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Cloning and Nucleotide Sequence of *opdA*, the Gene Encoding Oligopeptidase A in *Salmonella typhimurium*

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The *opdA* gene (formerly called *optA*) of *Salmonella typhimurium* encodes a metallopeptidase, oligopeptidase A (OpdA), first recognized by its ability to cleave and allow utilization of *N*-acetyl-L-Ala_4 (E. R. Vimr, L. Green, and C. G. Miller, J. Bacteriol. 153:1259–1265, 1983). Derivatives of pBR328 carrying the *opdA* gene were isolated and shown to express oligopeptidase activity at levels approximately 100-fold higher than that of the wild type. These plasmids complemented all of the phenotypes associated with *opdA* mutations (failure to use *N*-acetyl-L-Ala_4, defective phage P22 development, and diminished endopeptidase activity). The *opdA* region of one of these plasmids (pCM127) was defined by insertions of Tn*1000* (γδ), and these insertions were used as priming sites to determine the nucleotide sequence of a 2,843-bp segment of the insert DNA. This region contained an open reading frame coding for a 680-amino-acid protein, the N terminus of which agreed with that determined for purified OpdA. This open reading frame contained both a sequence motif typical of Zn^{2+} metalloproteases and a putative σ^{32} promoter. However, no induction was detected upon temperature shift by using a β-galactosidase operon fusion. The predicted OpdA sequence showed similarity to dipeptidyl carboxypeptidase, the product of the *S. typhimurium* gene *dcp*, and to rat metallopeptidase EC 3.4.24.15., which is involved in peptide hormone processing.

Oligopeptidase A (OpdA) was originally identified by Vimr and Miller (52, 53) as one of two activities in extracts of *Salmonella typhimurium* which hydrolyzed *N*-acetyl-L-Ala_4 ($AcAla_4$). Mutations in the gene encoding OpdA (*opdA* [formerly *optA*]) were isolated from a dipeptidyl carboxypeptidase (Dcp)-deficient strain (carrying mutant allele *dcp-1*) by screening for mutants unable to use $AcAla_4$ as a nitrogen source. (Dcp will also hydrolyze $AcAla_4$ and allow it to be used as a sole nitrogen source [53].) When first isolated, mutations in the gene encoding OpdA were called *optA*. It was later learned that a different locus in *Escherichia coli* already had that mnemonic. To avoid confusion, we propose changing the name of the *S. typhimurium optA* locus to *opdA*. These *opdA* mutations were shown to map between 75 and 78 min on the *S. typhimurium* chromosome and to have only a small effect on bulk protein degradation and no effect on cell growth (52). Surprisingly, strains with *opdA* mutations were unable to support normal development of phage P22. P22 formed tiny plaques on lawns of *opdA* strains. This phenomenon was correlated with a 10-fold reduction in burst size in these hosts (6, 51).

Recent work by Novak and Dev (35) and Novak et al. (36) showed that OpdA from *E. coli* is an endoprotease and that it is the major soluble activity in *E. coli* able to hydrolyze the lipoprotein signal peptide in vitro. They suggested that OpdA may play a specific role in the degradation of signal peptides after they are released from precursor forms of secreted proteins.

Because OpdA appears to be an endoprotease which plays specific roles in both signal peptide degradation and P22 development, we have begun to characterize the *opdA* locus and its product in more detail. We report here the cloning and nucleotide sequence of the *Salmonella opdA* gene, a more precise localization of the gene's position on the *S. typhimurium* chromosome, and the purification and N-terminal amino acid sequence of OpdA.

MATERIALS AND METHODS

Bacteria, phage, plasmids, and media. Strains of *S. typhimurium* were derived from LT2 and are listed in Table 1. *E. coli* K-12 strain JM109 (58) was used to propagate plasmids for purification of plasmid DNA. *E. coli* K-12 CSR603, obtained from Robert Hogg, was used to produce maxicells. Phages P22(HT12/4 *int-3*) and P1(*clr100*) were used as generalized transducing phages. P22(H5), a clear plaque mutant, was used to characterize plaque size. All plasmids were derivatives of pBR328 or pBR322 and are listed in Table 1. Bacteria were routinely grown at 37°C in LB (27). E medium (54) was used as the minimal medium and supplemented with appropriate amino acids at 0.4 mM and glucose at 4 g/liter. The medium described by Gutnick et al. (11), here referred to as NN, was used to test for use of peptides as sole nitrogen sources as described previously (53). Antibiotics were added to rich media at the following concentrations in micrograms per milliliter: tetracycline, 25; ampicillin, 50 for solid media, 100 for liquid media; kanamycin, 50; and chloramphenicol, 20.

Construction and screening of *S. typhimurium* plasmid libraries. Plasmid libraries containing 8- to 12-kb fragments of *S. typhimurium* chromosomal DNA in pBR328 were made as previously described (17). Plasmids carrying *opdA* were obtained from the following three different screens. (i) Plasmids which allowed strain TN1201 (*opdA1 dcp-1*) to use $AcAla_4$ (1.5 mM) as a nitrogen source on NN glucose agar were selected. Increased hydrolysis of $AcAla_4$ was confirmed by high-performance liquid chromatography analysis

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description
S. typhimurium strains	
TN1201	*dcp-1 zcf-845*::Tn*10 zhg-848*::Tn*5 opdA1*
TN1246	*leuBCD485 pepN90 pepA16 pepB11 supQ302* (Δ*proAB pepD*) *pepP1 pepQ1*
TN1379	*leuBCD485*
TN2183	*leuBCD485 metA15 pepN90 pepA16 pepB11 pepP1 pepQ1 pepT7*::Mud1X
TN2373 (TR6612)	*ara-9 polA2*
TN2540 (DB4926)	*metE551 metA22 ilv-452 trpB2 hisC527 galE496 xyl-404 rpsL120 flaA66 hsdL6 hsdSA29 H1-b nml H2-e,n,x* (Fels2$^-$)
TN3097	as TN1246 with pCM144
TN3101	*leuBCD485 opdA10*::MudJ
TN3192	(Fels2$^-$) *leuA414* r$^-$ m$^+$ *opdA10*::MudP
TN3193	(Fels2$^-$) *leuA414* r$^-$ m$^+$ *opdA10*::MudQ
TN3220	(Fels2$^-$) *leuA414* r$^-$ m$^+$ *opdA10*::MudP *zhg-3073*::Tn*10* dTc
TN3221	(Fels2$^-$) *leuA414* r$^-$ m$^+$ *opdA10*::MudQ *zhg-3073*::Tn*10* dTc
TN3227	*leuBCD485 zhg-1635*::Tn*10* dCm
TN3262 (JP120)	*asd-1*
TN3263 (JP104)	*glpD105*
TN3737	As TN2540 with *opdA10*::Mud*J*
TN3945	*xylB26*
TN3946	*his-644*
AK3073	As TN2540 with *zhg-3073*::Tn*10* dTc
AK3082	As TN2540 with *zhg-3082*::Tn*10* dTc
AK3108	As TN2540 with *zhg-3108*::Tn*10* dTc
AK3216	As TN2540 with *zhg-3216*::Tn*10* dTc
AK3223	As TN2540 with *zhg-3223*::Tn*10* dTc
Plasmids	
pJG13	7-kb *Sau*3A fragment of TN1246 containing *opdA* in *Bam*HI site of pBR328
pJG70	6.9-kb *Sau*3A fragment of TN1246 containing *opdA* in *Bam*HI site of pBR328
pJG73	7.3-kb *Sau*3A fragment of TN2294 containing *opdA* in *Bam*HI site of pBR328
pCM127	8.3-kb *Sau*3A fragment of TN1246 containing *opdA* in *Bam*HI site of pBR328
pCM128	7.1-kb *Sau*3A fragment of TN1246 containing *opdA* in *Bam*HI site of pBR328
pCM144	As pCM127 but containing the *opdA10*::Mud*J* allele

of products produced when cell extracts were incubated with $AcAla_4$ for 30 min at 37°C (32, 52). (ii) Plasmids which allowed the multiply peptidase-deficient strain TN2183 (*pepN pepA pepB pepD pepP pepQ pepT dcp opdA*) to use Met-Gly-Gly as a methionine source were selected (32). (iii) A library of plasmids containing DNA from strain TN1246 (*pepN pepA pepB pepD pepP pepQ*) was screened for plasmids which conferred elevated levels of benzyloxycarbonyl-Ala-Ala-Leu *p*-nitroanilide (Z-AAL-pNA) hydrolysis on strain TN1246. OpdA cleaves Z-AAL-pNA between the two Ala residues and does not release the chromophore, *p*-nitroaniline. Since the product of OpdA cleavage contains a free amino group, it can be hydrolyzed by the aminopeptidase peptidase N to yield free chromophore. This coupled assay was used to detect clones that overproduced OpdA by screening microcultures grown in the wells of plastic depression plates basically as described by Carter and Miller (2). Plasmid-containing strains were transferred from agar plates to wells of plastic depression plates each containing 0.2 ml of LB-chloramphenicol. Cells were grown overnight at 37°C, and replica cultures for storage were inoculated onto LB-chloramphenicol plates. Cells in the depression plate were pelleted by centrifugation and washed with 0.2 ml of 50 mM Tris-HCl (pH 7.6). The pellet was resuspended in 0.03 ml of lysis solution (1 mg of lysozyme per ml in 50 mM Tris-HCl [pH 7.6]) and subjected to three cycles of freezing (−70°C) and thawing (37°C). To each well was added 0.15 ml of a solution containing 50 mM Tris-HCl (pH 7.6) and 0.5 mM cobalt chloride. Then 0.017 ml of Z-AAL-pNA (0.5 mg/ml in dimethyl formamide) was added, and the plates were incubated at 37°C for 1 h. To each well was added 0.005 ml of partially purified *S. typhimurium* aminopeptidase N (4.5 mg of protein per ml, 20 absorbance U/min/mg). Aminopeptidase N activity was determined by Ala-β-naphthylamide hydrolysis assayed spectrophotometrically as described by Lee et al. (24). The plates were incubated at 37°C for 10 min. To enhance detection of free *p*-nitroaniline, the reaction product was diazotized and coupled to *N*-(1-naphthyl)-ethylenediamine to produce an intense purple color (37). Positive isolates were purified from the master plate. To confirm increased Z-AAL-pNA-hydrolyzing activity, extracts of these isolates were assayed spectrophotometrically. Cell extracts were added to a reaction mixture containing 0.07 mM Z-AAL-pNA, 44 mM *N*-methyl-diethanolamine-HCl (pH 8.0), 0.44 mM $CoCl_2$, and aminopeptidase N (0.8 absorbance U/min/ml). The reaction mixtures were incubated at room temperature, and release of *p*-nitroaniline was monitored at 410 nm. Extracts were diluted so that the assay was linear with added protein.

Temperature shift induction of *opdA10*::MudJ. An overnight culture of TN3101 grown in LB medium at 30°C was diluted 1:100 into fresh LB medium or E medium with 0.1% Casamino Acids and grown at 30°C until it reached an optical density at 600 nm of 0.3. Half of the culture was then transferred to a 42°C water bath, and half was maintained at 30°C. Samples were taken every 15 min for 90 min and assayed for β-galactosidase activity as described by Miller (33).

Recombinant DNA techniques. Restriction enzymes were obtained from New England Biolabs and Bethesda Research Laboratories and used according to the manufacturers' instructions. T4 DNA ligase was from Bethesda Research Laboratories. Fragments for subcloning were isolated by gel electrophoresis in low-melting-temperature agarose (GTG SeaPlaque agarose; FMC Bioproducts) and ligated in agarose according to the manufacturer's protocol. Other DNA manipulations were done by standard techniques (27).

Genetic techniques. Transductions with phage P22 (44) or phage P1 (15) were carried out as described elsewhere. Mu*dJ* (Mu*dI*1734 [Kan^r] *lac* operon fusion [3]) insertions into plasmid pCM127 were obtained by the procedure of Hmiel et al. (16). Tn*1000* (γδ) insertions in pCM127 were produced by the procedure of Guyer (12). Insertion *opdA10*::Mu*dJ*(Kan^r), originally present on pCM144, was returned to the chromosome by transducing TN1379 to kanamycin resistance on MacConkey lactose agar with a P22 lysate grown on TN3097 (which contains pCM144). When carried on the plasmid, the *opdA10*::Mu*dJ* insertion produced enough β-galactosidase to make the colony red on MacConkey lactose agar. A strain carrying *opdA10*::Mu*dJ* in single copy in the chromosome, however, is white on MacConkey agar. To detect chromosomal replacement, white kanamycin-resistant transductants

on MacConkey kanamycin agar were picked and checked for loss of plasmid-encoded antibiotic resistance markers (ampicillin and chloramphenicol resistance). Replacement of the chromosomal *opdA* gene by the *opdA*::Mu*dJ* insertion was confirmed by Southern hybridization (49). Integration of entire plasmids into the chromosome of a *polA* strain (57) was carried out as described previously (16). The map position of the integration was determined by P22 transduction with linked transposon *zhg-860*::Tn*5* (52). Tn*10* dTc (Tn*10*Δ16Δ17, tetracycline-resistant, transposition defective Tn*10* [56]) insertions near *opdA10*::Mu*dJ* were identified in the set of insertions constructed by Kukral et al. (21) by screening for Tn*10* dTc insertions which simultaneously conferred tetracycline resistance and caused loss of β-galactosidase activity on LB–tetracycline–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) agar plates. Tn*10* dTc insertions near *asd* were identified in the same collection by screening for insertions which simultaneously conferred tetracycline resistance and ability to grow without added diaminopimelic acid on strain TN3262 (*asd*). To isolate a Tn*10* dCm (chloramphenicol-resistant, transposition defective Tn*10* [9]) insertion near *opdA*, a population of random Tn*10* dCm insertion derivatives of TN1201 (Tn*5* near *opdA1*) was prepared (9). A P22 transducing lysate of this population was used to transduce TN1379 to both chloramphenicol and kanamycin resistance simultaneously. MudP22 mapping was performed as described by Youderian et al. (63). Maxicell analysis of plasmid-encoded proteins was performed essentially as described by Sancar et al. (45) with the modifications of Snavely et al. (48).

DNA sequencing. All nucleotide sequence was obtained by using the dideoxy method (47), alkaline denatured double-stranded templates (4), and Sequenase (United States Biochemicals) according to the manufacturer's instructions. Primers for sequencing from the ends of the Tn*1000* insertions were those described by Liu et al. (26), primers for pBR322 sequencing were from New England Biolabs, and other oligonucleotide primers were synthesized on an Applied Biosystems nucleic acid synthesizer. Sequences were compared by using the University of Wisconsin Genetics Computing Group package of programs (8).

Purification of OpdA and N-terminal amino acid sequencing. *S. typhimurium* OpdA was purified from a strain containing pCM128. OpdA from *E. coli* has been previously purified from a single chromosomal copy by a different procedure (36). Ten liters of cells grown overnight at 37°C in LB-chloramphenicol was harvested by centrifugation and lysed by sonication, and the extract was cleared by centrifugation at 200,000 × *g* for 2 h. The cleared extract was applied to a DE52 anion exchange column (Whatman) in 10 mM Tris-HCl (pH 7.9)–5 mM $MgCl_2$–0.1 M NaCl and eluted with a NaCl gradient (0.1 to 0.35 M). Active fractions detected by using the Z-AAL-pNA microassay were pooled and concentrated by ultrafiltration with an Amicon YM-30 membrane. Concentrated fractions were applied to an Ultrogel AcA34 gel filtration column. Active fractions were again pooled and concentrated with an Amicon Centriprep-10. A chloramphenicol caproate-agarose affinity column (Sigma) was used to remove a major contaminant, chloramphenicol-acetyltransferase encoded by the vector pBR328 (64). OpdA was adsorbed in 10 mM Tris-HCl (pH 7.6) and eluted with 0.2 M NaCl. The peak fractions were concentrated, applied to a MonoQ column (Pharmacia), and eluted with a NaCl gradient. Peak fractions were again concentrated, reapplied to the MonoQ column, and eluted with a NaCl gradient. The most active fractions were applied to an FPLC Sepharose 12 column (Pharmacia). The purity of the final product as estimated by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) was greater than 95%. Final purification, based on increase in specific activity for $AcAla_4$ hydrolysis, was about 3,000-fold, compared with levels from extracts of strains containing a single chromosomal copy of *opdA*. The amino acid sequence of the N-terminal region was determined with an Applied Biosystems Model 470A protein sequencer.

Nucleotide sequence accession number. The GenBank accession number for *opdA* is M84574.

RESULTS

Isolation of plasmids carrying the *opdA* gene. The gene for *opdA* was cloned by using three different selections. It was cloned directly from a pBR328 library of *Sau*3A partial-digest fragments from a multiply peptidase-deficient strain, TN1246, by screening for clones that allowed a *dcp opdA* strain to use $AcAla_4$ as a sole nitrogen source. Plasmid pJG70 was identified by this screen. It was integrated into the chromosome of a *polA* mutant, and the linkage of the plasmid-encoded chloramphenicol resistance to a transposon insertion (*zhg-860*::Tn*5*) near *opdA* was tested by P22 transduction. The cotransduction frequency of pJG70 and *zhg-860*::Tn*5* was 51%, suggesting that pJG70 carried insert DNA homologous to the *opdA* region. As part of another project, similar libraries were screened for plasmids which allowed a multiply peptidase-deficient strain (TN2183) to use Met-Gly-Gly as a methionine source. Plasmids pJG13 and pJG73 were identified by this screen. When integrated into the chromosome, plasmids pJG13 and pJG73 were 47 and 44% cotransducible with *zhg-860*::Tn*5*, respectively, suggesting that these plasmids also contained insert DNA with homology to the *opdA* region. In still another line of investigation, a similar library was screened for plasmids that led to overproduction of an activity that hydrolyzed the chromogenic endoprotease substrate Z-AAL-pNA. The assay used in this screen detected activities that cleaved Z-AAL-pNA between Ala and Leu. Plasmids pCM127 and pCM128, obtained from this screen, also integrated into the chromosome by homology with the *opdA* region. The integrated plasmids showed 45 (pCM127) and 38% (pCM128) cotransduction with *zhg-860*::Tn*5*.

Although they were isolated by different screens, all of these plasmids (pJG13, pJG70, pJG73, pCM127, and pCM128) contained inserts from the same region of the chromosome and conferred the same set of phenotypes: $AcAla_4$ utilization, Met-Gly-Gly utilization, and elevated Z-AAL-pNA hydrolysis. Assays of $AcAla_4$-hydrolyzing activity in extracts of strains carrying these plasmids showed levels approximately 100-fold higher than those in extracts of strains containing a single chromosomal copy (specific activity, 0.05 μmol min^{-1} mg^{-1}). In addition, all of these plasmids conferred the ability to support normal growth of phage P22 on an *opdA* strain. Phage P22 forms tiny plaques on *opdA* hosts (51), whereas *opdA* hosts carrying these plasmids showed normal, large plaques. Restriction mapping showed that these plasmids shared a common 4-kb *Eco*RI-*Kpn*I fragment (Fig. 1). Additionally, a Mu*dJ* insertion in pCM127 (see below) simultaneously eliminated its ability to complement the $AcAla_4$ utilization defect, the Z-AAL-pNA hydrolysis defect, and the phage P22 small-plaque phenotype. These results argue that these plasmids carry the *opdA* gene and that this gene is responsible for the phenotypes conferred by these plasmids.

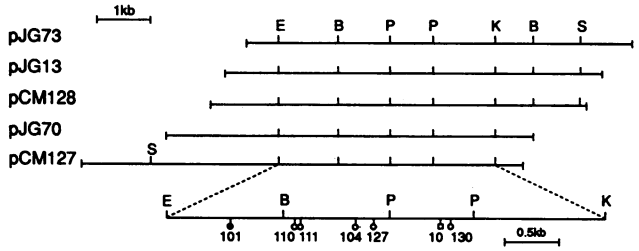

FIG. 1. Restriction endonuclease maps of *opdA*$^+$ plasmids and sites of insertions in pCM127. Horizontal lines represent chromosomal DNA carried on the plasmid. Restriction endonuclease sites: E, *Eco*RI; B, *Bgl*II; P, *Pvu*II; K, *Kpn*I; S, *Sal*I. The enlarged region of pCM127 shows the locations and allele numbers of insertions in or near *opdA*. Open symbols indicate insertions which reduced Z-AAL-pNA-hydrolyzing activity; insertion 101 (closed circle) did not. Tn*1000* insertions are represented by circles; the Mu*dJ* insertion is represented by a square.

Analysis of insertions in the cloned *opdA* gene. In order to define the location of the *opdA* gene in the cloned DNA, insertions of both the Mu*dJ* mini-Mu*d* element and Tn*1000* (γδ) were generated in pCM127. Strains containing plasmids that carry these insertions were screened for loss of their ability to rapidly hydrolyze the chromogenic substrate Z-AAL-pNA. Plasmids containing such insertions were restriction mapped. All insertions that led to loss of Z-AAL-pNA hydrolysis were localized to a 2-kb region of pCM127, a region shared by all of the plasmids (Fig. 1).

Maxicell analysis of pCM127 and pCM128 showed that both plasmids encoded a 68-kDa protein (Fig. 2B). In addition, this 68-kDa protein could be seen as a major Coomassie blue-stained protein after SDS-PAGE of crude soluble-protein extracts of strains carrying each of the *opdA* plasmids, and this protein was not present in an extract of a strain containing only the pBR328 vector (data not shown).

Maxicell analysis showed that the insertion *opdA10*::Mu*dJ* in plasmid pCM127 eliminated the 68-kDa band (Fig. 2B), as did insertions *opdA104*::Tn*1000* and *opdA130*::Tn*1000* (Fig. 2A). These insertions also eliminated Z-AAL-pNA-hydrolyzing activity. Insertion *opdA101*::Tn*1000*, however, did not affect either Z-AAL-pNA-hydrolyzing activity or the 68-kDa band. The presence of a new 23-kDa band in *opdA104*::Tn*1000* and a new 50-kDa band in *opdA130*::Tn*1000* indicated that *opdA* is transcribed in the direction from *opdA104*::Tn*1000* to *opdA130*::Tn*1000*. This is the same direction as *lac* transcription in *opdA10*::Mu*dJ*. This was later confirmed by sequencing from the end of *opdA10*::Mu*dJ*.

Mapping *opdA* on the *Salmonella* chromosome. The results of conjugation crosses had suggested that *opdA* is located between *asd* and *xylB* (52). To confirm this independently and to map the chromosomal location of *opdA* more accurately, we first returned insertion *opdA10*::Mu*dJ* to the chromosome by P22 transduction. We confirmed the replacement of the chromosomal locus by testing for the *opdA* phenotypes and by Southern hybridization (data not shown). The chromosomal *opdA10*::Mu*dJ* was exchanged with the MudP22 elements (MudP and MudQ) of Youderian et al. (63). These elements form locked-in P22 prophage which, when induced, cannot excise but still make phage particles and preferentially package chromosomal DNA from the pac site. MudP packages in the same direction as *lacZ* is transcribed in Mu*dJ*; MudQ packages in the opposite direction. MudP and MudQ transducing lysates were used in crosses

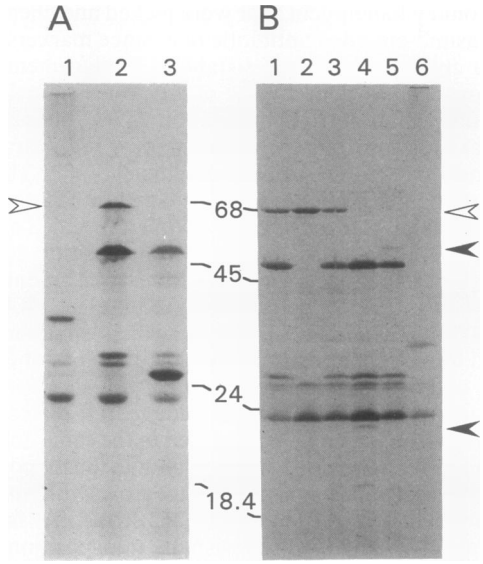

FIG. 2. Gene products expressed by *opdA* plasmids and *opdA* plasmids containing insertions. Gene products of plasmids in *E. coli* CSR603 were expressed in maxicells labelled with [^{35}S]methionine. Proteins were separated on SDS-10% polyacrylamide gels and visualized by autoradiography. Open arrowheads indicate the *opdA* gene product. Closed arrowheads indicate fragments generated by Tn*1000* insertions in pCM127. (A) Lanes: 1, pBR322; 2, pCM127; 3, pCM127 *opdA10*::Mu*dJ*. (B) Lanes: 1, pCM127; 2, pCM128; 3, pCM127 *opdA101*::Tn*1000*; 4, pCM127 *opdA104*::Tn*1000*; 5, pCM127 *opdA130*::Tn*1000*; 6, pBR328. Numbers between the panels indicate molecular mass standards in kilodaltons. Note that proteins of ~50 and 26 kDa are produced by pCM127 but not by pCM128. These proteins must be coded by the region of pCM127 to the left of *opdA* (Fig. 1) that is not carried by pCM128.

with strains carrying *asd* and *xylB* markers. The numbers of Asd$^+$ and Xyl$^+$ transductants were determined and normalized to the number of His$^+$ transductants (Table 2). Because MudP transduced *asd* much more frequently than *xylB* and because MudQ transduced *xylB* more frequently than *asd*, *opdA* must lie between *asd* and *xylB*. Additionally, because MudP packages in the same direction as *lac* is transcribed in Mu*dJ*, which is also the same direction as *opdA* transcription, *opdA* must be transcribed toward *asd*.

Although *opdA* is between *asd* and *xylB*, it is unlinked by P22 transduction to either of them. Eight Tn*10* dTc insertions linked to *opdA* by P22 transduction were isolated by screening a collection of random Tn*10* dTc insertions (21). A

TABLE 2. Relative efficiencies of transduction of *asd*$^+$ and *xylB*$^+$ by induced *opdA10*::Mu*dJ*P22 lysates

Selected marker	Efficiency of transduction with donor lysate[a]			
	TN3192(MudP)	TN3193(MudQ)	TN3220(MudP)	TN3221(MudQ)
asd$^+$	146.2 (4824)	3.7 (100)	382.8 (2565)	4.7 (200)
xylB$^+$	3.3 (110)	161.3 (4355)	17 (1140)	132.6 (5700)
his$^+$	1 (33)	1 (27)	1 (67)	1 (43)

[a] Values are efficiencies with which the selected marker was transduced by the MudP22 lysate relative to the efficiency with which *his*$^+$ was transduced. Numbers in parentheses are the numbers of transductants per microliter of lysate. TN3192 and TN3220 are independent replacements of *opdA10*::Mu*dJ* by MudP. TN3193 and TN3221 are independent replacements of *opdA10*::Mu*dJ* by MudQ.

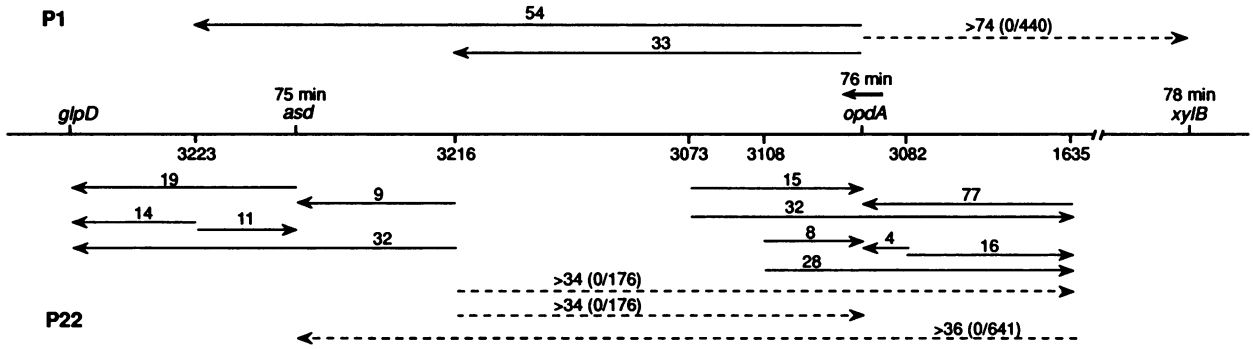

FIG. 3. Map position of *opdA* on the *S. typhimurium* chromosome. Linkage data above and below the line were obtained with phage P1 and with phage P22, respectively, as indicated. Arrows point from the selected marker to the unselected marker. Distances above the arrows are in kilobases calculated from cotransduction frequencies by using the formula in reference 46. Mapped genes with their positions in map units are indicated. The *opdA* allele used in all crosses was *opdA10*::Mu*dJ*. Transposon insertions are indicated by their allele numbers. Insertion 1635 is a Tn*10* dCm; all others are Tn*10* dTc. The arrow above *opdA* indicates direction of transcription of the gene.

Tn*10* dCm linked to *opdA* by P22 transduction was isolated from a random Tn*10* dCm population made on strain TN1201. None of these elements was linked to *asd* or to *xylB* by P22 transduction. Three Tn*10* dTc insertions that were P22 cotransducible with *asd* were isolated from a collection of random Tn*10* dTc insertions. None of these was cotransducible with *opdA* by using P22. However, two of these insertions, *zhf-3223*::Tn*10* dTc and *zhf-3216*::Tn*10* dTc, were cotransducible with *opdA10*::Mu*dJ* by using phage P1. P1 transducing particles carry approximately twice as much DNA as those formed by P22 and so are able to cotransduce markers too distant to be carried on a single P22 transducing fragment. These results clearly place *opdA* at 76 map units on the *Salmonella* chromosome (Fig. 3).

Purification and N-terminal sequence of OpdA. *S. typhimurium* OpdA was purified to near homogeneity from a strain containing plasmid pCM128. Though the yield was low (1.4%), an approximately 3,000-fold purification, compared with the level of activity in an extract of a strain containing a single chromosomal copy, was achieved (specific activities against $AcAla_4$ were 180 μmol min^{-1} mg^{-1} and 0.05 μmol min^{-1} mg^{-1}, respectively). The purest fraction obtained was subjected to N-terminal amino acid sequencing, and the first 18 amino acids were determined to be (Met)-Thr-Asn-Pro-Leu-Leu-Thr-Ser-Phe-(?)-Leu-Pro-Pro-Phe-Ser-Ala-Ile-Lys. Sequencing indicated that two species which differed only by the presence of an N-terminal methionine were present. Apparently, the level of overproduction exceeds the cell's capacity for N-terminal methionine removal. Purified OpdA was stimulated by 0.01 mM Co^{2+} and to a lesser extent by 0.1 mM Mn^{2+}. It was completely inhibited by 0.1 mM Zn^{2+}, 1 mM Ni^{2+}, 0.1 mM Cu^{2+}, and 1 mM EDTA. It hydrolyzed $AcAla_4$ and Z-AAL-pNA but not insulin A or B chain. Curiously, even though *opdA*-containing plasmids allowed a peptidase-deficient strain to use Met-Gly-Gly as a Met source, purified OpdA failed to hydrolyze this peptide. The rate of hydrolysis of Met-Gly-Gly was at least 10^5-fold less than that of $AcAla_4$.

Nucleotide sequence of the *opdA* gene. The nucleotide sequence for the gene *opdA* was determined by dideoxy sequencing techniques on double-stranded plasmid template DNA. Oligonucleotide primers were used to sequence from both ends of Tn*1000* insertions and from the L end of Mu*dJ*. To sequence a difficult region, a 700-bp *Pvu*II fragment from pCM127 was cloned into the *Bam*HI site of pBR328 and sequenced with primers homologous to the pBR328 sequence around the *Bam*HI site. Additionally, five primers which allowed the sequencing of regions which were inaccessible by using the Tn*1000* insertions were synthesized. Both strands were sequenced throughout, with the exception of 34 nucleotides on the 5′ end and 89 nucleotides on the 3′ end. The complete sequence of *opdA* is shown in Fig. 4.

The sequence contains a 680 amino acid open reading frame (ORF) starting at nucleotide 603 with an N-terminal sequence identical to that determined by directly sequencing OpdA and with codon usage typical of other *S. typhimurium* genes (55). This ORF is preceded by a possible ribosome binding site. The ORF predicts a 77-kDa protein with a pI of 5.0. The calculated pI is consistent with the following behavior of OpdA on anion exchange columns: elution from a DE52 column at pH 7.9 and 0.2 M NaCl and from a MonoQ column at pH 8.2 and 0.3 M NaCl. Both the purified protein and the protein observed in maxicells and crude extracts display a mobility on SDS-PAGE indicating a molecular weight of 68,000.

There is no strong consensus σ^{70} −10 or −35 region upstream of the start of translation, which is consistent with the low level of expression assayed from the chromosomal Mu*dJ* insertion (37 U of β-galactosidase). However, there is a near consensus σ^{32} heat shock promoter at nucleotides 504 to 540 (7). To determine whether this is an active heat shock promoter, β-galactosidase activity of a culture of TN3101 (*opdA10*::Mu*dJ*) was assayed at 15-min intervals after shifting the culture from 30 to 42°C. No increase in β-galactosidase levels was observed after the temperature shift (37 ± 5 U). Yano et al. (59) have used *lac* operon fusions to assay transcription from other σ^{32} promoters, *rpoD* and *groE*.

Examination of the sequence revealed a site [amino acids 467 to 475; (L)(F)HE(F)(G)H(G)(L)] with considerable similarity to a proposed metalloproteinase Zn^{2+} binding site motif: (uncharged)-(uncharged)-H-E-(uncharged)-(uncharged)-H-(uncharged)-(hydrophobic) (19). This is consistent with the observed inhibition of OpdA by EDTA.

The predicted amino acid sequence of OpdA shows considerable similarity to the predicted amino acid sequence of Dcp (13). Both ORFs are 680 amino acids long and display 33% amino acid identity over their entire lengths. In addition, there are two regions of >50% identity. One region is centered around the putative Zn^{2+} binding site and extends from residue 440 to 512. The other is between amino acids 590 and 677, the C-terminal eighth of the proteins.

A search of GenBank by the FASTA program (41) re-

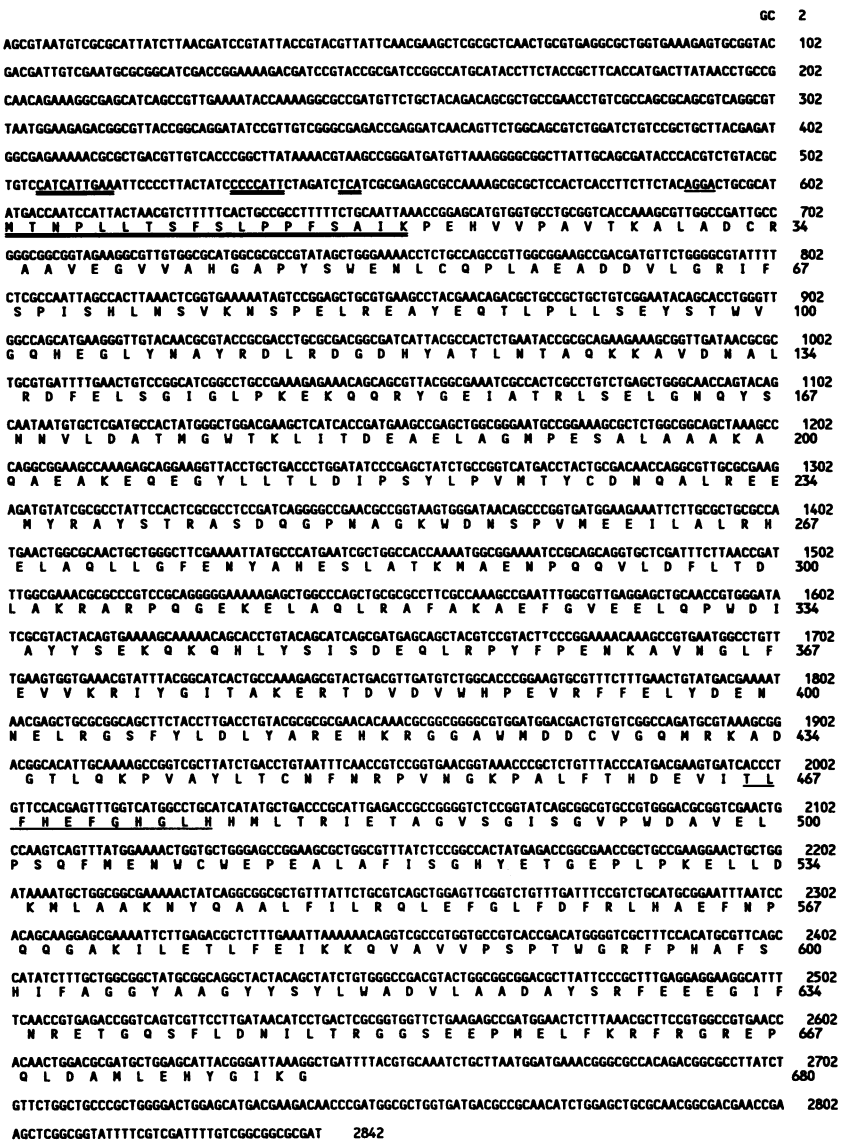

FIG. 4. Nucleotide sequence of the *opdA* region and predicted amino acid sequence of OpdA. Doubly underlined DNA sequences indicate potential σ^{32} promoter (7). Singly underlined DNA sequence indicates putative ribosome binding site. Singly underlined amino acid sequence indicates metalloprotease Zn^{2+} binding site motif (19). Doubly underlined amino acid sequence indicates sequence determined directly from purified OpdA protein.

vealed significant similarity of both OpdA and Dcp to rat metalloendopeptidase EC 3.4.24.15 (EP 24.15) (42). EP 24.15 is a zinc metalloendopeptidase found in brain, testes, and pituitary, and it appears to play a role in peptide hormone processing and degradation (23, 40). OpdA and EP 24.15 showed 28% amino acid identity in a 565-residue overlap, while Dcp and EP 24.15 displayed 24% amino acid identity in that region. Figure 5 shows the multiple alignments of OpdA, Dcp, and EP 24.15. The region around the putative zinc binding site is very similar in all three. Whereas ORFs for OpdA and Dcp are both 680 amino acids long, EP 24.15 is only 645 amino acids long and lacks the extended region of similarity shared by the C-terminal eighth of OpdA and Dcp. Except for the minimal Zn^{2+} binding site motif (HEXXH), no similarity to the other *E. coli* zinc metallopeptidase (aminopeptidase N) or to any other metalloprotease was found.

DISCUSSION

Clones carrying the *opdA* gene have arisen from three different screens. The first, intended for the isolation of *opdA*, involved complementing the $AcAla_4$ utilization defect of an *opdA* mutant strain. The second, intended to identify new endoprotease activities, involved screening libraries for plasmids conferring elevated Z-AAL-pNA-hydrolyzing activity. When this work was begun, studies with extracts of *opdA* and *opdA*$^+$ strains and purified protein had suggested that OpdA was a carboxypeptidase rather than an endopeptidase (36, 52). Thus, the chromogenic substrate Z-AAL-pNA, which is blocked at both the amino and carboxyl termini, was not expected to be an OpdA substrate. Subsequently, Novak and Dev (35) showed that purified OpdA from *E. coli* cleaves lipoprotein signal peptide in vitro at several internal sites, usually on one side or the other of an

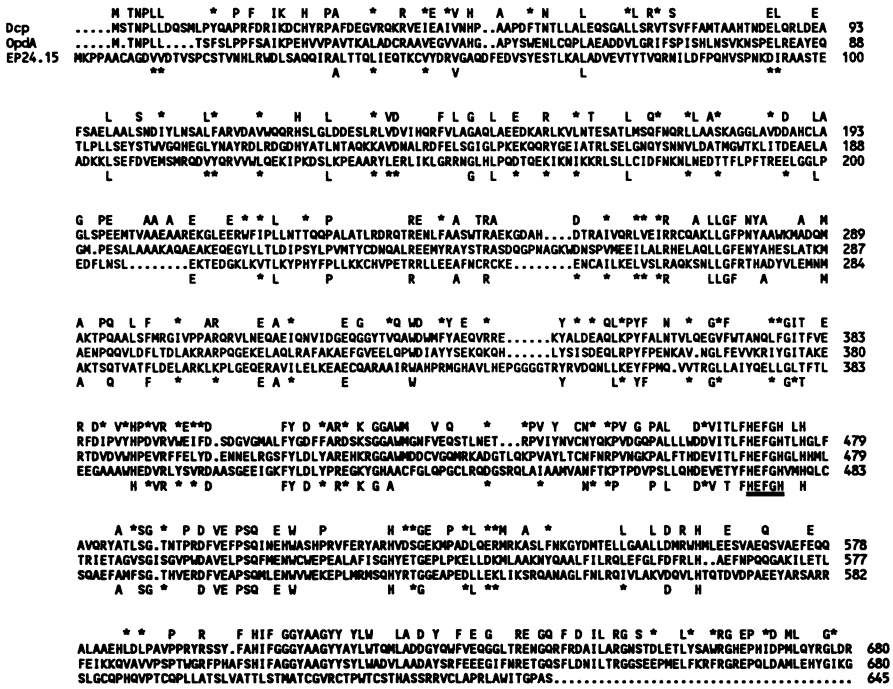

FIG. 5. Alignment of deduced amino acid sequences of OpdA, Dcp, and rat metalloendopeptidase EC 3.4.24.15 (EP 24.15). Alignment was performed with the Pileup program in the University of Wisconsin Genetics Computing Group package by using a comparison matrix based on PAM250, a gap penalty of 4, and a gap extension penalty of 0.1. Amino acid residues common to OpdA and Dcp are shown above the Dcp sequence. Residues common among all three are shown below the EP 24.15 sequence. Conservative amino acid replacements, represented by asterisks, are as follows: D or E; L or I or V; K or R; F or Y or W; S or T. The doubly underlined region beginning at OpdA residue 469 is the putative metalloprotease Zn^{2+} binding site motif. The Dcp sequence is from reference 13. The EP 24.15 sequence is from reference 42.

Ala or Gly residue, demonstrating that OpdA is an endoprotease. It is therefore not surprising that OpdA cleaves Z-AAL-pNA between Ala and Leu. The third screen was intended to identify clones carrying *pepM*, the gene encoding the activity responsible for N-terminal methionine removal (32). This screen also produced plasmids which were eventually shown to carry *opdA*. Since purified OpdA does not hydrolyze Met-Gly-Gly to free methionine, it is not clear how plasmids carrying *opdA* allow a peptidase-deficient strain to use this peptide as a source of methionine. One possibility is that overproduction of OpdA leads to the degradation of peptides that are inhibitors of the activity which does hydrolyze Met-Gly-Gly. Peptides are known to accumulate in peptidase-deficient strains (61, 62). Another possibility is that the in vitro conditions used to assay OpdA may not mimic those under which it acts in the cell. It has been shown that striking differences in the specificities of another bacterial peptidase can be generated by changing the metal ion present in the assay (14).

Phage P22 makes small plaques on an *opdA*-deficient host, a defect complemented by the *opdA*-containing plasmids. The small plaques are not the result of a general growth defect in the host, since an *opdA* strain and an $opdA^+$ strain have the same doubling time (24 min at 37°C) in LB medium. The plaque size appears to result from a 10- to 20-fold decrease in burst size when phage are grown on an *opdA* strain, compared with an $opdA^+$ strain (6, 51). Although phage P22 is similar to phage lambda in many respects, there are no known proteolytic steps in P22 development as there are in lambda development (43). Recent work in our laboratory strongly suggests that OpdA is required for an essential proteolytic modification that has escaped detection (6). The results of these experiments will be reported elsewhere.

Novak et al. have shown that *E. coli* OpdA is the major soluble activity in this bacterium able to cleave lipoprotein signal peptide in vitro (36). However, in *S. typhimurium*, elimination of *opdA* has no effect on cell growth. Apparently, there are multiple signal peptide-degrading activities in vivo. Just as both *S. typhimurium* and *E. coli* have three aminopeptidases with overlapping broad specificities (30, 31), at least three activities for *E. coli* which can degrade cleaved lipoprotein signal peptides in vitro have been identified. In addition to OpdA, both protease IV, a membrane-bound signal peptide-degrading activity encoded by *sppA* (18), and protease So, a soluble enzyme (5), have been shown to attack this signal peptide in vitro (35, 36). The So activity is weak, however, and under the conditions used, OpdA constitutes about 90% of the soluble lipoprotein signal peptide-degrading activity. It is possible that in vivo, the broad specificity aminopeptidases, peptidases N, A, and B and Dcp, are involved in signal peptide degradation as well. These enzymes have been shown to play a role in protein degradation (28, 29).

Inspection of the predicted amino acid sequence of *opdA* revealed a thermolysinlike metalloproteinase Zn^{2+} binding site. This site has been found in a number of Zn^{2+} metalloproteins from a variety of sources including human collagenase, crayfish digestive protease, *Bacillus spp.* thermolysin and neutral protease, and *E. coli* peptidase N (19). OpdA shows no extended amino acid similarity to any of these enzymes. Comparison of the *opdA* sequence with the sequence for *dcp* showed considerable similarity between the

two. Although both enzymes will hydrolyze $AcAla_4$, the sequence similarity is somewhat surprising, as Dcp is a true carboxypeptidase which removes dipeptides from the C termini of polypeptides (60) and OpdA is an endoprotease. Dcp is inhibited by captopril, whereas OpdA is not (51). In addition, even when Dcp is overproduced from a plasmid, it cannot substitute for OpdA in allowing normal P22 plaque formation (6).

Such sequence similarity is not typical of *E. coli* and *Salmonella* peptidases. Although at least 14 *E. coli* or *S. typhimurium* peptidolytic enzymes have been sequenced, only *Salmonella opdA* and *dcp* and *E. coli pepP* and *pepQ* (34) have been shown to be similar to each other. In addition to their similarity to each other, OpdA and Dcp share considerable similarity with the rat metallopeptidase EP 24.15. There are several other examples of a mammalian peptidase exhibiting sequence similarity to an *E. coli* or an *S. typhimurium* peptidase. *E. coli* peptidase Q (*pepQ*) shows 29% amino acid identity to human prolidase (34). Aminopeptidase A (*pepA*) from *E. coli* shows 35% amino acid identity to bovine lens leucine aminopeptidase (50). Protease III (*prt*) from *E. coli* shows 26% identity to human insulin-degrading enzyme (1), and *E. coli* aminopeptidase N (*pepN*) shows 21% amino acid identity in a 361-residue overlap with human aminopeptidase N (38).

In addition to their sequence similarity, OpdA and EP 24.15 have similar specificities and possibly similar functions. EP 24.15 cleaves peptides on the carboxyl side of hydrophobic amino acids, including Gly and Ala (40). It has an extended substrate binding site able to contain at least five amino acids. Additionally, EP 24.15 will not cleave polypeptides longer than about 20 amino acids (39, 40). On the basis of the available evidence, OpdA appears to prefer Gly or Ala on either side of the scissile bond (35, 52), and it requires substrates at least five amino acids long with two to three amino acids on either side of the scissile bond (35, 52). OpdA will hydrolyze the lipoprotein signal peptide only after it has been removed from the precursor protein, suggesting that in this case, OpdA will not attack the larger polypeptide (35).

OpdA and EP 24.15 both appear to be involved in posttranslational processing of polypeptides and degradation of bioactive peptides. EP 24.15 is found in peptide hormone-producing tissues such as brain, pituitary, and testes (40). It hydrolyzes several enkephalin-containing peptides in vitro, releasing the active peptide hormone (40). It is also the major activity from these tissues able to degrade gonadotropin-releasing hormone in vitro (40). Additionally, studies with a specific inhibitor of EP 24.15 have shown that EP 24.15 is active in the degradation of gonadotropin-releasing hormone in vivo (23). Although in vitro studies with *E. coli* OpdA demonstrated that it would hydrolyze only free lipoprotein signal peptide (35), recent work in our laboratory has suggested that OpdA is required for the posttranslational removal of 20 amino acids from the N terminus of a phage P22 protein (6). We also have evidence which indicates that *opdA* is the *Salmonella* homolog of the *E. coli* gene *prlC*, a site of suppressors of LamB signal sequence mutations (6). This result, along with the observation that *E. coli* OpdA degrades free lipoprotein signal peptide, suggests that OpdA may play a role in signal peptide processing or degradation in vivo.

The degree of sequence similarity among OpdA, Dcp, and EP 24.15 suggests that they represent a previously unidentified subclass of zinc metallopeptidases. The regions shared by all three could serve as a profile to identify other members of this subclass. The similarity between OpdA and Dcp suggests that they may have arisen from a duplication of an earlier peptidase gene. More data would be required to determine whether the branching which ultimately led to EP 24.15 occurred before or after this duplication. However, on the basis of the greater similarity of OpdA than Dcp to EP 24.15 and the similar specificities and perhaps similar functions of OpdA and EP 24.15, one might speculate that the branching which led to EP 24.15 occurred after the duplication which led to Dcp. The apparent conservation of both functional and structural elements between OpdA and EP 24.15 and of protease III and human insulin-degrading enzyme (1) suggests that functional similarity with their counterparts in higher organisms may be a common theme among *Salmonella* and *E. coli* peptidases. This may provide insights into the largely uncharacterized roles of these enzymes in vivo.

The *opdA* sequence contains a near consensus σ^{32} promoter at position 504. The consensus σ^{32} promoter is TNtC NCCCTTGAA(13- to 15-bp spacer)CCCATtTaNNNNNtcA (7); *opdA* has gtcCatCaTTGAA(15-bp spacer)CCCCATTct agatcTCA. Several σ^{32}-dependent heat shock proteases, including Lon (10) and ClpP (20), have been identified. Our failure to observe an increase in β-galactosidase levels from the *opdA10*::Mu*dJ* fusion after heat shock does not rule out the possibility that *opdA* is a heat shock gene but might be the result of the transient nature of the response or suggest that the cell maintains a relatively constant level of OpdA whether heat stressed or not. This would be similar to *topA* (encoding topoisomerase I) which, although it has an active σ^{32} promoter, shows no increase in protein levels upon temperature shift (25). Lesley et al. (25) found that after a temperature shift, transcription from the *topA* σ^{32} promoter increases and that transcription from one of the σ^{70} promoters decreases, thus leading to a relatively constant level of expression of the topoisomerase. It is possible that a similar mechanism may be operating with *opdA*, and experiments are in progress to examine the induction of *opdA* mRNA after heat shock.

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