JOURNAL OF BACTERIOLOGY, Mar. 1992, p. 1626–1630
0021-9193/92/051626-05$02.00/0

Vol. 174, No. 5

Cloning and Nucleotide Sequence of the *Salmonella typhimurium dcp* Gene Encoding Dipeptidyl Carboxypeptidase

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Received 16 September 1991/Accepted 20 December 1991

Plasmids carrying the *Salmonella typhimurium dcp* gene were isolated from a pBR328 library of *Salmonella* chromosomal DNA by screening for complementation of a peptide utilization defect conferred by a *dcp* mutation. Strains carrying these plasmids overproduced dipeptidyl carboxypeptidase ≈50-fold. The nucleotide sequence of a 2.8-kb region of one of these plasmids contained an open reading frame coding for a protein of 77,269 Da, in agreement with the 80-kDa size for dipeptidyl carboxypeptidase (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration). The N-terminal amino acid sequence of dipeptidyl carboxypeptidase purified from an overproducer strain agreed with that predicted by the nucleotide sequence. Northern (RNA) blot data indicated that *dcp* is not cotranscribed with other genes, and primer extension analysis showed the start of transcription to be 22 bases upstream of the translational start. The amino acid sequence of *dcp* was not similar to that of a mammalian dipeptidyl carboxypeptidase, angiotensin I-converting enzyme, but showed striking similarities to the amino acid sequence of another *S. typhimurium* peptidase encoded by the *opdA* (formerly *optA*) gene.

Dipeptidyl carboxypeptidase (Dcp), originally purified and characterized from *Escherichia coli* by Yaron and coworkers (26, 27), is the only cytoplasmic C-terminal exopeptidase known for this organism. The enzyme is a Zn^{2+} metallopeptidase which removes dipeptides from the C termini of N-blocked tripeptides, tetrapeptides, and larger peptides. Dcp has a fairly broad specificity but will not cleave bonds in which the nitrogen is supplied by a proline, nor will it cleave a Gly-Gly peptide bond (27). Dcp has enzymatic properties similar to those of a mammalian enzyme, angiotensin I-converting enzyme (ACE) (24), and both activities are inhibited by the antihypertensive drug captopril (2, 3). Dcp has been used in procedures for determining the primary structures of polypeptides (11).

E. coli (3) and *Salmonella typhimurium* (24) mutations leading to loss of Dcp have been isolated. The properties of strains carrying these mutations indicate that this enzyme can function both in peptide catabolism (3, 24) and in intracellular protein degradation (23). To further characterize *dcp*, we report the cloning of the gene, its nucleotide sequence, and some characterization of its mRNA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unless otherwise indicated, bacterial strains (Table 1) are derivatives of *S. typhimurium* LT2. Minimal media used in this work (E medium and NN [no nitrogen] medium) have been described previously (5, 25). LB broth (GIBCO) was used as rich medium. When required, LB medium was supplemented with tetracycline (25 μg/ml), chloramphenicol (10 μg/ml), ampicillin (100 μg/ml), or kanamycin (50 μg/ml). Unless otherwise indicated, cultures were grown by shaking aeration at 37°C.

Cloning of *dcp*. A plasmid library containing *Sau*3A fragments of *Salmonella* DNA in the *Bam*HI site of pBR328 was transformed into TN1201 (*dcp-1*; 24), and transformants able to use *N*-acetyl-Ala-Ala-Ala ($AcAla_3$) as sole nitrogen source were selected. The two plasmids obtained (pJG68 and pJG69) contained approximately 5-kb inserts with essentially the same restriction maps. The chromosomal fragment of pJG69 is slightly longer than that of pJG68.

Isolation of insertion mutations. Insertions of transposon Tn*1000* (γδ) into pJG68 were isolated as described by Guyer (7). Briefly, pJG68 was transformed into TN2658, a *Salmonella* strain carrying a *finP* F*lac* plasmid. The resulting strain was used as donor in a conjugation cross with TN1186 selecting resistance to tetracycline (conferred by a Tn*10* insertion in the chromosome of TN1186) and to chloramphenicol and ampicillin (conferred by pJG68). Exconjugants were screened for their ability to use $AcAla_3$ as sole nitrogen source. Strains that were unable to use $AcAla_3$ were presumed to have a γδ insertion in the plasmid *dcp* gene. All of these strains, as well as some strains that were able to use $AcAla_3$, were purified and saved.

Insertions of the Mu*dJ* element (MudI1734) (1) into the *dcp* gene carried by pJG68 were isolated as described previously (8). These plasmid insertions (*dcp-101*::Mu*dJ*, *-102*::Mu*dJ*, *-103*::Mu*dJ*, and *-104*::Mu*dJ* in pCM131 to -134, respectively) were returned to the chromosome by selection for strains carrying the insert antibiotic resistance (Kan^r) and by screening for the absence of vector resistance markers (Amp^r and Cam^r) (8). The approximate locations of the γδ and Mu*dJ* insertions were determined by restriction mapping.

DNA manipulations and sequencing. Standard methods (17) were used for DNA manipulations. DNA sequencing by the dideoxy method (18) used double-stranded plasmid templates and Sequenase according to the directions of the manufacturer (U.S. Biochemicals). Primers for sequencing from the ends of Tn*1000* were those described by Liu et al.

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TABLE 1. Bacterial strains

Strain	Genotype
TN1186	*dcp-1 zcf-845*::Tn*10*
TN1201	*dcp-1 zcf-845*::Tn*10 opdA1 zhg-848*::Tn*5*
TN1246	*leuBCD485 pepB11 pepN90 pepA16 supQ302* (*proAB pepD*) *pepP1 pepQ1*
TN1379	*leuBCD485*
TN2262	*leuBCD485 pepT7*::MudJ
TN2373	*ara-9 polA2*
TN2488	TN1201/pJG68
TN2625	*leuBCD485 metA15 pepN90 pepA16 pepB11 pepP1 pepQ1 opdA1 zhf-848*::Tn*5 pepT7*::Mud*1*(X) *pepD3 dcp-1 zcf-845*::Tn*10*
TN2658	purC7/F'42 *finP301* (SA2197)
TN2676	TN2625/pJG68
TN3309	*leuBCD485 dcp-102*::MudJ
TN3311	*leuBCD485 dcp-104*::MudJ
TN3412	*leuBCD485 supQ302* (*proAB pepD*) *pepN90 pepT1 zhf-848*::Tn*5 opdA1 zcf-845*::Tn*10 dcp-1*/pJG8 (*pepD*)
TN3518	TN1186/pJG68.296 (*dcp-296*::Tn*1000*)

(13): γ end primer 5'-TCAATAAGTTATACCAT-3' and δ end primer 5'-GAATTATCTCCTTAACG-3'. The primer 5'-TTCGTACTTCAAGTGAAT-3' (8a) was synthesized to sequence from the attL end of the Mu*dJ* insertion (10). Two other primers complementary to *dcp* sequences were used in sequencing (Fig. 1). The locations of the insertions used for sequencing and the sequencing strategy are shown in Fig. 1. Except for 177 nucleotides at the 5' end of the sequence shown in Fig. 2, the sequences of both strands were determined.

RNA methods. RNA was prepared by the hot phenol extraction method of Gerendasy and Ito (4) from strains grown to an A_{600} of approximately 0.3. For Northern (RNA) blot analysis, RNA samples were electrophoresed on 1% agarose formaldehyde gels (17) and vacuum blotted to nitrocellulose filters. A portion of the filter containing RNA molecular weight markers (0.24 to 9.5 kb) (Bethesda Research Laboratories) was cut out and stained with 0.04% methylene blue in 0.05 M sodium acetate. The remainder of the filter was probed with a single-stranded probe generated by polymerase chain reaction (22). pJG68.181 (γδ insertion 181 in pJG68; Fig. 1) was digested with *Sal*I and subjected to 50 cycles of polymerase chain reaction in the presence of the δ end γδ primer (13) and [α-^{32}P]CTP. This produces a labelled single-stranded DNA extending from the δ end of Tn*1000* to the *Sal*I site located within the *dcp* open reading frame near the N terminus.

For primer extension analysis (17), the primer PE (5'-GGCAACATACTCTGGTCTAA-3', corresponding to bases 445 to 464 in Fig. 2) and the γ end primer (90 to 180 ng) were 5' end labelled with T4 polynucleotide kinase and [γ-^{32}P] ATP. The labelled primers were separated from unincorporated ATP by electrophoresis on an 8% acrylamide gel and elution of the appropriate band by overnight incubation at 42°C in a solution containing 250 mM sodium acetate–20 mM Tris base (pH 7.5), 1 mM EDTA, and 0.25% sodium dodecyl sulfate (SDS), followed by phenol-chloroform extraction. The primer was then ethanol precipitated with yeast tRNA as carrier and resuspended in water.

For primer extensions, 45 μg of RNA and 1 μl of the appropriate labelled primer (2.5×10^5 cpm) were combined in 250 mM KCl–10 mM Tris (pH 8.3) in a total volume of 10 μl, and the solution was incubated at 95°C for 3 min. Samples were then annealed at 60°C for 10 min and chilled on ice, and 8 μl of 2.5× deoxynucleoside triphosphate mix (1 mM each dATP, dCTP, dGTP, and dTTP; 40 mM $MgCl_2$; 20 mM dithiothreitol; 60 mM Tris [pH 8.3]; 125 μg of actinomycin D per ml) was added. Avian myeloblastosis virus reverse transcriptase (1 μl, 10 U/μl; Life Sciences) was added, and the samples were incubated at 55°C for 1 h.

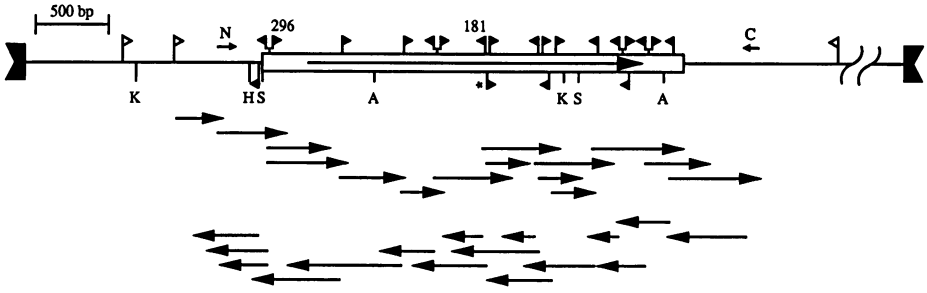

FIG. 1. γδ and Mu*dJ* insertions into pJG68 and sequencing strategy. The thin line represents the chromosomal DNA cloned into pJG68. The box represents the *dcp* open reading frame, with the inset arrow indicating the direction of transcription. The symbols above the line show the positions of γδ insertions, with the arrows pointing from γ to δ showing the orientation. The positions of insertions 181 and 296, discussed in the text, are indicated. Closed symbols indicate that the insertion destroys the ability of pJG68 to complement a *dcp* mutation, while insertions indicated by open symbols do not affect complementation. Symbols below the line show the positions of Mu*dJ* insertions, with arrows pointing toward the attL end of the element and an asterisk indicating the insertion (*dcp-104*::Mu*dJ*) in the expressing orientation. Restriction sites used in the initial mapping of the insertions are indicated (A, *Ava*I; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I). N and C indicate the positions of two primers used in sequencing. The arrows at the bottom of the figure indicate the extents of the sequences obtained from the various priming sites.

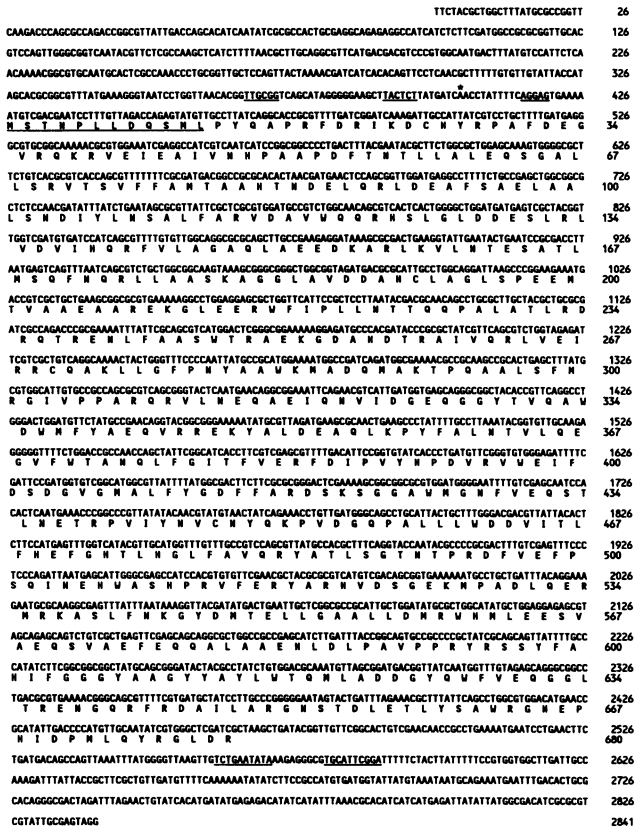

FIG. 2. Nucleotide sequence of the *dcp* region and amino acid sequence of Dcp. The proposed −35 (368 to 373) and −10 (392 to 397) positions and ribosome binding site (415 to 419) are underlined, as is the determined N-terminal amino acid sequence. Asterisk, the transcription start indicated by primer extension. An inverted repeat (underlined at 2562 to 2571 and 2581 to 2590) followed by a run of Ts is proposed as a transcription terminator.

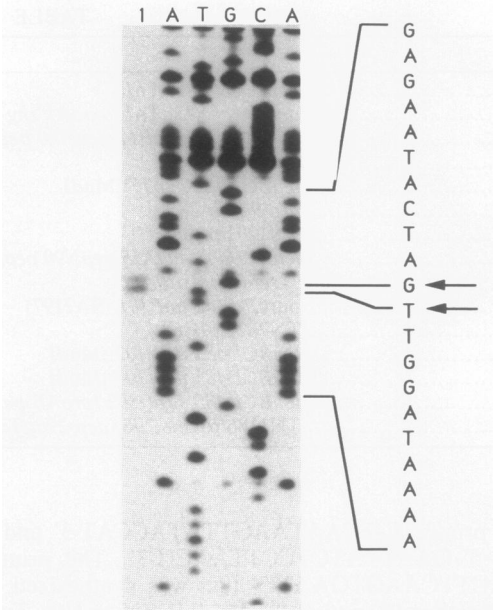

FIG. 3. Primer extension analysis. RNA from strain TN2488 was primer extended with reverse transcriptase and a 5′-end-labelled primer PE (lane 1). The same primer was used to generate the sequencing ladder with pJG68 DNA as template. Arrows indicate the sizes of the two primer extension products. The product corresponding to the indicated G (C in mRNA) is believed to be an artifact. The sequence indicated to the right of the autoradiogram is that of the noncoding strand. The sequencing ladder corresponds to the coding strand.

Template RNA was then hydrolyzed by adding 1/10 volume of 3 N NaOH and incubating for 1 h at 65°C. After neutralizing with a 1/10 volume of 3 N HCl, the samples were phenol-$CHCl_3$ extracted and precipitated with 20 μg of yeast tRNA as carrier. The end-labelled primer PE and γ end primer were used in sequencing reactions (as described above) with plasmid templates from TN2488 and TN3518, respectively, to generate a ladder for comparison with the primer extension products.

Dcp purification and N-terminal amino acid sequencing. Strain TN2676(pJG68) was grown to stationary phase in LB medium. Cells (34 g [wet weight]) were collected by centrifugation, resuspended in 30 ml of 0.05 M *N*-methyldiethanolamine buffer (pH 8.1) (DEA), and disrupted by sonication. Debris was removed by centrifugation (1 h at 26,000 × *g*), and the extract was further clarified by centrifugation (2 h at 200,000 × *g*). The supernatant (24 ml) was loaded on a Q-Sepharose (Pharmacia) column (100 ml), which was washed with 300 ml of DEA and 350 ml of DEA plus 0.2 M NaCl, and eluted with a linear gradient (2-liter total volume, 0.22 to 0.42 M NaCl in DEA). Active fractions were identified by the microtiter well assay described below, pooled (140 ml), and concentrated (to 2.3 ml) with a Centriprep 10 concentrator (Amicon). The concentrated material was applied to a gel filtration column (500 ml) (Ultrogel AcA34) which was eluted with DEA. The activity eluted at a position corresponding to a molecular mass of approximately 80 kDa, and the active fractions (25 ml) were pooled and concentrated as described above (to 2.7 ml). This material (1 ml) was further purified by chromatography on MonoQ (Pharmacia) and Superose 12 (Pharmacia). Approximately 360 μg of protein was obtained, and this material showed a single Coomassie blue-stained band at approximately 80 kDa after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined with the bicinchoninic acid protein assay reagent according to the directions of the manufacturer (Pierce). Purified Dcp (30 μg) was subjected to N-terminal sequence analysis (14) with an Applied Biosystems 470A microsequencer.

Enzyme assay. Dcp activity was detected in some cases by using $AcAla_3$ as substrate and high-performance liquid chromatography (HPLC) after derivatization with trinitrobenzene sulfonate for product analysis (23; modifications as described in Table 2). For purification of Dcp, a rapid, semiquantitative assay with Met-Gly-Met-Met as substrate and L-amino acid oxidase–peroxidase–*o*-dianisidine (12) to detect free amino acids was used. Dcp hydrolyzes this substrate to Met-Gly and Met-Met. To produce free amino acids from these dipeptides, the dipeptidase PepD (15) (from a crude extract of TN3412 which lacks all relevant peptidases as a result of chromosomal mutations and overproduces PepD from a *pepD* plasmid) was included in the reaction mixture. The assay was carried out by placing 5 to 10 μl of column fraction into a well of a plastic depression plate which contained 15 μl of assay mix (0.1 μmol of Met-Gly-Met-Met, 2.5 nmol of $CoCl_2$, and 93 ng of protein from a crude extract of TN3412 in DEA). After 20 min at 37°C, 25 μl of amino acid oxidase reagent (0.68 mg of

TABLE 2. Enzyme levels

Strain	Relevant genotype	Plasmid	Sp act (μmol/min/mg)[a]
TN1379	*dcp*$^+$	None	0.09
TN1201	*dcp*	None	0.004
TN2488	*dcp*	pJG68	4.9
TN2489	*dcp*	pJG69	6.1

[a] The assay mix contained 0.033 μmol of $AcAla_3$, 0.003 μmol of $CoCl_2$, and crude extract (0.1 to 50 μg of protein) in a 30-μl total volume of barbital buffer (0.05 M, pH 8.1). Bovine serum albumin was present in assays containing <5 μg of extract protein. Since these extracts contained active dipeptidases, the primary Dcp product (Ala_2) is partially hydrolyzed to free Ala, and this was taken into account in calculating the hydrolysis rate.

L-amino acid oxidase, 0.68 mg of *o*-dianisidine, and 1.36 mg of horseradish peroxidase [all Sigma] per ml of water) was added. After 10 min at room temperature, activity was noted by the presence of reddish brown color in the wells.

Nucleotide sequence accession number. The GenBank accession number for the *dcp* sequence is M84575.

RESULTS AND DISCUSSION

Properties of *dcp* plasmids. pJG68 and pJG69 were isolated by their ability to restore growth on $AcAla_3$ to a *dcp* mutant. Extracts of strains carrying these plasmids were assayed for hydrolysis of $AcAla_3$ (Table 2). The level of activity in these strains is approximately 50-fold higher than in the wild-type strain. This level of expression allows the Dcp protein to be visualized in a Coomassie blue-stained SDS-PAGE gel of a crude extract. With an extract from a strain carrying the plasmid and lacking dipeptidase activity (TN2676), the only product produced was identified by HPLC as Ala-Ala (data not shown). Plasmid pJG68 was transduced into a *polA* strain (TN2373) to force its insertion into the chromosome by homologous recombination (6). The inserted plasmid was shown to be cotransducible with transposons (*zcf-3314*::Tn*10*Δ*16*Δ*17* and *zcf-845*::Tn*10*) known to be cotransducible with the *dcp* locus (data not shown). (Previous results had indicated that *dcp* is located at approximately 25 map units on the *Salmonella* chromosome [24].) Our results indicate that pJG68 and pJG69 encode Dcp and that they carry the segment of the chromosome previously shown to contain the *dcp* gene. Since strains carrying pJG68 and pJG69 grow normally, there appears to be no deleterious effect of overproduction of Dcp.

Restriction mapping of the two plasmids showed that pJG68 carried about 5.2 kb of chromosomal DNA. Plasmid pJG69 contained a slightly larger, 5.9-kb insert. All restriction sites found in pJG68 (Fig. 1) were also present in pJG69. Tn*1000* insertions into pJG68 were isolated, and the resulting plasmids were tested for loss of *dcp* complementation ($AcAla_3$ utilization). The localization of these insertions (Fig. 1) defines the location of *dcp* in pJG68, and this region was sequenced with primers that hybridize to the ends of Tn*1000* (13).

Nucleotide sequence of *dcp*. The nucleotide sequence of a 2,836-bp segment of pJG68 that includes *dcp* is shown in Fig. 2. This sequence contains an open reading frame coding for a 77,269-kDa protein. The N-terminal amino acid sequence of this open reading frame agrees with that determined for Dcp (Materials and Methods). The major sequence obtained was MSTNPLLDQSML. A slightly weaker signal, reading STNPLLDQSMLP, was observed. It appears that the purified material contains both Dcp which has been processed to remove N-terminal Met (approximately 60%) and material which has not undergone this processing. A reasonable Shine-Dalgarno sequence (19) is located 6 bp 5′ to the first AUG of the open reading frame. Possible −10 and −35 sequences for a σ^{70} promoter are within 50 bp upstream of the start of translation (Fig. 2). An inverted repeat followed by a T-rich region suggestive of a rho-independent transcription terminator begins at nucleotide 2562, downstream from the end of the open reading frame.

Four insertions of Mu*dJ* into the *dcp* gene were isolated. Only one of these, *dcp-104*::Mu*dJ*, was inserted in the orientation that allows transcription of the *lac* operon from the *dcp* promoter. A strain (TN3311) carrying this insertion in the chromosome was weakly Lac$^+$ on MacConkey agar and expressed β-galactosidase at a level of approximately 10 Miller units. In preliminary experiments, this level was not significantly altered by growth under anaerobic conditions, by growth with a poor nitrogen (10 mM glutamate) or carbon (0.4% succinate) source, or by growth after a shift to 42°C.

Analysis of the *dcp* mRNA. The transcriptional start site of *dcp* was determined by primer extension with two different primers. One primer (primer PE) was complementary to a sequence near the N-terminal end of the *dcp* coding region. This primer was extended with mRNA prepared from TN2488(pJG68). The major extension product terminated at an A residue (base 405; Fig. 2) 22 bp upstream from the proposed start of translation. A somewhat weaker extension product contained one additional base, terminating at C-404. We believe it is likely that this second product is artifactual, since most mRNAs start with purines and reverse transcriptase is known to sometimes add a single additional base beyond the end of the template. A second primer complementary to a sequence in the γ end of γδ was extended with mRNA from strain TN3518. This strain carries plasmid pJG68.296 (carrying γδ insertion 296). This γδ-296 is inserted between nucleotides 457 and 458, 30 nucleotides downstream from the start of the *dcp* coding region (Fig. 2). The extension product from this primer terminated at the same residue as primer PE (data not shown). These results suggest that the −35 and −10 regions identified from inspection of the sequence are indeed used in transcription of *dcp*. No evidence of additional transcripts was obtained.

Northern blot analysis of mRNA from strains TN2488 (containing pJG68), TN3309 (*dcp-102*::Mu*dJ*), and TN1379 (*dcp*$^+$) indicated that the *dcp* mRNA was approximately 2,050 bases in length (data not shown). Transcripts of identical size were observed for RNA prepared from the plasmid-containing strain and the strain carrying a single copy of the chromosomal *dcp* gene. No transcript was observed in the strain carrying a plasmid with an insertion in *dcp*. A transcript initiating at the A-405 as indicated by primer extension and terminating at the inverted repeat downstream from the end of the open reading frame would be approximately 2,200 bases long. The *dcp* gene does not seem to be cotranscribed with any other genes. The failure to observe significant levels of longer transcripts in the RNA prepared from the plasmid-containing strain suggests that the increase in levels of *dcp* expression in such strains is not the result of transcription from vector promoters.

Sequence similarities between Dcp and another *Salmonella* peptidase. Although *E. coli* Dcp and ACE, a mammalian Dcp, have similarities in substrate specificity and in sensitivity to captopril (3, 20, 26, 27), no significant similarities between the amino acid sequences of *S. typhimurium* Dcp and ACE (21), aside from a thermolysin type Zn^{2+} binding motif, were uncovered (9). ACE shows significant internal

amino acid sequence similarity and is thought to have arisen by duplication of an earlier sequence (21). Comparison of the Dcp sequence with those of the N- and C-terminal halves of ACE again uncovered no significant similarity.

Comparisons with other *E. coli* and *S. typhimurium* peptidase amino acid sequences revealed that the sequence deduced for Dcp is strikingly similar to that of oligopeptidase A (23) encoded by the *opdA* (formerly *optA*) gene (1a). Both sequences are 679 amino acids long with 33% identical residues along their entire lengths. Both sequences contain a thermolysin type Zn^{2+} binding site motif (near amino acid 469) (9), and the sequences in this region are very similar (24 of 35 identities). The C-terminal regions of the two proteins are also quite similar (42 of 80 identities). It is interesting, in light of suggestions that the enterobacteriaceal chromosome arose by successive duplication of an earlier, smaller genome (16), that the *S. typhimurium dcp* and *opdA* genes are located approximately 180° from each other on the circular map. The amino acid sequences of both Dcp and OpdA also show significant similarity to a mammalian metalloendoprotease. A more detailed comparison of these sequences is presented in the accompanying communication (1a).

ACKNOWLEDGMENTS

This work was supported by grant AI10333 from the National Institute for Allergy and Infectious Diseases.

We gratefully acknowledge the contributions of Lazarus Astrachan, Chris Conlin, Jane Glazebrook, Judy Miller, and Todd Golde to this paper.

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