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A Periplasmic D-Alanyl-D-Alanine Dipeptidase in the Gram-Negative Bacterium *Salmonella enterica*

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The VanX protein is a D-alanyl-D-alanine (D-Ala-D-Ala) dipeptidase essential for resistance to the glycopeptide antibiotic vancomycin. While this enzymatic activity has been typically associated with vancomycin- and teicoplainin-resistant enterococci, we now report the identification of a D-Ala-D-Ala dipeptidase in the gram-negative species *Salmonella enterica*. The *Salmonella* enzyme is only 36% identical to VanX but exhibits a similar substrate specificity: it hydrolyzes D-Ala-D-Ala, DL-Ala-DL-Phe, and D-Ala-Gly but not the tripeptides D-Ala-D-Ala-D-Ala and DL-Ala-DL-Lys-Gly or the dipeptides L-Ala-L-Ala, *N*-acetyl-D-Ala-D-Ala, and L-Leu-Pro. The *Salmonella* dipeptidase gene, designated *pcgL*, appears to have been acquired by horizontal gene transfer because *pcgL*-hybridizing sequences were not detected in related bacterial species and the G+C content of the *pcgL*-containing region (41%) is much lower than the overall G+C content of the *Salmonella* chromosome (52%). In contrast to wild-type *Salmonella*, a *pcgL* mutant was unable to use D-Ala-D-Ala as a sole carbon source. The *pcgL* gene conferred D-Ala-D-Ala dipeptidase activity upon *Escherichia coli* K-12 but did not allow growth on D-Ala-D-Ala. The PcgL protein localizes to the periplasmic space of *Salmonella*, suggesting that this dipeptidase participates in peptidoglycan metabolism.

Vancomycin is a glycopeptide antibiotic that inhibits peptidoglycan synthesis by binding to the peptidoglycan precursor UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala at the D-Ala-D-Ala terminus (32). Acquired resistance to glycopeptides in enterococci requires VanX (36), a dipeptidase that cleaves D-Ala-D-Ala and allows the synthesis of D-Ala-D-lactate mediated by the *vanH* and *vanA* gene products (6). This decreases the rate of synthesis of the pentapeptide precursor UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala and increases the levels of a pentadepsipeptide precursor, UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Lac, which exhibits decreased binding to glycopeptide antibiotics (34). Vancomycin resistance also requires the D,D-carboxypeptidase VanY (2) and the regulatory system VanR-VanS, which is responsible for transcriptional control of the *vanA*, *vanH*, *vanX*, and *vanY* genes (3; see reference 35 for a recent review). While D-Ala-D-Ala dipeptidase activity has been associated typically with vancomycin-resistant enterococci, we now describe the identification of a D-Ala-D-Ala dipeptidase in the gram-negative bacterium *Salmonella enterica*.

S. enterica is a facultative intracellular pathogen that is responsible for several disease syndromes in humans, including gastroenteritis, typhoid fever, and bacteremia (23). There is a single species in the genus *Salmonella* which encompasses over 2,300 serotypes that differ in host specificity and the disease condition that they promote in susceptible hosts. During growth within host tissues, *Salmonella* modifies its peptidoglycan (33) and sheds part of its lipopolysaccharide (LPS) (12). Moreover, *Salmonella* can introduce modifications in the lipid A portion of its LPS that decrease the ability of the LPS to induce expression of tumor necrosis factor alpha by adherent monocytes (21) and increase resistance to cationic peptide antibiotics (22). While changing the peptidoglycan and the LPS may be a way of preventing activation of the host immune system, the virulence role and molecular basis of these cell surface alterations remain largely unknown.

The PhoP-PhoQ two-component regulatory system governs several aspects of *Salmonella* virulence, including intramacrophage survival, resistance to antimicrobial peptides, and invasion of epithelial cells (16). The PhoQ protein is a membrane-bound sensor that modifies the transcriptional activity of the PhoP protein in response to the extracytoplasmic levels of Mg^{2+}: PhoP-activated genes are transcriptionally induced during growth in micromolar concentrations of Mg^{2+} and repressed when bacteria are grown in the presence of millimolar concentrations of Mg^{2+} (10, 39). *Salmonella* appears to reside in a low-Mg^{2+} environment within host tissues because PhoP-activated genes are transcriptionally induced to high levels within host cells (1, 11, 41). The PhoP-PhoQ system controls expression of some 40 different proteins, including two distinct Mg^{2+} transporters, a UDP-glucose dehydrogenase, and a nonspecific acid phosphatase, as well as proteins required for modification of the lipid A in the LPS (20, 39).

In this paper, we identify a D-Ala-D-Ala dipeptidase in *S. enterica*. We establish that this peptidase is encoded by a gene that is specific to *Salmonella* and regulated by the PhoP-PhoQ regulatory system. We also demonstrate that the *Salmonella* dipeptidase localizes to the periplasmic space, suggesting a role for this enzyme in peptidoglycan metabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacterial genetic techniques, and growth conditions. The strains and plasmids used in this study are listed in Table 1. All *Salmonella* strains are derived from wild-type *S. enterica* serovar Typhimurium 14028s, except for strain AA3007 which is derived from LT2. Plasmids are derived from pUC19, pBR322, or Mud5005. Bacteria were grown at 37°C in either Luria-Bertani (LB) (29) or N-minimal medium (38) supplemented with

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source[a]
S. enterica		
14028s	Wild type	ATCC
MS7953s	*phoP7953*::Tn*10*	9
EG9331	*pcgL*::MudJ	39
EG10277	*sugR1*::MudJ	4
EG10981	*phoP7953*::Tn*10 ugtL*::*lac-kan*	This work
EG11250	*ugtL*::*lac-kan*	This work
E. coli		
DH5α	F′/*endA1 hsdr17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacIzYA-argF*)*U169 deoR* [ϕ80*dlac*Δ(*lacZ*)M15]	13
JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*e14*$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96 endA1 hsdr17*	43
EG10358	TK2316Mucts/pEG9129	This work
EG10421	DH5α/pEG9125	This work
TK2316Mucts	F$^-$ *hsdR thi thr lacZ*(Am) *nagA kdp rha* Mucts	15
Plasmids		
pBR322	rep_{pMB1} Tet^r Ap^r	5
pEG5005	pBC0::Mud5005	14
pEG9122	pBR322::*pcgL*$^+$	This work
pEG9123	pBR322::*pcgL*$^+$	This work
pEG9125	pUC19::*pcgL*$^+$ *ugtL*$^+$	This work
pEG9129	Mud5005::*pcgL*$^+$ *ugtL*$^+$	This work
pEG9130	pUC19::*ugtL*$^+$	This work
pEG9131	pUC19::*pcgL*$^-$	This work
pEG9132	pUC19::*pcgL*$^+$ *ugtL*$^+$	This work
pUC19	rep_{pMB1} *lacZ*α Ap^r	43

[a] ATCC, American Type Culture Collection.

0.1% Casamino Acids, 38 mM glycerol, and different concentrations of $MgCl_2$. Experiments that evaluated the ability of strains to use D-Ala-D-Ala as a sole carbon source were carried out in N-minimal medium supplemented with 0.2% D-Ala-D-Ala, 0.1 mg of thiamine per liter, and $MgCl_2$ (10 mM). Ampicillin and kanamycin were used at 50 μg/ml, and tetracycline was used at 10 μg/ml. Phage P22-mediated transductions were carried out as described before (8). Plasmids were transformed into bacteria either by electroporation with a Bio-Rad apparatus or by chemical transformation using standard procedures.

Molecular biological techniques. The nucleotide sequence of a 3.8-kb region that harbors the *pcgL* and *ugtL* genes was determined on both strands by using plasmid pEG9129 as the template and newly synthesized primers. Plasmid pEG9129 DNA was extracted from strain EG10358 and purified by use of the Qiagen plasmid midi kit, and a segment of it was sequenced with the dichlororhodamine dye terminator cycle sequencing kit and an ABI 310 automatic sequencer. DNA sequence analysis and protein sequence alignments were performed with the GeneWorks (IntelliGenetics) and GCG (University of Wisconsin) software packages.

Southern hybridization analysis was carried out on chromosomal DNA that had been digested with *Eco*RI, size fractionated on a 1% agarose gel, and transferred to a nylon membrane by capillary blotting. To investigate the presence of *pcgL*-hybridizing sequences, a 782-bp PCR fragment containing the *pcgL* coding region was generated with primers 858 (5′-GGGTCTCTGCTTAACGG-3′) and 906 (5′-GCGAGGTGTAACATATGG-3′), labeled with [α-^{32}P]dCTP with the Ready to Go kit (Pharmacia Biotech), and used as a probe under previously described conditions (19). To investigate the presence of $vanX_{E.\ coli}$ (termed *ecovanX* by Lessard et al. [26])-hybridizing sequences, a 473-bp PCR fragment containing a segment of the $vanX_{E.\ coli}$ coding region was generated by using primers 744 (5′-AATTGAAATACGCCTGCGCTG-3′) and 745 (5′-GAGCAG AGGGTAACTCGCTGC-3′) and labeled as described above for the *pcgL* probe. Hybridization experiments with the $vanX_{E.\ coli}$ probe were carried out under both high- and low-stringency conditions, as previously described (17).

Cloning of the *pcgL* gene. We used the in vivo cloning procedure with the mini-Mu replicon Mud5005 (14) to construct a library from wild-type *S. enterica* serovar Typhimurium. Kanamycin-resistant transductants of strain TK2316Mucts were screened by colony hybridization using as a probe a 202-bp DNA fragment located immediately adjacent to the MudJ transposon in the *pcgL*::MudJ strain EG9331. The 202-bp fragment was generated by the PCR using primers 633 (5′-GCGTGGGCCAAAGATCCTTCT-3′) and 634 (5′-ACGCAGTACAATTC ACCAGTG-3′) and labeled with [α-^{32}P]dCTP with the Ready to Go kit (Pharmacia Biotech). Four hybridizing clones were identified, and one of them, EG10358, harboring plasmid pEG9129 with a 24-kb insert (see Fig. 1), was used for further studies.

Construction of a *ugtL* mutant. An 11-kb *Hin*dIII fragment from plasmid pEG7125 (18) harboring a promoterless *lac* operon and *kan* resistance gene was introduced into the *Afl*II site within the *ugtL* gene in plasmid pEG9124. (Plasmid pEG9124 was derived from plasmid pEG9125 by digestion with *Aat*II and *Sma*I followed by religation). Plasmid pFH1, harboring the *lac* operon in the same orientation as the *ugtL* gene, was used to transfer the *ugtL* mutation back to the chromosome as described previously (19). The structure of the *ugtL* locus in the resulting mutant was confirmed by Southern hybridization analysis using as a probe a 610-bp fragment containing the *ugtL* gene that was generated by the PCR using primers 758 (5′-GAACACGTCGATTGTCGGCGC-3′) and 759 (5′-ACGATTAGCTGACGGCTTTG-3′).

Overproduction and purification of the PcgL protein. The PcgL protein was overproduced in *Escherichia coli* K-12 DH5α cells harboring the *pcgL*-containing plasmid pEG9125. Bacterial cells were grown in LB broth, harvested, and dissolved in 50 mM HEPES buffer (pH 7.7). After sonication and centrifugation, the supernatant was precipitated with ammonium sulfate to 80% saturation. After desalting, the protein was purified in a two-step procedure using a high-pressure liquid chromatography apparatus. First, we used an ion-exchange column (Toyopearl SP column) and 200 mM KCl to elute the protein, and then we used a size exclusion column (Waters Protein Pak 300SW). The purity of the PcgL protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The N-terminal sequence of the first 10 residues of the PcgL protein was determined with Edman degradation by Midwest Scientific Laboratory (St. Louis, Mo.).

Enzyme and virulence assays. Crude extracts from N-minimal medium-grown bacteria were prepared as follows: bacteria were grown in 3 to 5 ml of medium, harvested, washed twice with 50 mM HEPES buffer (pH 7.7), resuspended in 500 μl of the same buffer, and sonicated. The supernatants were used for total protein determination with the bicinchoninic acid protein assay kit (Sigma), with bovine serum albumin as a standard. Periplasmic and cytoplasmic fractions were prepared by osmotic shock as described previously (31). β-Galactosidase and nonspecific acid phosphatase activities were determined as described before (24, 29).

Peptide hydrolysis was determined quantitatively by measuring the release of free amino acids by a modification of the cadmium-ninhydrin method (42). Briefly, substrates (10 mM) were incubated with 10 μg of protein from crude cell extracts or with 1.8, 9, or 18 ng of purified PcgL protein, for 30 min at 37°C in 50 mM HEPES buffer (pH 7.7). Then, 7.5 times the volume of cadmium-ninhydrin solution (0.01 g of ninhydrin/ml of ethanol, 12.5 ml of acetic acid, 1 g of $CdCl_2$/ml of bidistilled water) was added, and the samples were incubated for 5 min at 85°C for color development. The optical density at 505 nm was then determined. Standards of 0.1, 0.5, and 1 mM D-alanine were used, as well as blanks containing 10 mM of the corresponding substrate resuspended in 50 mM HEPES buffer. All substrates were from Sigma.

Macrophage survival assays with the macrophage-like cell line J774 and invasion assays of canine kidney epithelial (MDCK) cells were conducted as described previously (25). Virulence assays were performed with 7- to 8-week-old female BALB/c mice inoculated orally or intraperitoneally with 100 μl of bacteria diluted in phosphate-buffered saline.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database under accession no. AF120672.

RESULTS

The *pcgL* gene encodes a novel *Salmonella* protein. The *Salmonella pcgL* locus was originally identified as a PhoP-activated *lac* gene fusion generated with the promoter probe transposon MudJ (39). To determine the function of the *pcgL* gene, we isolated the wild-type copy of *pcgL* by screening a plasmid library by colony hybridization using as a probe a 202-bp DNA segment located immediately adjacent to the MudJ transposon in the *pcgL* mutant EG9331 (Fig. 1). Sequence analysis of a positive clone revealed that the MudJ had inserted within a novel *Salmonella* gene encoding a 256-amino-acid protein with a predicted molecular mass of 28,995 Da and an isoelectric point of 6.98.

The PcgL protein is 36% identical over 192 residues to the 202-amino-acid VanX, a D-Ala-D-Ala dipeptidase encoded within the *Enterococcus faecium* transposon Tn*1546* and required for vancomycin resistance (36). The similarity between PcgL and VanX extends over the entire length of these proteins (Fig. 2A). Conserved amino acids include VanX residues His116, Asp123, and His184, which have been implicated in the coordination of Zn^{2+}, and Glu181, which was shown to be the

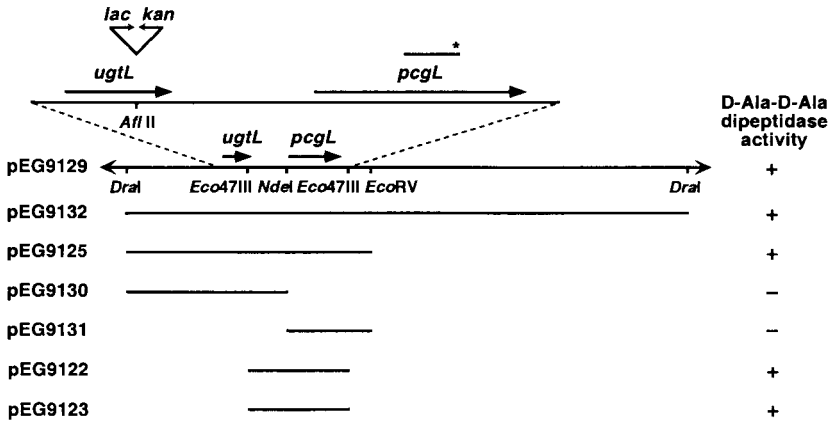

FIG. 1. Physical and genetic map of *pcgL*-containing region and localization of *pcgL*-encoded D-Ala-D-Ala dipeptidase activity. The short horizontal line with an asterisk above indicates the position of the PCR-generated fragment used to screen the wild-type library. Plasmids pEG9122 and pEG9123 are pBR322 derivatives that harbor the same *Eco*47III fragment in opposite orientations. The *pcgL* gene is in the same orientation as the *tet* gene of pBR322 in plasmid pEG9123 and in the opposite orientation in plasmid pEG9122. Plasmid pEG9129 is based on Mud5005, and plasmids pEG9125, pEG9130, pEG9131, and pEG9132 are derived from plasmid pUC19.

catalytic base (7, 28). The PcgL protein is larger than VanX due to a 20-amino-acid cleavable signal sequence at the N terminus (see below) and to 23 additional residues located between a position equivalent to amino acids 130 and 131 of the VanX protein. The PcgL protein also exhibits similarity to uncharacterized open reading frames from *Mycobacterium tuberculosis* and *Synechocystis* sp. and to an *E. coli* K-12 protein that displays D-Ala-D-Ala dipeptidase activity (26).

The *pcgL* gene product is required for D-Ala-D-Ala dipeptidase activity. Despite the low-level sequence identity between the PcgL and VanX proteins, we detected high levels of D-Ala-D-Ala dipeptidase activity in crude extracts prepared from wild-type *Salmonella* grown under conditions that promote expression of PhoP-activated genes (i.e., 25 μM Mg^{2+}) (Fig. 3A). This activity was very much reduced in extracts prepared from wild-type cells grown under conditions that repress transcription of PhoP-activated genes (i.e., 25 mM Mg^{2+}) and from extracts prepared from a *phoP* mutant, and it was absent from extracts prepared from the *pcgL* strain (Fig. 3A).

To determine the location of the *pcgL* gene within plasmid pEG9129, we examined the ability of different subclones to restore D-Ala-D-Ala dipeptidase activity to the *pcgL* mutant and localized the *pcgL* gene to the 1.5-kb *Eco*47III fragment in plasmids pEG9122 and pEG9123, which differ in the relative orientation of the insert (Fig. 1). This fragment includes the complete *pcgL* open reading frame and could complement the *pcgL* mutant independent of its orientation, suggesting that it also harbors the regulatory signals necessary for *pcgL* expression.

Substrate specificity of the PcgL protein. To ascertain the physiological role of PcgL, we purified the PcgL protein and determined its substrate specificity. The PcgL protein was overproduced in an *E. coli* K-12 strain harboring the multicopy number plasmid pEG9125 and purified to near homogeneity

A

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PcgL  MVKCVSSFLLFSLLSVQAMSAENHIDLHQPKDFVDITTVAPDVQVDMRYFSSHNFIGRPIKGYNAPVCLLTRPAANAVKQVADRLRPFGLTLKIYDCYRP  100
VanX  ..........................MEIGFTFLDEIVHGVRWDAKYATWDNFTGKPVDGYEVNRIVGTYELAESLLKAKELAATQGYGLLLWDGYRP   72

PcgL  QSAVNDFIAWAKDPSQNQMKNEFYPQVEKNRLFEEGYLAARSGHSRGSTLDLTIVPLDSKIPIYDPGRPLVNCTASAAQRSPDNSLDFGTGFDCFSPLSH  200
VanX  KRAVNCFMQWAAQPENNLTKESYYPNIDRTEMISKGYVASKSSHSRGSAIDLTLYRLD.....................TGELVPMGSRFDFMDERSH  149

PcgL  PDNVILTAQQRANRLLLQTLMRDAGFTPLDTEWWHFSLTHEPYPNTWFDFPVKQRP  256
VanX  HAANGISCNEAQNRRRLRSIMENSGFEAYSLEWWHYVLRDEPYPNSYFDFPVK...  202
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B

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UgtL  SDGEIHEKTASWGILQSEWLRKCGRLLLLLLY.............RFVIGWAFFQLLAMIVAGIFLLGILLFHPIIFVQTIAITEKLNHASLDLWHI..  87
Chs1  ADTDVPDRIPEFVLQRRRWLNGSFFAAAYAICHYYRFFRTSHTISRKFMLSIEFIYQLATIVFGWFNIGNFFIIFYILTSSLASTSANFLPGEILFRIAI 586

UgtL  ............LKLCLWH.............YGIIAGFI.....FMAECTLSKSIRQVQRLSKKFGAQDVSSRP 132
Chs1  WIYASLLVTCFVLALGNRPHGSPNFYLSMVIMYSILMGYLLFCSGWIAYRAISDAIHNASSTSSSYTSALLNSNV 661
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FIG. 2. (A) Alignment of the PcgL protein of *S. enterica* and the VanX protein of the *Enterococcus faecium* transposon Tn*1546*. (B) Alignment of the *S. enterica ugtL* gene product with a segment from a chitin synthetase from *Schizosaccharomyces pombe*.

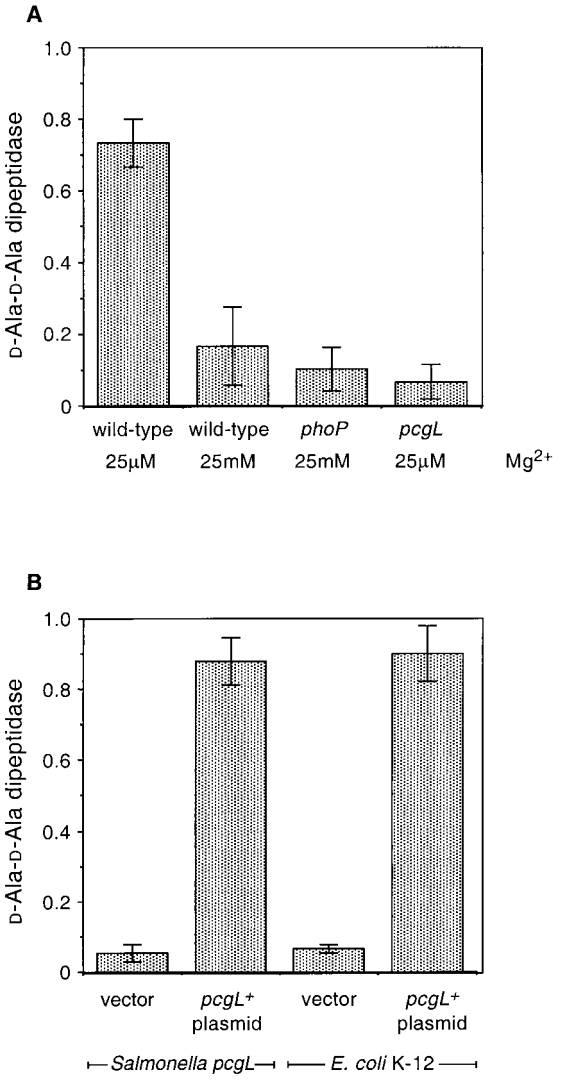

FIG. 3. (A) D-Ala-D-Ala dipeptidase activity of crude extracts from wild-type and *pcgL* and *phoP* mutant *Salmonella* strains grown in N-minimal medium with 25 μM or 25 mM $MgCl_2$. Data correspond to mean values of 20 independent assays. (B) D-Ala-D-Ala dipeptidase activity of *S. enterica pcgL* mutant strain EG9331 and of *E. coli* K-12 harboring the *pcgL*-containing plasmid pEG9122 or the vector pBR322. Data correspond to mean values of three independent assays. D-Ala-D-Ala dipeptidase activity indicates micromoles of D-Ala produced per milligram of protein per minute.

(*E. coli* K-12 lacks the *pcgL* gene and does not express D-Ala-D-Ala dipeptidase activity under the tested growth conditions; see below). Like VanX, the PcgL protein hydrolyzed D-Ala-D-Ala and D-Ala-Gly but not the N-blocked D-Ala-D-Ala species *N*-acetyl-D-Ala-D-Ala and the tripeptide D-Ala-D-Ala-D-Ala. The dipeptides L-Ala-L-Ala and L-Leu-Pro and the tripeptide DL-Ala-DL-Lys-Gly were not substrates of PcgL either. On the other hand, the PcgL protein hydrolyzed DL-Ala-DL-Phe but not DL-Ala-DL-Val, and this distinguishes it from VanX, which has activity towards both D-Ala-D-Phe and DL-Ala-DL-Val (26, 42). Thus, despite the low-level sequence identity between PcgL and VanX, these proteins have very similar substrate specificities. These results indicate that the *pcgL* gene encodes a D,D-dipeptidase that uses D-Ala-D-Ala as its preferred substrate.

Subcellular location of the PcgL protein. The PSORT protein localization program (30) predicted PcgL to be a periplasmic protein and to have a 20-amino-acid cleavable N-terminal signal sequence. Consistent with the notion that PcgL is a periplasmic protein, the N-terminal sequence of the first 10 residues of the purified PcgL protein was determined by Edman degradation and found to be A-E-N-H-I-D-L-H-Q-P, a perfect match to residues 21 through 30 of the deduced amino acid sequence of the *pcgL* gene. To further examine the subcellular location of the PcgL protein, we analyzed periplasmic and cytoplasmic fractions from wild-type *Salmonella* for D-Ala-D-Ala dipeptidase activity (membrane fractions were not tested because the PcgL protein does not have long stretches of hydrophobic residues that could constitute transmembrane domains). We also determined the β-galactosidase and nonspecific acid phosphatase activities of these fractions as prototypical cytoplasmic and periplasmic enzymes, respectively. (Because *Salmonella* does not harbor the *lac* operon, these experiments were performed with strain EG10277, which carries the *E. coli lac* operon.) Despite the predicted periplasmic location of PcgL, only 22.6% of the D-Ala-D-Ala dipeptidase activity localized to the periplasm (Fig. 4). The low recovery of D-Ala-D-Ala dipeptidase activity in the periplasmic space is not unusual for osmotically released enzymes, and similar findings have been reported for the periplasmic proteases DegP and Prc (37, 40). The periplasmic location of PcgL distinguishes it from the cytoplasmic VanX proteins of enterococci and *E. coli* K-12 and suggests that the *Salmonella* enzyme acts on D-Ala-D-Ala originating from a peptidoglycan-derived fragment and/or peptidoglycan precursors released into the periplasmic space rather than interacting directly with the cytoplasmic pool of D-Ala-D-Ala.

The *pcgL* gene is specific to *Salmonella*. We mapped the *pcgL* locus to the 37- to 42-min region in the *S. enterica* serovar Typhimurium chromosome by hybridizing a *pcgL*-specific probe to an ordered library of the *Salmonella* genome. We analyzed the DNA sequences flanking the *pcgL* gene and determined that *pcgL* is part of a 3.8-kb region with a G+C content of only 41%, which is much lower than the overall G+C content of the *Salmonella* chromosome (52%). Because unusual G+C contents are often indicative of horizontally acquired sequences, we examined the distribution of the *pcgL* gene among related bacterial species. We used the *pcgL* gene as a probe in Southern hybridization experiments carried out under stringent conditions and detected *pcgL*-hybridizing sequences in *S. enterica* but not in 14 other microbial species examined (Fig. 5A). Taken together, these data indicate that the *pcgL* gene is specific to

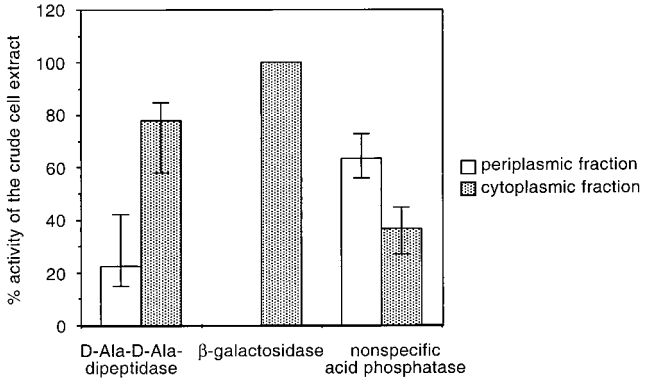

FIG. 4. Localization of the D-Ala-D-Ala peptidase activity to different subcellular compartments. The β-galactosidase and nonspecific phosphatase activities were determined as prototypical cytoplasmic and periplasmic enzymes, respectively. Data correspond to mean values of three independent assays.

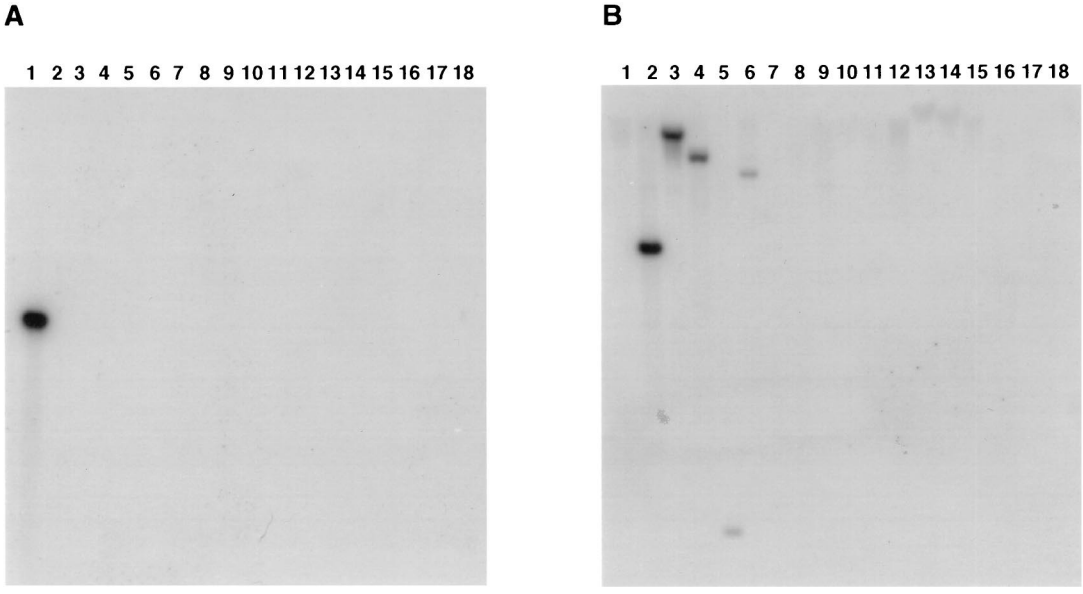

FIG. 5. Southern hybridization experiments using *pcgL* (A) and *ecovanX* (B) probes were carried out as described in Materials and Methods with DNA from *Salmonella enterica* serovar Typhimurium (lane 1), *E. coli* K-12 (lane 2), *Shigella flexneri* (lane 3), *Citrobacter freundii* (lane 4), *Enterobacter aerogenes* (lane 5), *Enterobacter cloacae* (lane 6), *Klebsiella pneumoniae* (lane 7), *Serratia marcescens* (lane 8), *Serratia oediferus* (lane 9), *Proteus mirabilis* (lane 10), *Proteus vulgaris* (lane 11), *Erwinia herbicola* (lane 12), *Yersinia enterocolitica* (lane 13), *Yersinia pseudotuberculosis* (lane 14), *Yersinia pestis* (lane 15), *Pseudomonas aeruginosa* (lane 16), *Mycobacterium avium* (lane 17), and *Saccharomyces cerevisiae* (lane 18).

Salmonella and that it was likely incorporated into the *Salmonella* chromosome by horizontal gene transfer. Acquisition of the *pcgL*-containing region appears to have occurred early in the evolution of *Salmonella* because *pcgL*-hybridizing sequences were detected in all eight subspecies that comprise *S. enterica* (data not shown).

Molecular and functional characterization of the *pcgL* region. At a position 523 bp upstream from the *pcgL* gene, we identified a 132-codon open reading frame, encoding a product designated UgtL that exhibits sequence similarity to a chitin synthetase from *Schizosaccharomyces pombe* (26% identity and 53% similarity over 65 residues to the 859-amino-acid chitin synthetase) (Fig. 2B). Because chitin is the yeast equivalent of bacterial peptidoglycan and D-Ala-D-Ala is produced only for its incorporation into the peptidoglycan, the UgtL protein could function together with the PcgL protein in some aspect of peptidoglycan metabolism. Yet, the region of sequence similarity between UgtL and the chitin synthetase is limited to the predicted transmembrane domains of these putative integral membrane proteins.

To examine the function of the *ugtL* gene, we constructed a *ugtL* mutant by introducing a promoterless *lac* operon and a kanamycin resistance cassette into the chromosomal copy of the *ugtL* gene (see Materials and Methods). The *ugtL* mutant was viable and produced wild-type levels of D-Ala-D-Ala dipeptidase activity, consistent with the notion that the *ugtL* and *pcgL* genes are not part of the same transcriptional unit and that UgtL is not necessary for D-Ala-D-Ala hydrolysis. Nevertheless, the PcgL and UgtL proteins may participate in the same cellular pathway because transcription of the *ugtL* gene is regulated by the Mg^{2+} concentration in the medium and is dependent on a functional PhoP-PhoQ two-component system (Fig. 6). That the *ugtL* mRNA is likely to be translated is suggested by the presence of an excellent Shine-Dalgarno sequence (AGGA) 9 bp upstream from the *ugtL* open reading frame.

The *pcgL* gene is necessary for growth on D-Ala-D-Ala but dispensable for virulence in mice. The PcgL-mediated hydrolysis of D-Ala-D-Ala produces D-Ala, an amino acid that can serve as a sole carbon source in *E. coli* and, presumably, in other enteric species. This raised the possibility of the periplasmic PcgL protein allowing *Salmonella* to use D-Ala-D-Ala as a sole carbon source, and consistent with this notion, wild-type *Salmonella* grew on N-minimal liquid medium containing D-Ala-D-Ala (7.5 mM) as a sole carbon source whereas the *pcgL* mutant did not (Fig. 7). This growth defect is specifically due to the absence of a functional *pcgL* gene because the *pcgL* mutant harboring the *pcgL*$^+$ plasmid grew on D-Ala-D-

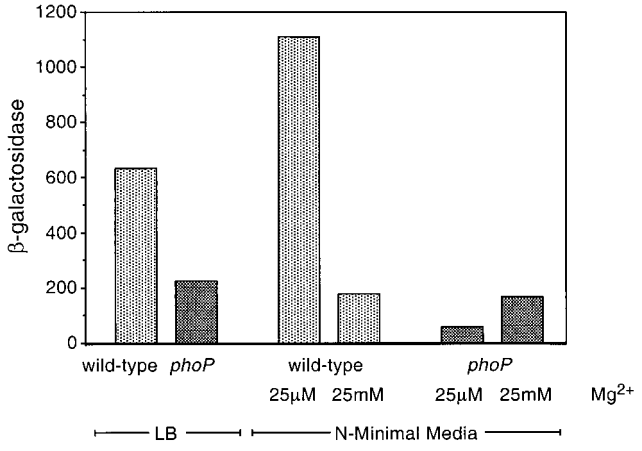

FIG. 6. β-Galactosidase activity (in Miller units [29]) from a *ugtL-lac* transcriptional fusion of *Salmonella* strains grown in LB or N-terminal medium with 25 μM or 25 mM $MgCl_2$. These results demonstrate that expression of *ugtL* is regulated by the PhoP-PhoQ system. Data correspond to a single experiment of two independent assays that gave similar results.

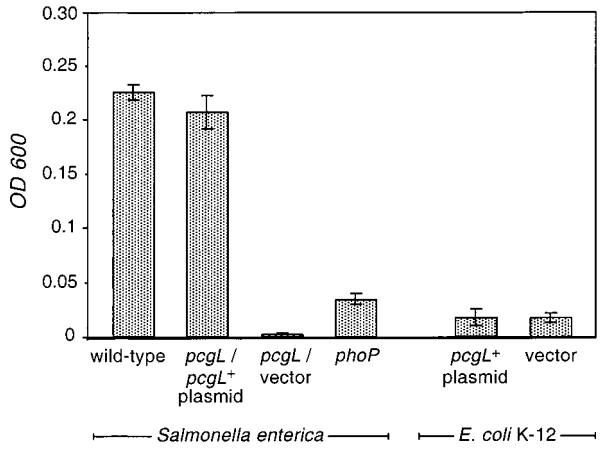

FIG. 7. Growth of wild-type and *phoP* and *pcgL* mutant *Salmonella* strains harboring either the *pcgL*-containing plasmid pEG9122 or the vector pBR322 and of *E. coli* K-12 harboring the *pcgL*-containing plasmid pEG9122 or the vector pBR322 in N-minimal medium with D-Ala-D-Ala as a sole carbon source. The final optical density at 600 nm (*OD 600*) of the bacterial cultures was determined after 24 h of incubation. Similar results were obtained after 48 h of incubation except that the *phoP* mutant *Salmonella* strain grew better than the *E. coli* K-12 strains. Data correspond to mean values of two independent experiments.

Ala like wild-type *Salmonella* did (Fig. 7). The *phoP* mutant was also defective for growth on D-Ala-D-Ala but not to the same extent as the *pcgL* strain, and this may reflect the residual level of D-Ala-D-Ala dipeptidase activity exhibited by the *phoP* mutant. On the other hand, the *ugtL* mutant grew on D-Ala-D-Ala like wild-type *Salmonella* did (data not shown), consistent with the notion that the *ugtL* gene product is not required for the hydrolysis and/or transport of D-Ala-D-Ala.

As described above, the *pcgL* gene is specific to *Salmonella* and requires the PhoP-PhoQ virulence regulatory system for expression (39). This raised the possibility that PcgL may be necessary for *Salmonella* pathogenesis and/or may be responsible for phenotypes associated with mutations in the *phoP* locus. However, the *pcgL* mutant was as virulent as wild-type *Salmonella* when inoculated into BALB/c mice by either the oral or intraperitoneal route. Likewise, the *pcgL* mutant displayed wild-type levels of invasion into the epithelial cell line MDCK, survival within the macrophage-like cell line J774, and growth in low-Mg^{2+} defined medium.

The *pcgL* gene confers D-Ala-D-Ala dipeptidase activity upon *E. coli* K-12. The *E. coli* K-12 genome (GenBank accession no. D90789) harbors an open reading frame coding for a 193-amino-acid protein that exhibits sequence similarity to the enterococcal VanX protein (40% identity over 141 residues). However, several lines of evidence argue against this open reading frame, $vanX_{E.\ coli}$ (designated *ecovanX* by Lessard et al. [26]), encoding the functional homolog of the *Salmonella* PcgL protein: (i) $VanX_{E.\ coli}$ is only 40% identical to PcgL, whereas homologous proteins in *E. coli* and *Salmonella* typically exhibit >85% sequence identity; (ii) unlike PcgL, $VanX_{E.\ coli}$ does not appear to have a signal sequence and is likely a cytoplasmic protein; (iii) no D-Ala-D-Ala dipeptidase activity was detected in extracts prepared from *E. coli* K-12 when cells were grown under conditions that promote expression of the *Salmonella* *pcgL* gene (Fig. 7); and (iv) sequences hybridizing to the $vanX_{E.\ coli}$ gene were detected in *Shigella flexneri*, *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae* (Fig. 5B), bacterial species lacking *pcgL*-hybridizing sequences (Fig. 5A). Cumulatively, these data demonstrate that the $vanX_{E.\ coli}$ and *pcgL* genes have different phylogenetic distributions and suggest that these products play different physiological roles.

To gain further insight into the function of the *Salmonella* *pcgL* gene, we investigated the behavior of an *E. coli* K-12 strain harboring the *pcgL*-containing plasmid pEG9122. As expected, the *E. coli* strain exhibited D-Ala-D-Ala dipeptidase activity (Fig. 3B). Moreover, this activity was regulated by the PhoP protein since reduced D-Ala-D-Ala dipeptidase levels were present in extracts prepared from an isogenic *phoP* mutant of *E. coli* K-12 (data not shown). On the other hand, the *E. coli* K-12 strain harboring the $pcgL^+$ plasmid could not grow on D-Ala-D-Ala (Fig. 7). Because the PcgL protein was normally exported to the periplasmic space in *E. coli* K-12 (see above), these data indicate that, in addition to *pcgL*, other genes are necessary for *E. coli* K-12 to use D-Ala-D-Ala as a sole carbon source.

DISCUSSION

D-Ala-D-Ala dipeptidase activity was first described by Reynolds and coworkers in vancomycin-resistant enterococci (36). In these organisms, the VanX protein cleaves D-Ala-D-Ala, which decreases the cytoplasmic pool of this dipeptide and allows the incorporation of D-Ala-D-lactate into peptidoglycan precursors. The resulting pentadepsipeptide precursor, UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Lac, exhibits lower binding to glycopeptide antibiotics than the normal pentapeptide precursor, UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala, and this accounts for vancomycin resistance (35).

The glycopeptide antibiotic-producing organisms *Streptomyces toyocaensis* NRRL 15009 and *Amycolatopsis orientalis* C329.2 contain proteins that exhibit 61 to 64% sequence identity to the VanX protein of vancomycin-resistant enterococci (27). However, *Streptomyces toyocaensis* NRRL 15009 and *A. orientalis* C329.2 do not appear to have been the source of glycopeptide resistance genes in vancomycin-resistant enterococci because the G+C contents of the resistance gene clusters in *Streptomyces toyocaensis* NRRL 15009 and *A. orientalis* C329.2 are 65.3 and 63.6%, respectively, much higher than those of vancomycin-resistant enterococci (27).

Unexpectedly, VanX-like proteins have been recently identified in three gram-negative species (i.e., *E. coli* K-12 and *Synechocystis* sp. during genome projects and *S. enterica* in the present study), and D-Ala-D-Ala dipeptidase activity has been demonstrated for the purified *Salmonella* PcgL protein (this work) and for maltose-binding protein fusion derivatives of the *E. coli* K-12 and *Synechocystis* VanX homologs (26). Because the outer membrane of gram-negative bacteria prevents access of vancomycin to its target, these VanX-like proteins must have roles other than mediation of vancomycin resistance. Then, what is the physiological function(s) of D-Ala-D-Ala dipeptidases in gram-negative bacteria?

A periplasmic D-Ala-D-Ala dipeptidase in *S. enterica*. We have purified the PcgL protein of *S. enterica* serovar Typhimurium, a D-Ala-D-Ala dipeptidase that exhibits a substrate specificity similar to that displayed by the enterococcal VanX proteins, with which it has only 36% sequence identity. The *Salmonella* protein localizes to the periplasmic space (Fig. 4), and this distinguishes it from the enterococcal VanX proteins, which are cytosolic enzymes. Thus, the PcgL protein is likely to act on D-Ala-D-Ala released from pentapeptide precursors and/or peptidoglycan-derived fragments rather than directly control the cytoplasmic pool of D-Ala-D-Ala.

The *pcgL* gene appears to have been incorporated into the *Salmonella* genome by horizontal gene transfer because *pcgL*-hybridizing sequences were not detected in related bacterial

species (Fig. 5A) and the G+C content of the *pcgL*-containing region is only 41%, very different from that of the rest of the *Salmonella* chromosome (52%). This suggests that the *pcgL* locus endows *Salmonella* with unique abilities not present in related enteric species. The identification of an open reading frame closely linked to *pcgL* whose product exhibits similarity to a chitin synthetase from *Schizosaccharomyces pombe* and is also regulated by the PhoP-PhoQ system suggests that the *pcgL* region participates in some aspect of peptidoglycan metabolism. While no significant differences in peptidoglycan structure were detected between wild-type and *pcgL* strains grown in laboratory media (our unpublished results), these findings are consistent with the notion that the PcgL protein acts on a substrate (i.e., D-Ala-D-Ala) liberated by an unidentified endopeptidase cleaving between diaminopimelic acid and D-Ala.

While there are several potential functions for the *Salmonella* PcgL protein, we are now entertaining three possibilities: nutrient acquisition, virulence, and resistance to a toxic compound. In contrast to wild-type *Salmonella*, the *pcgL* mutant was unable to use D-Ala-D-Ala as a sole carbon source, suggesting that the PcgL protein may play a role in nutrient acquisition. D-Ala-D-Ala could originate from peptidoglycan-derived fragments of dying microorganisms or, as suggested by Lessard and colleagues for *E. coli* K-12 (26), as a result of peptidoglycan turnover in the same microorganism. However, it is presently unknown whether, in its natural environment, *Salmonella* encounters levels of D-Ala-D-Ala which are high enough to sustain bacterial growth.

While the *pcgL* gene is *Salmonella* specific (Fig. 5A) and transcriptionally controlled by the PhoP-PhoQ virulence regulatory system (39), a *pcgL* mutant retained its ability to cause a lethal infection in BALB/c mice. However, our experiments do not rule out the possibility that the PcgL protein may be required for virulence in other animal species known to be natural hosts for *Salmonella* or for other aspects of the pathogen-host interaction such as chronic infection. Finally, the periplasmic location of the PcgL protein suggests that it may play a defensive role, mediating the detoxification of a noxious compound that *Salmonella* may encounter in soil or water.

A cytoplasmic D-Ala-D-Ala dipeptidase in *E. coli* K-12. *E. coli* K-12 harbors a protein, $VanX_{E.\ coli}$ that is 40% identical to the enterococcal VanX and exhibits D-Ala-D-Ala dipeptidase activity (26). Yet, the $VanX_{E.\ coli}$ and PcgL proteins are not homologs because they exhibit low-level sequence identity, localize to different subcellular compartments, and are encoded by genes with different phylogenetic distributions (Fig. 5). Moreover, transcription of *pcgL* is governed by the PhoP-PhoQ regulatory system (39) and does not require RpoS (our unpublished results), the alternative sigma factor controlling transcription of several genes expressed during stationary phase. This is in contrast to $vanX_{E.\ coli}$, which is transcriptionally regulated by RpoS (26). Finally, whereas wild-type *Salmonella* could use D-Ala-D-Ala as a sole carbon source, wild-type *E. coli* K-12 did not (Fig. 7) unless the $vanX_{E.\ coli}$ gene was artificially transcribed from the *lac* promoter or in derivatives expressing the enterococcal *vanX* gene (26).

The $vanX_{E.\ coli}$ gene is immediately adjacent to an operon encoding proteins exhibiting similarity to dipeptide permeases (26). Interestingly, transcription of both $vanX_{E.\ coli}$ and the putative permease genes is under RpoS control. And growth of *E. coli* on D-Ala-D-Ala was enhanced when the putative dipeptide permease was coexpressed with $vanX_{E.\ coli}$. This led Lessard and coworkers to hypothesize that D-Ala-D-Ala generated as a result of peptidoglycan turnover may be transported by this putative peptide transporter into the cytosol, where it would be cleaved by the $VanX_{E.\ coli}$ protein into D-Ala, providing a source of carbon and energy during stationary phase (26). However, two findings argue against this hypothesis: first, as stated above, wild-type *E. coli* K-12 cannot grow on D-Ala-D-Ala (26) (Fig. 7). And second, very small amounts of D-Ala-D-Ala are generated from peptidoglycan turnover to sustain bacterial growth.

Conclusions. The PcgL and $VanX_{E.\ coli}$ proteins are likely to play different physiological roles in their respective organisms. Yet, these proteins exhibit certain features in common. (i) These enzymes are D-Ala-D-Ala dipeptidases and not carboxypeptidases. Thus, they presumably act on D-Ala-D-Ala generated by equivalent periplasmic endopeptidases cleaving between diaminopimelic acid and D-Ala on peptidoglycan that was not cross-linked and/or on peptidoglycan precursors. And (ii), *pcgL* and $vanX_{E.\ coli}$ exhibit a limited phylogenetic distribution (Fig. 5), raising the possibility that these genes may have been incorporated into the *Salmonella* and *E. coli* K-12 genomes as a result of horizontal gene transfer. In the case of the *pcgL* gene, this view is supported by the usually low G+C content of the *pcgL*-containing region. On the other hand, the G+C content and codon usage of $vanX_{E.\ coli}$ are similar to those of ancestral *E. coli* K-12 genes, suggesting that if $vanX_{E.\ coli}$ was acquired by lateral gene transfer, it must have originated from an organism with a G+C content and a codon usage similar to those of *E. coli* K-12. Further experiments will be required to determine the specific role that these enzymes play in *Salmonella* and *E. coli* K-12.

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