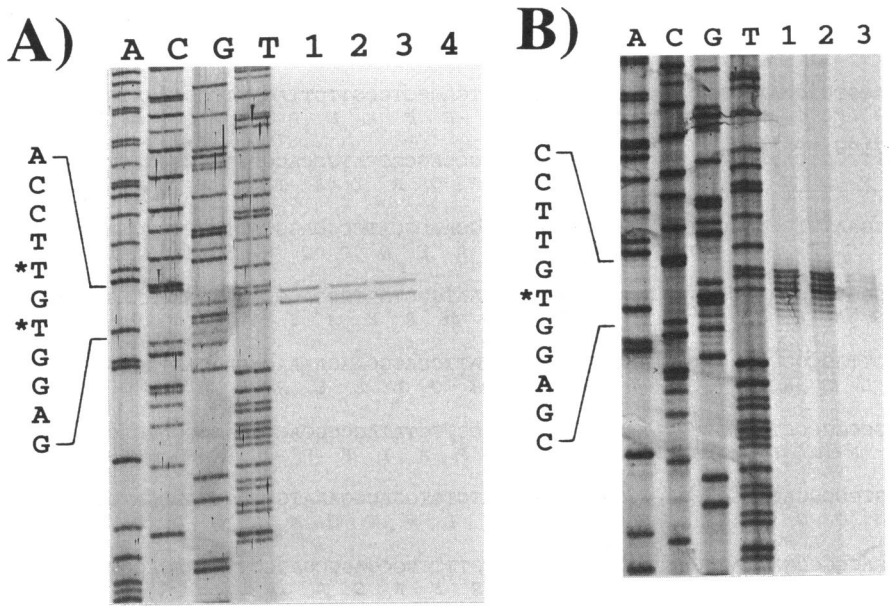

FIG. 4. Mapping of the 5′ end of *dcp* transcripts. (A) Primer extension with primer 2 (Fig. 3) and RNA from strain ER1562 harboring plasmid pBS12 (lane 1), pBS10 (lane 2), pBS15 (lane 3), or pLG339 (lane 4). Reference sequencing reactions (lanes A, C, G, and T) were initiated by the same primer on a single-stranded M13 template containing the 1.8-kb *Kpn*I-*Acc*I fragment of pBS10. Accordingly, the sequence is the complement of the one shown in Fig. 3. The sequence of the coding strand, encompassing the 3′ ends of the major extension products (indicated by asterisks), is shown to the left of the panel. (B) S1 nuclease mapping with a single-stranded probe covering positions 499 to 773 (Fig. 3) and RNA from strain ER1562 carrying plasmid pBS10 (lane 1), pBS15 (lane 2), or pLG339 (lane 3). Reference sequencing reactions (lanes A, C, G, and T) were performed with the same primer and the same template as those used to synthesize the probe (see Materials and Methods). The sequence encompassing the 3′ end of the protected probe fragment (indicated by an asterisk) is given on the left.

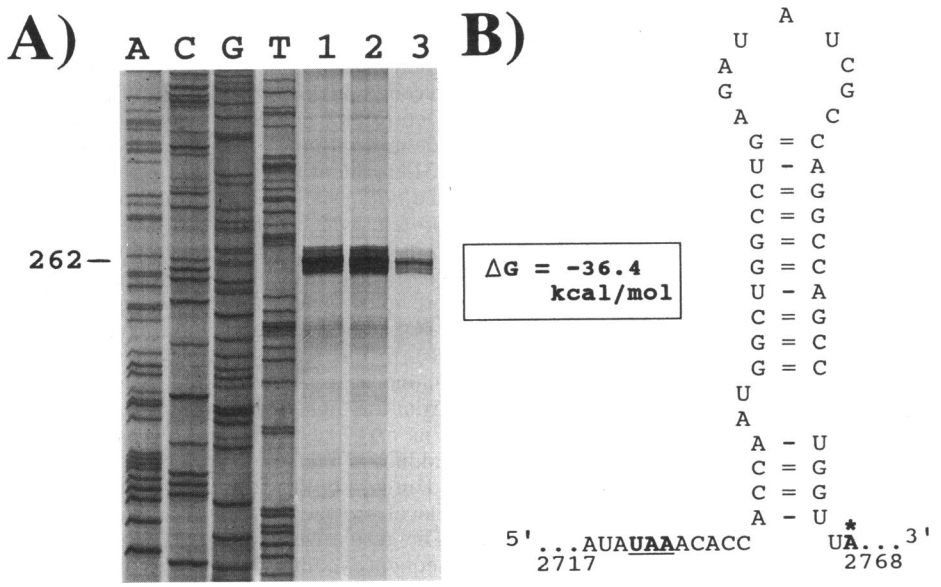

FIG. 5. Termination of *dcp* transcripts. (A) S1 nuclease mapping with a single-stranded probe covering positions 2507 to 2862 (Fig. 3) and RNA from strain ER1562 carrying plasmid pBS10 (lane 1), pBS12 (lane 2), or pLG339 (lane 3). The reference sequencing ladder (lanes A, C, G, and T) is the same as that shown in Fig. 4B. The size of the reference band (in base pairs) corresponding to the protected probe fragment is indicated. It was calculated from the hybridization site of primer 1, which had been used to generate the reference sequencing ladder. (B) Potential stem-loop structure at the 3′ end of *dcp* mRNA. Nucleotide numbering is as in Fig. 3. The stop codon for *dcp* translation is underlined. The position of most frequent transcription termination, as determined by S1 nuclease mapping, is indicated by an asterisk.

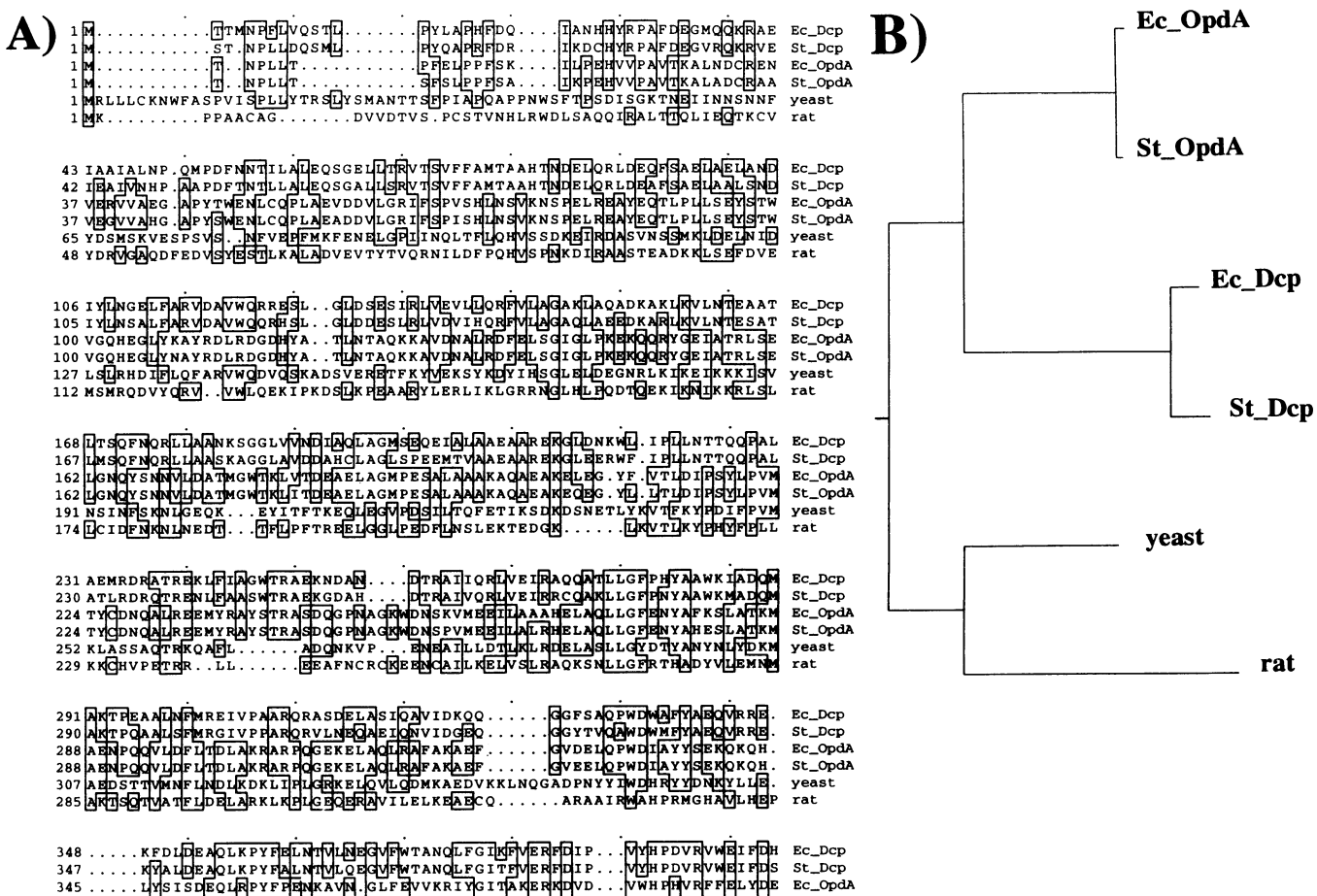

FIG. 6. Comparison of related zinc-dependent metalloproteinases. (A) Multiple alignment of the deduced amino acid sequences of *E. coli* Dcp (Ec_Dcp) and OpdA (11) (Ec_OpdA), *S. typhimurium* Dcp (24) (St_Dcp) and OpdA (10) (St_OpdA), an *S. cerevisiae* zinc metalloproteinase (6) (yeast), and rat metalloendopeptidase EP24.15 (45) (rat). The alignment was constructed with the Clustal program of the Geniusnet Husar package by using a gap penalty of 10. Residues which are identical in at least 50% of the aligned sequences are boxed. Three regions, displaying 42.9% (motif 1), 63.6% (motif 2), and 61.5% (motif 3) identity of the six sequences, are indicated. The putative catalytic site (motif 2) contains two zinc-binding residues (*) and an essential glutamic acid residue (#). (B) Dendrogram deduced from a progressive sequence alignment by use of the Tree program of the Geniusnet Husar package. Phylogenetic relationships were calculated according to the work of Feng and Doolittle (21). Sequences are designated as in Fig. 6A. The lengths of the horizontal branches directly correlate with the frequencies of base exchanges in the respective coding regions.

TABLE 2. Purification of Dcp from *E. coli*

Purification step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)
Crude extract	5,000	10.5	0.084	1.0
Q-Sepharose Fast Flow	256	3.29	0.41	4.9
Fraktogel TSK HW 55(s)	29	2.57	1.0	11.8
Isoelectric focusing	0.53	2.22	6.38	77.0

[a] One unit of Dcp liberates 1 μmol of hippuric acid from Hip-His-Leu in 1 min at 37°C.

of a signal peptide from native Dcp, however, must be excluded as the N terminus of purified Dcp corresponds to the 5′ end of the *dcp* gene. Moreover, the average hydropathy of Dcp (−0.321 [33]) is suggestive of a hydrophilic protein. The hydropathic profile of the *E. coli* enzyme, which was found to be almost identical to that of Dcp from *S. typhimurium*, also did not show any extended stretches of marked hydrophobicity to which a membrane-spanning potential (32) could be assigned. Additionally, no segments with the potential to form membrane-linked helices (20) were detected. Thus, the hydropathic properties of Dcp are indicative of a soluble protein with a cytoplasmic location.

Biochemical and biophysical properties of Dcp. The initial report on the purification of Dcp from *E. coli* B (63) also determined some properties of the enzyme. To further investigate the influence of various biochemical and biophysical parameters on the enzymatic activity, we attempted to overexpress the *dcp* gene by subcloning it into pBR322. As shown by densitometric analysis of electrophoretically separated plasmid preparations, the copy number of the resulting plasmid pUS39, however, was considerably reduced compared with the vector pBR322. We applied a three-step procedure to enrich Dcp about 80-fold from a crude extract of strain UK191(pUS39) (Table 2).

In addition to the list of known Dcp substrates (63), we showed that the *E. coli* enzyme releases C-terminal dipeptides from two *p*-nitro-benzoylated tripeptides which were used previously to assay mammalian ACE. These are the fluorogenic substrate *p*-NO_2-Z-Gly-Phe-Gly (43) and the chromogenic substrate *p*-NO_2-Z-Gly-(S-NBD)-Cys-Gly (44).

The Lineweaver-Burk plot with Hip-His-Leu as substrate gave a K_m value of 1.55 mM for the *E. coli* K-12 enzyme, which is in the same order of magnitude as the values obtained for Dcp of *E. coli* B (63). The isoelectric points calculated from the deduced amino acid sequences of the *E. coli* and *S. typhimurium* enzymes are 5.29 and 5.06, respectively. To determine the pH optimum for Dcp activity, we used the phosphate buffer system (pH 5.0 to 9.2) and the Tris-HCl buffer system (pH 7.2 to 9.0). Maximum activity of the *E. coli* K-12 enzyme was observed at pH 7.5, compared with pH 8.2 for Dcp from *E. coli* B (63). The activity dropped sharply at alkaline pH, whereas at pH 5.0 the enzyme still retained about 50% of its maximum activity. The temperature of highest activity was 42°C. At 37°C, the enzyme showed about 75% of the maximum activity. We also examined the effects of protease inhibitors and divalent cations on Dcp activity (Table 3). Whereas the serine protease inhibitor phenylmethylsulfonyl fluoride had almost no effect, the cysteine protease inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), trypsin inhibitor, and pepstatin reduced the activity of Dcp to between 80 and 60% of its maximum. In contrast, chymostatin, an inhibitor of chymotrypsin-like enzymes (46), clearly affected Dcp activity, and the chelating agent 1,10-phenanthroline completely inhibited the enzyme. In agreement with earlier studies (63), EDTA, even if used at high concentrations (10 mM), was found to have only a minor effect on Dcp activity. We also verified a previously observed slight activation of Dcp by 1 mM Mn^{2+} or Mg^{2+} (63). Ca^{2+} and Co^{2+}, used at the same concentration, had a similar effect. However, a five- to eightfold stimulation by 5×10^{-5} M Co^{2+}, as demonstrated for Dcp from *E. coli* B (63), could not be reproduced for the *E. coli* K-12 enzyme. On the other hand, Cu^{2+}, Zn^{2+}, and Ni^{2+} strongly inhibited Dcp, reducing its activity to 16, 10, and 9% of its normal level, respectively. NaCl, known to stimulate ACE at a concentration of 300 mM (7), had an inhibitory effect on Dcp. In the presence of 100 mM NaCl, the enzyme retained only 20% of its maximum activity.

TABLE 3. Effects of proteinase inhibitors and divalent cations on Dcp activity

Supplement	Concn (mM)	Relative Dcp activity[a]
None (control)		1[b]
Proteinase inhibitors		
PMSF[c]	1	0.92
Trypsin inhibitor[d]	0.02	0.76
Trypsin inhibitor[e]	0.06	0.66
Chymostatin	0.2	0.1
E64	0.005	0.61
Pepstatin	0.001	0.81
1,10-Phenanthroline	5	0.02
EDTA	10	0.76
Divalent cations[f]		
Mn^{2+}	1	1.21
Ca^{2+}	1	1.2
Mg^{2+}	1	1.18
Co^{2+}	1	1.15
Cu^{2+}	1	0.16
Zn^{2+}	1	0.1
Ni^{2+}	1	0.09

[a] Purified Dcp was assayed with Hip-His-Leu as substrate.
[b] Control without inhibitor.
[c] PMSF, phenylmethylsulfonyl fluoride.
[d] From lima bean.
[e] From bovine pancreas.
[f] Divalent cations were added as Cl salts.

DISCUSSION

Expression of the *dcp* gene. Our failure to construct a high-copy-number plasmid containing the *dcp* gene suggested that overproduction of Dcp affects the viability of *E. coli*. This agrees with the relatively high frequency (9.5%) of rare codons (33) in the *dcp* reading frame, as rare codon usage in other *E. coli* genes has been considered a means to keep the corresponding protein products in low copy number (9, 33). In particular, the rare codons ACG (Thr), AAT (Asn), and CAA (Gln) seem to be equally dispersed throughout the *dcp* gene. On the other hand, peptidase genes such as *pepD*, *pepN*, *pepA*, and *pepP* which contain rare codons at moderate frequencies of 4.1, 5.7, 5.4, and 4.5%, respectively, could easily be overexpressed in *E. coli* (3, 27, 55, 64). The frequencies of rare and preferred codons in the *dcp* gene of *S. typhimurium* (9.25% rare codons; optimal codon usage frequency of 0.55) were found to be very similar to those compiled from the *E. coli* gene. Elevated intracellular Dcp concentrations, however, seem to be innocuous to *S. typhimurium*, since the *dcp* gene was recently overexpressed about 50-fold from a pBR328

derivative in this organism (24). Thus, the *E. coli* enzyme might have accessory functions that require effective control of its copy number.

The positions and the spacings of the putative sigma^{70} promoter elements and the Shine-Dalgarno sequences are almost identical for the *dcp* genes of *E. coli* and *S. typhimurium*. Accordingly, the degree of homology between the available *dcp* upstream sequences of both organisms (75% identity within 426 nucleotides) is similar to the degree of homology (72.5%) between the respective coding regions. Following the TAA stop codons of both genes, the similarity between the 177 nucleotides of available 3′-flanking sequences drops sharply to the less significant level of 41.2%. Reduced sequence similarity might be the reason for the different positions of transcription terminators proposed for the *dcp* genes of *E. coli* (6 nucleotides downstream of the stop codon) and *S. typhimurium* (93 nucleotides downstream of the stop codon) (24). Alternatively, the inverted repeat indicated by Hamilton and Miller (24), because of its unfavorable ΔG value (+0.6 kcal/mol), may not represent the functional terminator of *dcp* transcription in *S. typhimurium*. Another potential terminator (5′-CGGTTGTTCGGCACTGTCGAACAACCG-3′) with the more reasonable free energy of −14.6 kcal/mol can be identified at a distance of only 8 nucleotides after the stop codon. The position of this palindrome, which, however, is not followed by the usual stretch of T residues (46), would also agree with the estimated length (2,050 nucleotides) of *dcp* transcripts from *S. typhimurium* (24).

Catalytic activity of Dcp. pBS13, one of the deletion derivatives of the recombinant plasmid pBS10 (Fig. 1), lacks the C-terminal 71 codons of the *dcp* gene. The protein product of the truncated gene, which retains the three highly conserved regions indicated in Fig. 6A, was sufficient to support slow growth of *dcp hisG*::Tn*10* mutants on agar plates containing Hip-His-Leu as histidine source. This indicates that the C terminus, although important for full activity, is dispensable for the peptidolytic action of Dcp. Dcp exhibits a much higher susceptibility to the chelating agent 1,10-phenanthroline than to EDTA. The known preference of 1,10-phenanthroline for Zn^{2+} (47), together with the presence of a potential Zn^{2+}-binding site in the deduced amino acid sequence, suggests that Dcp is a zinc-dependent metallopeptidase. However, we observed strong inhibition of Dcp activity after addition of Zn^{2+} to a final concentration of 1 mM. This effect probably results from the formation of zinc monohydroxide ($[ZnOH]^+$) in the presence of elevated Zn^{2+} concentrations which might compete with the substrate by blocking the active center of the enzyme (36).

Dcps of *E. coli* and *S. typhimurium* closely resemble mammalian ACE in their substrate specificities and in their susceptibility to chemical inhibitors like captopril (13, 16, 60). From the failure of inhibitory anti-ACE antibodies to affect the *E. coli* enzyme, Deutch and Soffer (16) had already concluded that there was a lack of structural homology between the catalytically active antigenic determinants of Dcp and ACE. This earlier observation is now confirmed by sequencing data, indicating that neither Dcp and ACE nor the corresponding genes show any significant homologies. Thus, the strikingly similar enzymatic properties of Dcp and ACE obviously result from a common mechanism of peptide hydrolysis rather than from structural homologies. Sequence similarity, on the other hand, does not necessarily imply identical catalytic properties. OpdA and Dcp are clearly distinguishable on the basis of their substrate specificities (59, 63), although the respective amino acid sequences display extensive homology.

Cellular location of Dcp. Analysis of the deduced amino acid sequence of Dcp argued against both the presence of a signal peptide for protein export and any interactions of the enzyme with the cytoplasmic membrane. This calls into serious question the earlier observation that a significant fraction of Dcp activity (thought to account for the utilization of Ac-Ala_3) is located in the periplasmic space from whence it was reported to be released by osmotic shock (16). The limitation of Dcp to the cytoplasmic compartment would also imply that N-blocked peptide substrates such as Ac-Ala_3 and Hip-His-Leu can be transported across the cytoplasmic membrane, contrary to previous suggestions (42).

ACKNOWLEDGMENT

We thank John Cullum for careful reading of the manuscript.

REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional gal promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256**:11905–11910.
2. **Bally, M., M. Foglino, M. Bruschi, M. Murgier, and A. Lazdunski.** 1986. Nucleotide sequence of the promoter and aminoterminal encoding region of the *Escherichia coli pepN* gene. Eur. J. Biochem. **155**:565–569.
3. **Bally, M., M. Murgier, and A. Lazdunski.** 1984. Cloning and orientation of the gene encoding aminopeptidase N in *Escherichia coli*. Mol. Gen. Genet. **195**:507–510.
4. **Becker, S., and R. Plapp.** 1992. Location of the *dcp* gene on the physical map of *Escherichia coli*. J. Bacteriol. **174**:1698–1699.
5. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
6. **Bolle, P. A., V. Gilliquet, G. Berben, J. Dumont, and F. Hilger.** 1992. Yeast sequencing reports: the complete sequence of K3B, a 7.9 kb fragment between PGK1 on chromosome III, reveals the presence of seven open reading frames. Yeast **8**:205–213.
7. **Bünning, P., B. Holmquist, and J. F. Riordan.** 1983. Substrate specificity and kinetic characteristics of angiotensin converting enzyme. Biochemistry **22**:103–110.
8. **Burke, J. F.** 1984. High-sensitivity S1 mapping with single-stranded [^{32}P] DNA probes synthesized from bacteriophage M13mp templates. Gene **30**:63–68.
9. **Carter, J. R., M. A. Franden, R. Aebershold, and C. S. McHenry.** 1992. Molecular cloning, sequencing, and overexpression of the structural gene encoding the δ subunit of *Escherichia coli* DNA polymerase III holoenzyme. J. Bacteriol. **174**:7013–7025.
10. **Conlin, C. A., and C. G. Miller.** 1992. Cloning and nucleotide sequence of *opdA*, the gene encoding oligopeptidase A in *Salmonella typhimurium*. J. Bacteriol. **174**:1631–1640.
11. **Conlin, C. A., N. J. Trun, T. J. Silhavy, and C. G. Miller.** 1992. *Escherichia coli prlC* encodes an endopeptidase and is homologous to the *Salmonella typhimurium opdA* gene. J. Bacteriol. **174**:5881–5887.
12. **Cushman, D. W., and H. S. Cheung.** 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. **20**:1637–1648.
13. **Cushman, D. W., H. S. Cheung, E. F. Sabo, and M. A. Ondetti.** 1977. Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. Biochemistry **16**:5484–5491.
14. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *E. coli* requiring methionine or vitamin B12. J. Bacteriol. **60**:17–28.
15. **Davis, B. J.** 1964. Disc electrophoresis. II. Ann. N.Y. Acad. Sci. **121**:404–427.
16. **Deutch, C. E., and R. L. Soffer.** 1978. *Escherichia coli* mutants defective in dipeptidyl carboxypeptidase. Proc. Natl. Acad. Sci. USA **75**:5998–6001.
17. **Doolittle, R. F., D. F. Feng, M. S. Johnson, and M. A. McClure.** 1986. Relationships of human protein sequences to those of other organisms. Cold Spring Harbor Symp. Quant. Biol. **51**:447–455.
18. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High

efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**2127–2145.
19. **Eckerskorn, C., W. Mewes, H. Goretzki, and F. Lottspeich.** 1988. A new siliconized-glass fiber as support for protein-chemical analysis of electroblotted proteins. Eur. J. Biochem. **176:**509–519.
20. **Eisenberg, D., R. M. Weiss, and T. C. Terwilliger.** 1984. The hydrophobic moment detects periodicity in protein hydrophobicity. Proc. Natl. Acad. Sci. USA **81:**140–144.
21. **Feng, D. F., and R. F. Doolittle.** 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. **25:**351–360.
22. **Foglino, M., S. Gharbi, and A. Lazdunski.** 1986. Nucleotide sequence of the *pepN* gene encoding aminopeptidase N of *Escherichia coli*. Gene **49:**303–309.
23. **Gerendasy, D., and J. Ito.** 1990. Nucleotide sequence and transcription of the right early region of bacteriophage PRD1. J. Bacteriol. **172:**1889–1898.
24. **Hamilton, S., and C. G. Miller.** 1992. Cloning and nucleotide sequence of *Salmonella typhimurium dcp* gene encoding dipeptidyl carboxypeptidase. J. Bacteriol. **174:**1626–1630.
25. **Henrich, B., U. Monnerjahn, and R. Plapp.** 1990. Peptidase D gene (*pepD*) of *Escherichia coli* K-12: nucleotide sequence, transcript mapping, and comparison with other peptidase genes. J. Bacteriol. **172:**4641–4651.
26. **Henrich, B., and R. Plapp.** 1986. Use of the lysis gene of bacteriophage ϕX174 for the construction of a positive selection vector. Gene **42:**345–349.
27. **Henrich, B., U. Schroeder, R. W. Frank, and R. Plapp.** 1989. Accurate mapping of the *Escherichia coli pepD* gene by sequence analysis of its 5′ flanking region. Mol. Gen. Genet. **215:**369–373.
28. **Ikemura, T.** 1981. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the *E. coli* translational system. J. Mol. Biol. **151:**389–409.
29. **Jongeneel, C. V., J. Bouvier, and A. Bairoch.** 1989. A unique signature identifies a family of zinc-dependent metallopeptidases. FEBS Lett. **242:**211–214.
30. **Klein, J., B. Henrich, and R. Plapp.** 1986. Cloning and expression of the *pepD* gene of *Escherichia coli*. J. Gen. Microbiol. **132:**2337–2343.
31. **Klein, J. R., B. Henrich, and R. Plapp.** 1990. Molecular cloning of the *envC* gene of *Escherichia coli*. Curr. Microbiol. **21:**341–347.
32. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815:**468–476.
33. **Konigsberg, W., and G. N. Godson.** 1983. Evidence for use of rare codons in the *dnaG* gene and other regulatory genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **80:**687–691.
34. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
35. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
36. **Larsen, K. S., and D. S. Auld.** 1988. The mechanism of zinc inhibition of bovine carboxypeptidase A. FASEB J. **2:**A1114.
37. **Lazdunski, A. M.** 1989. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. FEMS Microbiol. Rev. **63:**265–276.
38. **Meselson, M., and R. Yuan.** 1968. DNA restriction enzyme from *E. coli*. Nature (London) **217:**1110–1114.
39. **Miller, C. G.** 1987. Protein degradation and proteolytic modification, p. 680–691. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
40. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
42. **Payne, J. W.** 1980. Transport and utilization of peptides by bacteria, p. 211–256. *In* J. W. Payne (ed.), Microorganisms and nitrogen sources. John Wiley & Sons, Inc., New York.
43. **Persson, A., and I. B. Wilson.** 1977. A fluorogenic substrate for angiotensin-converting enzyme. Anal. Biochem. **83:**296–303.
44. **Persson, A. V., S. F. Russo, and I. B. Wilson.** 1978. A new chromogenic substrate for angiotensin-converting enzyme. Anal. Biochem. **91:**674–683.
45. **Pierotti, A., K.-W. Dong, M. J. Glucksman, M. Orlowski, and J. L. Roberts.** 1990. Molecular cloning and primary structure of rat testes metalloendopeptidase EC 3.4.24.15. Biochemistry **29:**10323–10329.
46. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
47. **Salvesen, G., and H. Nagase.** 1989. Inhibition of proteolytic enzymes, p. 83–104. *In* R. J. Beynon and J. S. Bond (ed.), Proteolytic enzymes, a practical approach. Oxford University Press, Oxford.
48. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
50. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
51. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 111–112. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Simmonds, S., and J. S. Fruton.** 1949. The utilization of amino acids and peptides by mutant strains of *Escherichia coli*. J. Biol. Chem. **180:**635–640.
53. **Soffer, R. L.** 1976. Angiotensin-converting enzyme and the regulation of vasoactive peptides. Annu. Rev. Biochem. **45:**73–94.
54. **Spector, T.** 1978. Refinement of the Coomassie blue method of protein quantitation. Anal. Biochem. **86:**142–146.
55. **Stirling, C. J., S. D. Colloms, J. F. Collins, G. Szatmari, and D. J. Sheratt.** 1989. *xerB*, an *Escherichia coli* gene required for plasmid ColE1 site-specific recombination, is identical to *pepA* encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase. EMBO J. **8:**1623–1627.
56. **Stoker, N. G., N. F. Fairweather, and B. G. Spratt.** 1982. Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. Gene **18:**335–341.
57. **Stormo, G. D., T. D. Schneider, and L. M. Gold.** 1982. Characterization of translational initiation sites in *Escherichia coli*. Nucleic Acids Res. **10:**2971–2996.
58. **Sugiura, M., Y. Ito, K. Hirano, and S. Sawaki.** 1977. Detection of dipeptidase and tripeptidase activities on polyacrylamide gel and cellulose acetate gel by the reduction of tetrazolium salts. Anal. Biochem. **81:**481–484.
59. **Vimr, E. R., L. Green, and C. G. Miller.** 1983. Oligopeptidase-deficient mutants of *Salmonella typhimurium*. J. Bacteriol. **153:**1259–1265.
60. **Vimr, E. R., and C. G. Miller.** 1983. Dipeptidyl carboxypeptidase-deficient mutants of *Salmonella typhimurium*. J. Bacteriol. **153:**1252–1258.
61. **Wilson, K.** 1987. Preparation of genomic DNA from bacteria, p. 2.4.1-2.4.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
62. **Yaron, A.** 1976. Dipeptidyl carboxypeptidase from *Escherichia coli*. Methods Enzymol. **50:**599–610.
63. **Yaron, A., D. Mlynar, and A. Berger.** 1972. A dipeptidyl carboxypeptidase from *E. coli*. Biochem. Biophys. Res. Commun. **47:**897–902.
64. **Yoshimoto, T., H. Tone, T. Honda, K. Osatomi, R. Kobayashi, and D. Tsuru.** 1989. Sequencing and high expression of aminopeptidase P gene from *Escherichia coli* HB101. J. Biochem. **105:**412–416.