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Cloning, Expression, and Purification of UDP-3-*O*-Acyl-GlcNAc Deacetylase from *Pseudomonas aeruginosa*: a Metalloamidase of the Lipid A Biosynthesis Pathway

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The *lpxC* (*envA*) gene of *Escherichia coli* encodes UDP-3-*O*-acyl-GlcNAc deacetylase, the second and committed step of lipopolysaccharide biosynthesis. Although present in all gram-negative bacteria examined, the deacetylase from *E. coli* is the only example of this enzyme that has been expressed and purified. In order to examine other variants of this protein, we cloned the *Pseudomonas aeruginosa* deacetylase structural gene from a λ library as a 5.1-kb *Eco*RI fragment. The LpxC reading frame encodes an inferred protein of 33,435 Da that is highly homologous to the *E. coli* protein and that possesses a nearly identical hydropathy profile. In order to verify function, we subcloned the *P. aeruginosa lpxC* gene into the T7-based expression vector pET11a. Upon induction at 30°C, this construct yielded active protein to approximately 18% of the soluble fraction. We devised a novel, rapid, and reproducible assay for the deacetylase which facilitated purification of the enzyme in three steps. The purified recombinant protein was found to be highly sensitive to EDTA yet was reactivated by the addition of excess heavy metal, as was the case for crude extracts of *P. aeruginosa*. In contrast, deacetylase activity in crude extracts of *E. coli* was insensitive to EDTA, and the extracts of the *envA1* mutant were sensitive in a time-dependent manner. The *lpxC* gene has no significant homology with amidase signature sequences. Therefore, we assign this protein to the metalloamidase family as a member with a novel structure.

The biosynthesis of lipid A has been studied extensively in *Escherichia coli*, and the genes encoding the majority of enzymes involved in this process have been identified (16). Less is known about the mechanistic enzymology of these enzymes, and little comparative enzymology across diverse genera has been explored. In *E. coli*, the second step of this pathway is catalyzed by UDP-3-*O*-acyl-GlcNAc deacetylase, encoded by the *lpxC* (*envA*) gene (Fig. 1) (24). This enzyme follows a thermodynamically unfavorable acyltransfer reaction (2) and is a point of regulation for lipopolysaccharide (LPS) biosynthesis (18). The enzyme is essential for bacterial viability (3) and represents an attractive target for the development of antibacterial substances. Indeed, a series of small-molecule inhibitors of the enzyme possessing antibacterial properties has recently been reported, validating this notion (15). The presence of a hydroxamic acid moiety in these inhibitors hints that the deacetylase may be a metalloamidase.

As a consequence of increased genomic sequencing efforts, an increasing number of bacterial DNA sequences homologous to several LPS biosynthetic enzymes from *E. coli* have been made available. Yet to date only two sequences, from *Haemophilus influenzae* and *Neisseria gonorrhoeae*, have appeared for the deacetylase, and the enzyme from *E. coli* remains the only one that has been functionally expressed and purified (24). We desired to obtain further members of the *lpxC* gene family from other genera for comparative studies in order to gain mechanistic insights into this interesting amidase. We herein report the cloning, expression, and purification of this enzyme from *Pseudomonas aeruginosa* and the development of a rapid and sensitive assay for monitoring the enzyme's activity. As seen with wild-type cell extracts of *P. aeruginosa*, the purified enzyme is rapidly and completely inhibited by EDTA but can be reactivated by addition of an excess of heavy metals such as zinc. In contrast, the deacetylase found in wild-type *E. coli* extracts is insensitive to EDTA, yet the presence of metal was revealed by the time-dependent inactivation of deacetylase activity by EDTA in extracts of an *envA1* mutant allele. The assignment of this enzyme to the metalloprotein family and the development of more advanced assay techniques now allow increasingly directed kinetic and mechanistic studies of this regulated step of lipid A biosynthesis.

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MATERIALS AND METHODS

Materials. Sequencing-grade α-^{35}S-labeled dATP was obtained from New England Nuclear. The Sequenase DNA sequencing kit, version 2.0, was from United States Biochemical Corporation. Restriction enzymes were from Promega (Madison, Wis.). Charcoal was obtained from Sigma Chemical Co. Resins polyamide, XAD-2, SP207, SP800, and HP20 were from Supelco.

Bacterial strains. Bacterial strains and plasmids used in this study are listed in Table 1. Strains BL21(DE3) and BL21(DE3)/pLysS were purchased from Novagen. Strain JB1104 has been described previously (4).

Preparation of cell extracts. Membrane extracts of *E. coli* JB1104 were used for development of the acetate release assay and were prepared as previously described (1). Protein concentrations were determined by the method of Smith et al. with bovine serum albumin (BSA) as a standard (17).

UDP-3-*O*-acyl-GlcNAc deacetylase activity assay. The assay directly measures the release of [^{3}H]acetate from [^{3}H]acetyl-UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc. Assay mixtures contained [^{3}H]acetyl-UDP-3-*O*-acyl-GlcNAc (3 μM, 3.25 Ci/mmol), Bis-Tris (pH 5.5, 40 mM), BSA (1 mg/ml), and 0.05 to 0.5 U of enzymatic activity in a final volume of 20 μl. One unit of activity is that amount of enzyme required to release 1 nmol of acetate per min at 30°C. After 5 min of assay, a 5-μl aliquot was removed and quenched by vortexing in 1 ml of 100 mM sodium acetate containing finely ground activated charcoal (10% [wt/vol]). This stops the reaction, while the substrate adheres to the charcoal by virtue of the acyl group and the uracil ring. Sodium acetate buffer blocks the adherence of radiochemical acetate to the charcoal. At this point, the samples were stable for several hours. The samples were clarified by centrifugation in a microfuge for 30 s, and a 0.5-ml portion was removed and quantitated in 10 ml of scintillant by liquid scintillation spectrometry against a standardized quench curve. A no-enzyme control was subtracted in order to eliminate background counts. Assay results were linear from 0.1 to 0.5 mg of wild-type membrane-free crude extract per ml over 10 min.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the progress of deacetylase purification. Electrophoresis was carried out with 12% polyacrylamide gels (Novex).

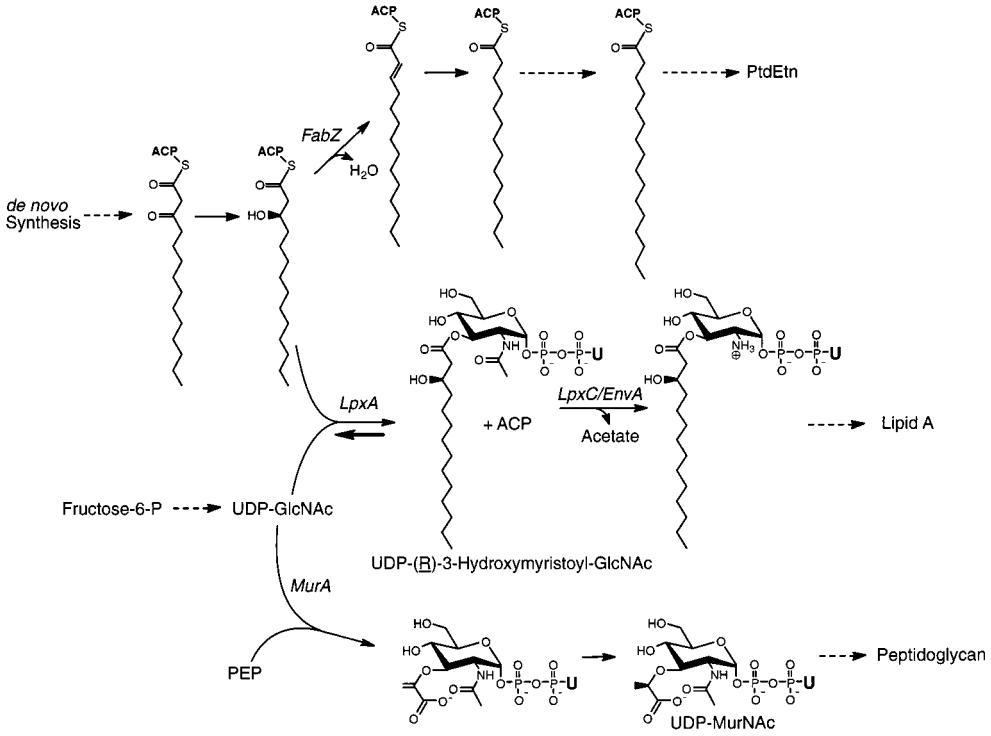

FIG. 1. Central role of UDP-3-*O*-acyl-GlcNAc deacetylase. Three major cell wall components arise from two precursors of intermediary metabolism. UDP-GlcNAc serves as a glucosamine source for both peptidoglycan and lipid A synthesis. β-Hydroxymyristoyl-ACP serves as a fatty acyl donor for both fatty acid synthesis and lipid A synthesis in *E. coli*. The deacetylase is the first thermodynamically favorable step of lipid A biosynthesis (2) and consequently is regulated (18). These reactions have been extensively reviewed (16). PtdEtn, phosphatidyl ethanolamine.

Cloning of the *P. aeruginosa lpxC* gene into pRL124. The *lpxC* gene was cloned by probing a *P. aeruginosa* λ insertion library (Clontech; DNA derived from strain ATCC 27853) with the *E. coli lpxC* gene. Preliminary experiments to find optimal hybridization conditions were conducted by probing small replicate digests of the *P. aeruginosa* genome under progressively less stringent conditions until a single band was observed in each. For this purpose, chromosomal DNA was isolated from *P. aeruginosa* MB3286 by the method of Goldberg and Ohman (9) and was digested separately with *Bam*HI, *Eco*RI, *Sal*I, *Sma*I, and *Xho*I. The digests were split into four replicate gels of 0.9% agarose, electrophoresed and blotted onto nitrocellulose with a Posiblot apparatus, and cross-linked with a UV-Stratalinker (Stratagene). Satisfactory conditions for hybridization were as follows: 42°C for 24 h in a medium consisting of 20% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 1% SDS, 150 μg of salmon sperm DNA per ml, and 1.5×10^6 cpm of ^{32}P-random-primer-labeled *E. coli lpxC* probe per ml. The blot was washed twice with 2× SSC–0.1% SDS at 42°C, followed by another wash for 10 min at room temperature. Filters were visualized by overnight autoradiography.

The complete gene was subsequently retrieved by probing 150-mm-wide nitrocellulose plaque lifts made from the library at a density of approximately 10,000 plaques per plate. Several positive plaques were purified, each containing a 5.1-kb *Eco*RI fragment. One plaque was chosen, from which a large-scale DNA preparation was made. This lambda DNA was cut with *Eco*RI, followed by heat inactivation of the enzyme. The insert was then directly ligated into pRL124 that had been previously digested with *Eco*RI and phosphatase treated. One clone carrying insert pRL124LpxC was used for further study.

Construction of the *lpxC* overproducing plasmid pET11aLpxC. Through Southern hybridization to the *E. coli lpxC* gene (as described above) and direct DNA sequencing into the ends of the insert, a 1.7-kb *Pst*I-*Eco*RI fragment was identified that contained all of the desired *P. aeruginosa lpxC* gene. This fragment was subcloned from pRL124LpxC into similarly cut and phosphatase-treated pBluescript SKII (+) (Stratagene). The insert of the resulting clone, pSAS1700, was sequenced in its entirety, revealing the complete reading frame of the *P. aeruginosa lpxC* gene. Based on this information, PCR primers to the N-terminal and C-terminal regions of this gene were designed to allow unidirectional liga-

TABLE 1. Properties of the bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
BL21(DE3)	T7 RNA polymerase lysogen	Novagen
BL21(DE3)/pLysS	T7 RNA polymerase lysogen/plasmid-encoded T7 lysozyme	19
LS620	*envA1*	Merck Culture Collection
P. aeruginosa strain		
MB3286		Merck Culture Collection
Plasmids		
pET11a	T7 expression vector, AMP^r	19
pRL124	Low-copy-number vector	ATCC[a]
pRL124LpxC	5.1-kb *Eco*RI $lpxC^+$ fragment in pRL124 replicon	This work
pET11aLpxC	$lpxC^+$ clone in pET11a	This work

[a] ATCC, American Type Culture Collection.

tion of the resulting PCR fragment into the T7-based expression vector pET11a. The 5′ primer incorporated a G/C clamp, an *Nde*I site, and 18 additional bases of coding sequence 5′-GGG CCC ATC GTA ATA TCG CAT ATG ATC AAA CAA CGC ACC-3′. Due to the presence of a *Bam*HI site in the target gene, this PCR fragment was created with a compatible *Bgl*II 3′ end. Accordingly, the 3′ primer incorporated a G/C clamp, a *Bgl*II site, tandem stop codons, and 16 additional bases of anticoding sequence 5′-GGG CCC ATC GTA GAT CTT TAC TAC ACT GCC GCC GCC-3′. PCR mixtures contained 10 ng of *Eco*RI-digested pSAS1700 plasmid DNA template, 1 μM concentrations of each primer, 200 μM concentrations of deoxynucleoside triphosphates and 5 U of *Taq* DNA polymerase in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2.5 mM $MgSO_4$. PCR conditions were 35 cycles of denaturation at 96°C for 30 s, annealing at 47°C for 30 s, and elongation at 72°C for 2 min. This was followed by a 10-min polishing at 72°C. One microliter of the resulting reaction mixture containing a single ~950-bp fragment was ligated into the pCRII vector (Invitrogen) and transformed into *E. coli* OneShot cells under ampicillin selection. One clone containing the desired insert was verified for fidelity by direct DNA sequencing. The *Nde*I-*Bgl*II fragment was then cut, gel purified, and ligated into *Nde*I-and-*Bam*HI-double-digested pET11a. The pET11aLpxC plasmid, identified as a transformant containing a 914-bp insert, was isolated by an alkaline lysis procedure (Wizard Minipreps DNA purification system; Promega) and reverified by DNA sequencing. This expression construct was transformed into chemically competent BL21(DE3)/pLysS cells (Novagen) with ampicillin selection just prior to use.

Purification of recombinant *P. aeruginosa* deacetylase. All procedures were performed at 4°C unless otherwise specified. Recombinant deacetylase protein was purified to >97% apparent purity by a combination of ion exchange, chromatofocusing, and gel exclusion chromatography. In this case, a 1-liter culture of cells growing at 30°C in Luria-Bertani (LB) medium and bearing the T7-based expression clone of the deacetylase, pET11aLpxC, was induced at an optical density at 600 nm of 0.6 with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The induction continued for 2 h, after which the cells were harvested by centrifugation at 5,000 × *g* for 10 min. The cell pellet was resuspended in 20 ml of 10 mM sodium phosphate, pH 7.0, containing 1 mM EDTA, hereafter referred to as buffer P. The resuspension was passed once through a French pressure cell at 18,000 lb/in^2, and the resulting debris was pelleted by centrifugation at 20,000 × *g* for 30 min.

The soluble fraction was diluted to 2 mg/ml and passed at 2.5 ml/min through a DEAE Sepharose ion exchange column (2.5 by 12 cm) which had been preequilibrated with buffer P. The column was washed with buffer P until absorbance returned to baseline and was eluted with a 200-ml-by-200-ml linear gradient from buffer P to buffer P containing 100 mM sodium chloride. The deacetylase eluted at approximately 20 to 30 mM salt, and the peak fractions totaling 20 ml were pooled. The protein was precipitated by bringing the solution to 75% saturation with solid ammonium sulfate and pelleting the flocculate at 20,000 × *g* for 20 min.

The solid was resuspended in 1 ml of buffer P, dialyzed against 25 mM histidine (pH 6.3), and applied to a chromatofocusing column of PBE94 (1.5 by 15.5 cm) which had been preequilibrated with 25 mM histidine (pH 6.3). The column was developed with 400 ml of polybuffer 74, pH 4.0, at a flow rate of 0.7 ml/min. Tubes for collecting fractions contained 1/10 vol of 10× buffer P in order to neutralize the fractions containing the deacetylase protein. Deacetylase activity eluted symmetrically at pH 4.76. Active fractions were concentrated by ammonium sulfate precipitation as described before and were resuspended in 1 ml of buffer P.

The sample was loaded in its entirety onto a column (1.6 by 65 cm) of Sephadex G-75 which was preequilibrated with buffer P. This buffer was also used as the eluate and the column was run at 6 ml/h, collecting 1.5-ml fractions. In our hands, the deacetylase protein had a propensity to irreversibly form catalytically inactive multimers. Use of this sizing column cleanly removes accumulated multimers at the void front, whereas active deacetylase elutes as a single monomeric peak. The LpxC protein was concentrated with a Centricon 10 microconcentrator (Amicon) to >20 mg/ml and was stored at 4°C. Yields were typically 4 to 8 mg of monomer per liter.

Synthesis of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN[^{3}H]Ac. This labeled substrate was prepared in three steps. In the first step, UDP-GlcN was prepared enzymatically from UTP and GlcN 1-P. Briefly, reactions were performed in parallel in each of two 25-mg GlcN 1-P bottles (Sigma). Reagents were added to a final volume of 3 ml in each bottle at the following final concentrations: 100 mM HEPES (pH 7.4), 32 mM UTP (pH 7.4), 32 mM GlcN 1-P, 20 mM $MgCl_2$, and 200 mM β-mercaptoethanol. The reaction was initiated by the addition of 25 U each of inorganic pyrophosphatase (Sigma) and UDP-Glc pyrophosphorylase (Sigma). The reaction proceeded at room temperature overnight. The reaction products were purified on Whatman DE52 anion-exchange resin by diluting the reaction mixture to 60 ml and loading it over a 20-ml column. The effluent was discarded, and the column was washed with 60 ml of water, which was also discarded. UDP-GlcN was recovered from the column with a 60-ml 50 mM triethyl ammonium bicarbonate wash, which was frozen and lyophilized. The sample was resuspended and relyophilized to a constant weight to yield 98 mg of UDP-GlcN (84% yield). The material was >90% pure as judged by thin-layer chromatography (TLC) and nuclear magnetic resonance analysis.

In the second step, UDP-GlcN[^{3}H]Ac was prepared from UDP-GlcN and

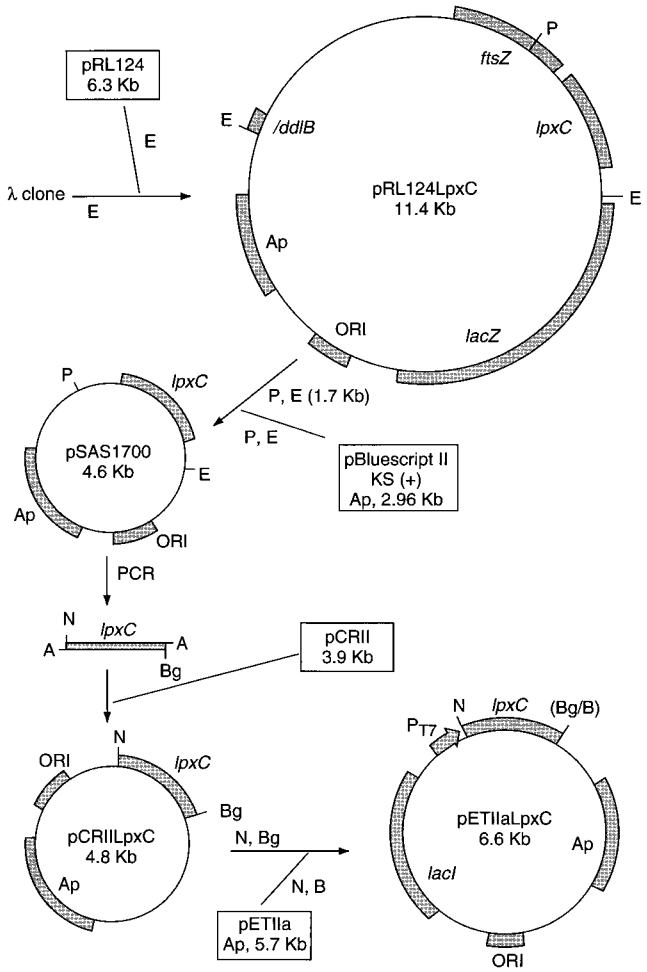

FIG. 2. Cloning of the *P. aeruginosa lpxC* gene and construction of the pET11aLpxC expression vector. Details of the construction are described in Materials and Methods. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Nco*I; P, *Pst*I; Ap, ampicillin resistance. In pET11aLpxC, ligation of the compatible *Bam*HI and *Bgl*II overlap (denoted Bg/B) destroys both sites.

[^{3}H]acetic anhydride (25 mCi, 6.5 Ci/mmol). The reaction was performed directly in the tube from the supplier (Amersham). The reaction mixture contained 300 μl of deionized water, 500 μl of methanol, 50 μl of freshly saturated sodium bicarbonate, and 115 μl of UDP-GlcN (2.26 mg, 3.85 mmol). The reaction proceeded for 10 min at room temperature and was terminated by heating the mixture at 100°C in a heating block for 2 min. The reaction mixture was diluted with 7 ml of deionized water and applied to a 5-ml DEAE column (DEAE Fast Flow; Sigma) previously equilibrated with water. The column was washed with 30 ml of deionized water followed by 20 ml of 100 mM triethylammonium bicarbonate. This cut was captured in a 50-ml screw-cap plastic tube, frozen, and lyophilized with an in-line sodium hydroxide pellet trap. The sample was relyophilized three times, resuspended in water, and titrated to pH 6.0 with base. The product was quantitated by scintillation spectrometry to yield 2.05 mCi of UDP-GlcN[^{3}H]Ac. This material was used without further purification.

In the third step, acylation of UDP-GlcN[^{3}H]Ac to form UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN[^{3}H]Ac was performed enzymatically in a reaction mixture consisting of 40 mM HEPES (pH 8.0), 1 mg of BSA per ml, 211 μM *R*-3-hydroxymyristoyl-acyl carrier protein (ACP), 211 μM (137 μCi) UDP-Glc[^{3}H]NAc, and 0.14 μg of purified UDP-GlcNAc 3-*O*-acyltransferase in a final volume of 200 μl. The sample was placed in a 600-μl Eppendorf tube with an equal volume of methanol-prewashed and water-equilibrated HP20 beads and incubated with shaking at 30°C for 1 h. After this time, the supernatant was removed and saved for later recycling. The pelleted beads were washed a total of five times with 200 μl of water, the beads being recovered each time by centrifugation. The acylated product was recovered by washing the beads a total of three times with methanol and drying the combined washes in a fresh tube. The dried product was resuspended in 30 μl of 10 mM Bis-Tris chloride, pH 5.5, and

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E. coli  MIKQRTLKRIVQATGVGLHTGKKVTLTLRPAPANTGVIYRRTDLNPPVDFPADAKSVRDTML  62
H. inf.  MIKQRTLKQSIKVTGVGLHSGEKVTLTLRPAMPNTGVVYYRTDLNPAVAFPADPNSVRDTML  62
P. aeru. MIKQRTLKNIIRATGVGLHSGEKVYLTLKPAPVDTGIVFCRTDLDPVVEIPARAENVGETTM  62
N. gon.   MLQRTLAKSIGVTGVGLHSGERVALTLHPAPENSGISFRRTDLDGEMGEQIKLNPYLDQRY  61

E. coli  CTCL...VNEHDVRISTVEHLNAALAGLGIDNIVIEVNAPEIPIMDGSAAPFVYLLLDAGID 121
H. inf.  CTAL...INEQGVRISTVEHLNAALAGLGIDNIIIEVDAPEIPIMDGSASPFIYLLLDAGIE 121
P. aeru. STTL...VKG.DVKVDTVEHLLSAMAGLGIDNAYVELSASEVPIMDGSAGPFVFLIQSAGLQ 120
N. gon.  PPFIQPSVTDKGLRVGTIEHIMSALSAYGIDNALIELNAPEIPIMDGSSLPFIYLLQDAGVV 123

E. coli  ELNCAKKFVRIKETVRVEDGDKWAEFKPYNGFSLDFTIDFNHPAIDSSNQRYAMNFSADAFM 183
H. inf.  EQNAAKKFIRIKQYVRVEDGDKWAEFKPYNGFRLDFTIDFDHPAIGKDVRNYEMNFSAQAFV 183
P. aeru. EQEAAKKFIRIKREVSVEEGDKRAVFVPFDGFKVSFEIDFDHPVFRGRTQQASVDFSSTSFV 182
N. gon.  DQKAQKRFLKILKPVEIKEAGKWVRFTPYDGFKVTLTIEFDHPVFNRSPPTFEIDFAGKSYI 185

E. coli  RQISRARTFGFMRDIEYLQSRGLCLGGSFDCAIVVDDYRVLNEDGLRFEDEFVRHKMLDAIG 245
H. inf.  HQISRARTFGFMKDIEYLQSQGLVLGGSLDNAIVLDDYRILNEDGLRFKDELVRHKMLDAIG 245
P. aeru. KEVSRARTFGFMRDIEYLRSQNLALGGSVENAIVVDENRVLNEDGLRYEDEFVKHKILDAIG 244
N. gon.  GEIARARTFGFMHEVEMMRAHNLGLGGNLNNAIVIGDTDVLNPEGLRYPDEFVRHKIXDAIG 247

E. coli  DLFMCGHNIIGAFTAYKSGHALNNKLLQAVLAKQEAWEYVTFQDDAELPLAFKAPSAVLA.  305
H. inf.  DLYMAGYNIIGDFKAYKSGHGLNNKLLRAVLANQEAWEFVTFEDKAQVPQGYVAPVQVLI.  305
P. aeru. DLYLLGNSLIGEFRGFKSGHALNNQLLRTLIADKDAWEVVTFEDARTAPISYMRPAAAV..  304
N. gon.  DLYIVGHPIVGAFEGYKSGHAVNNALLRAVLADETAYEWVEFADSDDLPDAFHELNIRNCG  309
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FIG. 3. Comparison of protein sequences for UDP-3-*O*-acyl-GlcNAc deacetylase. Sequences are from *E. coli* (P07652) (3) *H. influenzae* (*H. inf.*) (P45070) (8) and *P. aeruginosa* (*P. aeru.*) (U67855). A fourth sequence is inferred from a highly homologous reading frame from *N. gonorrhoeae* (*N. gon.*) (U58847; coding sequence 440 to 1367 with a 1-bp deletion, as described in the text). The quality of the sequence similarity between the protein inferred from this adjusted reading frame and the *P. aeruginosa* LpxC protein is 53 standard deviations away from random. The *Pseudomonas* protein harbors only one cysteine residue that is not conserved in the stack plot. The *H. influenzae* deacetylase, which overall is more like the protein from *E. coli* (77% identity) than that from *P. aeruginosa* (57% identity), utilizes preferentially the same replacements for cysteine as those used by *P. aeruginosa*. The proteins from *E. coli* and *P. aeruginosa* show 58% identity and 77% homology to each other. The reconstructed *N. gonorrhoeae* reading frame is 49% identical and 71% similar to the *P. aeruginosa lpxC* gene.

quantitated by scintilation spectroscopy. The average yield was ~14% (19 μCi) UDP-3-*O*-acyl-GlcN[^{3}H]Ac.

Demonstration of metal removal and reactivation. Extracts of *P. aeruginosa* MB3286 were incubated for 5 min at 0.125 mg/ml with 40 mM Bis-Tris (pH 6.5) containing 1 mg of BSA per ml and 2 μM EDTA at 30°C. Subsequently, a sample was removed and assayed for residual activity by the addition of substrate. The inactivation of activity was essentially instantaneous upon EDTA addition. The inactivated sample was aliquoted directly into tubes containing various metals or cations so as to bring the final metal or cation concentration to 6 μM. These metals were $Zn(II)SO_4$, $Co(II)Cl_2$, $Cu(II)Cl_2$, and $Mg(II)Cl_2$. Metal solutions were freshly prepared in Chelex-treated water in order to minimize environmental contaminants. After five min, the rescue of deacetylase activity was measured by initiating a reaction by the addition of 3 μM substrate. The recovered activities were linear. Longer incubations with metal did not alter the levels of restored activity.

Sequence analysis. All sequence comparisons and manipulations were performed using the Genetics Computer Group package, version 8.1 (Wisconsin Sequence Analysis Package; Genetics Computer Group, Inc.). LpxC peptide sequences were aligned as a single group with the PILEUP algorithm (7). Further database homologies were identified by searching SWISSPROT with PROFILEMAKE and PROFILESEARCH from this alignment (10, 11).

Nucleotide sequence accession number. The nucleotide sequence for *P. aeruginosa* described in this paper has been submitted to GenBank under the accession number U67855.

RESULTS

Cloning of the *P. aeruginosa* UDP-3-*O*-acyl-GlcNAc deacetylase gene. This gene was obtained from a λgt11 insertion library as described in Materials and Methods. The *lpxC* gene has toxic effects on cells in high-copy plasmids (3). Once a λ clone that contained a putative *lpxC* gene was identified, the *Eco*RI insert was placed into the promoterless, low-copy vector pRL124 (Fig. 2) in order to reduce the potential for rearrangements caused by selective pressure. DNA sequencing of a 1.7-kb *Pst*I-*Eco*RI fragment revealed a complete reading frame highly homologous to the *lpxC* gene in *E. coli* and *H. influenzae* and to an inferred reading frame from *N. gonorrhoeae* (Fig. 3). The hydropathy plots of these *lpxC* sequences are virtually identical (data not shown).

In preparation for protein expression experiments the LpxC reading frame was subcloned into the T7-based expression vector pET11a through the use of PCR. However, like the vector pET11a, the LpxC reading frame from *P. aeruginosa* contained a *Bam*HI site. In order to generate a compatible insert, the reading frame was amplified with a 3′ primer that contained an isoschizomeric *Bgl*II site as described in Materials and Methods (Fig. 2). The intermediate PCR product was cloned into pCRII and sequenced to verify fidelity of the *Taq*-generated reading frame prior to final subcloning into pET11a.

Fast and sensitive assay for deacetylase activity. In order to monitor the purification in a timely manner, it was desirable to improve the speed and sample-handling ability of the deacetylase assay (24). For this purpose, a new assay substrate radiolabeled in the acetate moiety was synthesized as shown in Fig. 4. In this preparation, the final acylation step was catalyzed by UDP-GlcNAc 3-*O*-acyltransferase. The equilibrium constant for this reaction strongly disfavors production of the acylated UDP-GlcNAc (2). We reasoned that the inclusion of appropriately chosen hydrophobic beads in the system might draw the reaction forward by binding UDP-3-*O*-acyl-GlcNAc as it was produced. The beads could then be washed free of major contaminants, and product could subsequently be eluted with a solvent. To test this, five bead types were investigated for their ability to enhance yield of the acylated product and for ease of this product's retrieval. Small test reactions were performed in the presence of the beads with the readily visualized substrate

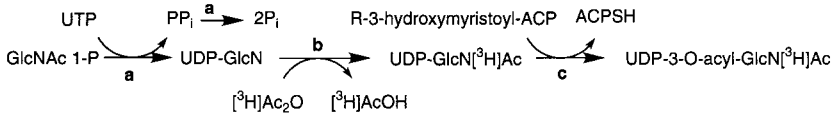

FIG. 4. Synthesis of the deacetylase assay substrate UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN[^{3}H]Ac. This radiolabeled compound was synthesized and purified as described in Materials and Methods. Steps: a, UDP-glucose pyrophosphorylase and inorganic pyrophosphatase catalysis; b, chemical acetylation; c, UDP-GlcNAc 3-*O*-acyltransferase catalysis.

TABLE 2. Enhancement of UDP-3-*O*-acyl-GlcNAc formation by hydrophobic beads

Resin type	Reaction yield (%)	Product recovered[a] (%)	Product contamination[b] (%)
No resin	10		
Polyamide	9	0.24	28
XAD-2	7	47	4.2
SP207	15	83	4.1
SP800	20	93	1.2
HP20	20	98	1.2

[a] Percentage of product recovered in MeOH eluate.
[b] Percentage of UDP-GlcNAc recovered in MeOH eluate.

[α-^{32}P]UDP-GlcNAc. After 1 h at 30°C, each bead set was washed three times with 10 mM Bis-Tris, pH 6.0, and was eluted three times with methanol. The results of each test were assessed by TLC (Table 2). The resins HP20 and SP800 each enhanced overall product formation twofold. However, the HP20 resin was somewhat more efficient in retrieving product from solution. This resin was selected for all future work.

UDP-3-*O*-acyl-GlcN[^{3}H]Ac was prepared by using this adaptation as described in Materials and Methods. The release of acetate from this radiolabeled compound was easily quantitated by quenching aliquots of the reaction mixture in a slurry of charcoal buffered in sodium acetate in order to eliminate nonspecific binding of this product. The starting material binds firmly to the charcoal. After clarification by centrifugation, the supernatant was quantitated by liquid scintillation spectrometry. The observed velocities of the deacetylase as measured with UDP-3-*O*-acyl-GlcN[^{3}H]Ac were highly comparable to those seen with the substrate [α-^{32}P]UDP-3-*O*-acyl-GlcNAc. Linearities with respect to time and protein concentration were excellent and highly reproducible (Fig. 5). Unlike previous TLC assays, data could be obtained shortly after the assay.

pH profiles of the deacetylase from *E. coli* and *P. aeruginosa*. The results of previous deacetylase activity assays employing cell extracts of *P. aeruginosa* implied that the enzyme from this organism possessed a significantly altered pH activity profile as compared to those seen in similar extracts made from several enterobacterial strains (24). For this reason, we further examined the pH activity profiles of both the *E. coli* and *P. aeruginosa* enzymes by the charcoal assay in order to compare the extent of these deviations and to determine an optimal pH at

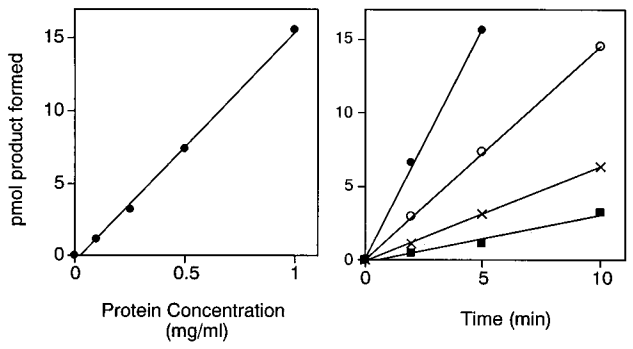

FIG. 5. Linearity of deacetylase assays with respect to time and protein concentration. The assays use membrane-free crude extracts of *E. coli* JB1104 (4). The operation of the assay is described in Materials and Methods. Left panel, Linearity with respect to protein concentration at 5 min; right panel, linearity with respect to time at the following concentrations of extract: 1 mg/ml (●), 0.5 mg/ml (○), 0.25 mg/ml (×), and 0.1 mg/ml (■).

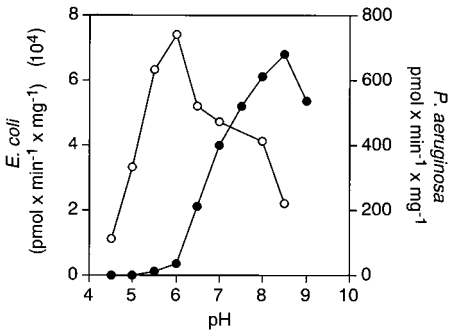

FIG. 6. pH profile of UDP-3-*O*-acyl-GlcNAc deacetylase from *P. aeruginosa* and *E. coli*. Activities were determined at a constant ionic strength of 100 mOS as described in the text. Data are from wild-type *P. aeruginosa* cell-free extracts (●) and purified recombinant *E. coli* deacetylase (○).

which to monitor the expression and purification of the cloned *P. aeruginosa* deacetylase. In cell-free crude extracts of *E. coli*, the deacetylase substrate is unstable at neutral and basic pH by virtue of the thermodynamically favored reverse reaction, catalyzed by the LpxA protein, from which this substrate is created (Fig. 1). The abundance of free ACP in crude extracts exacerbates this problem. However, at low pH, the LpxA enzyme is inactive and the deacetylase assay is facile. In contrast, the deacetylase from *P. aeruginosa* was easily assayed at any pH in cell-free crude extracts by using the *E. coli* substrate, because the LpxA protein in this organism greatly prefers a substrate containing an acyl group that is four methylene units shorter (21). Accordingly, deacetylase activity profiles were measured as a function of pH on the purified deacetylase from *E. coli* and in cell-free crude extracts prepared from *P. aeruginosa* in a constant-ionic-strength buffer system (I = 100 mOs) of ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid], Tris, and diethanolamine (6). The *P. aeruginosa* deacetylase showed a broad activity optimum peaking at pH 8.5, in marked contrast to the purified *E. coli* protein, whose optimum is at pH 6.0 under these conditions (Fig. 8). At the latter pH, the specific activity of the *Pseudomonas* protein was less than 5% of the maximal specific activity. Consequently, all subsequent deacetylase assays involving the *P. aeruginosa* protein were performed at pH 8.0.

Expression of recombinant *P. aeruginosa* UDP-3-*O*-acyl-GlcNAc deacetylase. The *lpxC* gene was expressed from pET11aLpxC with freshly transformed BL21(DE3)/pLysS cells (19). When grown in LB medium, the induced system directed massive overexpression of an apparent 38.6-kDa polypeptide, as indicated by SDS-PAGE on whole cells. This polypeptide was somewhat larger than both the 33.4-kDa polypeptide predicted from sequence data and the *E. coli* protein which migrates precisely at its true molecular mass of 34 kDa (24) (Fig. 6). Unfortunately, cell growth and induction at 37°C in LB medium with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) resulted in the retrieval of very little soluble deacetylase activity in extracts. SDS-PAGE analysis demonstrated a corresponding lack of soluble protein, with the vast majority residing in inclusion bodies (data not shown). Moderation of the growth temperature to 30°C greatly enhanced recovery of soluble, active protein. Results were similar in both rich (LB) and M9 minimal media. All further expression work was performed at this lower temperature in rich medium. The deacetylase represents approximately 18% of the soluble protein in such extracts as determined by SDS-PAGE, and this value represents a 900- to 1,000-fold overexpression of activity relative to

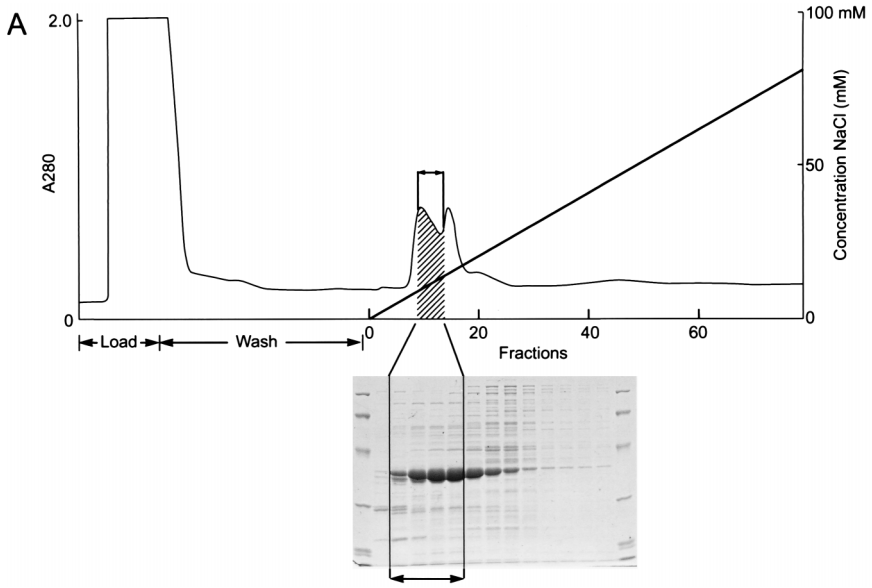

FIG. 7. Purification of *P. aeruginosa* deacetylase by DEAE Sepharose chromatography. Details of the purification are described in Materials and Methods. Fractions with the highest levels of activity were analyzed by SDS-PAGE on 12% acrylamide gels (Novex) with Coomassie staining. The double-headed arrow indicates pooled fractions. No activity was observed in the load and wash volumes.

that of the wild type. Cell-cracking buffer contained EDTA, which inhibits the activity of the *P. aeruginosa* deacetylase. Activity was easily restored by dilution into buffer containing 50 μM $ZnSO_4$ just prior to assay (see below). Recombinant deacetylase was purified from this starting point.

Purification of recombinant *P. aeruginosa* deacetylase. The enzyme was purified in three steps from a broken-cell lysate as described in Materials and Methods (Fig. 7 to 9). As seen from Table 3, the largest loss of yield was observed at the chromatofocusing step (Fig. 8). Though the loss of yield was undesirable, this step removed several otherwise persistent contaminants. On the subsequent size exclusion column, we often observed a large protein peak at the void front, the magnitude of which varied between preparations (Fig. 9). This material was identified by SDS-PAGE as multimeric LpxC protein. Only the LpxC protein eluting at apparent monomeric size was catalytically active. These multimers presumably formed during ammonium sulfate precipitation and/or during chromatofocusing and were not studied further.

Demonstration of a metal requirement for deacetylase activity. The deacetylase activity observed in cell extracts of wild-type *P. aeruginosa* was inhibitable by EDTA. This activity was restored by the addition of certain metals. Complete inactivation was obtained by as little as 2 μM EDTA, and activity was

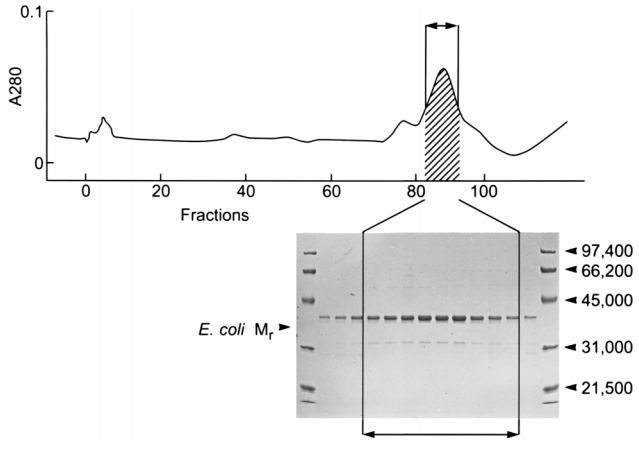

FIG. 8. Purification of *P. aeruginosa* deacetylase by chromatofocusing separation. Activity eluted at pH 4.76. The *P. aeruginosa* deacetylase migrates more slowly than the *E. coli* enzyme (arrowhead) on SDS-acrylamide gels. No evidence for contamination with the chromosomally encoded *E. coli* deacetylase was found at any protein loading (data not shown). The double-headed arrow indicates pooled fractions.

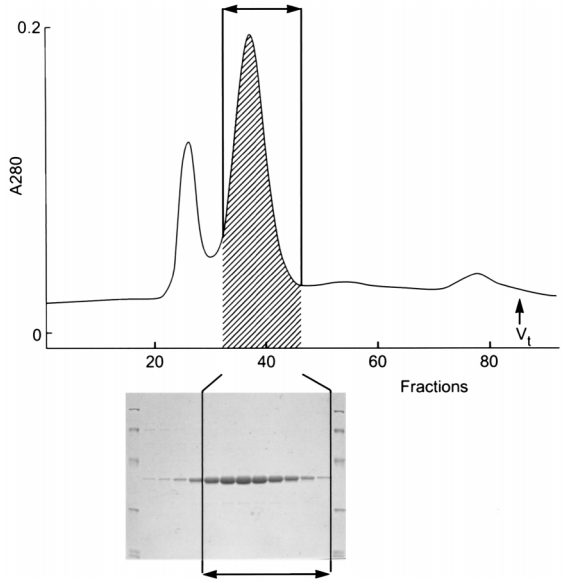

FIG. 9. Purification of *P. aeruginosa* deacetylase by Sephadex G-75 chromatography. The faster migrating peak consisted of inactive aggregated monomer, as determined by SDS-PAGE. The double-headed arrow indicates pooled fractions.

TABLE 3. Purification of *P. aeruginosa* UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase from *E. coli* BL21(DE3)/pLysS containing pET11aLpxC

Step	Total vol (ml)	Total protein (mg)	Total units (nmol × min^{-1})[a]	Sp act (nmol × min^{-1} × mg^{-1})	Fold purification (relative)	Yield[b] (%)
Crude extract	14	130	35,490	273	1	100
DEAE Sepharose	20	40	31,100	778	2.85	88
Chromatofocusing	28	5.6	6,412	1,147	4.2	18
G-75 Sepharose	12	3.7	4,944	1,330	4.9	14
Concentration	0.35	3.4				

[a] Activity measured at pH 8.0 in the presence of 50 μM $ZnSO_4$ as described in Materials and Methods.
[b] Yield of active monomer.

restored by the addition of excess (6 μM) metal to the reaction mixture (Table 4). Both inactivation and restoration of deacetylase activity were essentially instantaneous. In contrast, addition of either magnesium or calcium failed to restore significant activity, thus confirming a requirement for a heavy metal as opposed simply to a divalent cation. Combinations of metals did not give reactivation to any greater degree than that seen with zinc. Inactivation-reactivation experiments with purified enzyme gave similar results (Table 4), supporting the conclusion that the purified recombinant protein functioned as did the wild type.

In contrast, cell extracts of wild-type *E. coli* were unaffected by exposure to EDTA at concentrations of up to 0.5 mM for 30 min. Inclusion of zinc at low micromolar levels in deacetylase assays of such wild-type cell extracts did not significantly affect assay velocity (Table 4). Yet, it seemed possible that a judiciously mutated wild-type *E. coli* protein, perhaps one which had lost a metal ligand or one which caused distorted metal binding, might be more sensitive to chelators. One chromosomally encoded mutant in the *lpxC* gene encoding *E. coli* UDP-3-*O*-acyl-GlcNAc deacetylase has been described (3). This mutant, the chromosomally located *envA1* allele, harbors a missense mutation that converts His-19 to a tyrosine residue in an N-terminal region that is highly conserved in *Pseudomonas*. Cell-free crude extracts of this mutant possess greatly reduced residual activity compared to those of the wild type (ca. 5% of wild-type activity [24]). Interestingly, this residual deacetylase activity, for which there is no wild-type background, was found to be sensitive to EDTA in a time-dependent manner (Fig. 10). In the standard assay, concentrations of EDTA ranging from 200 to 700 μM resulted in observed inactivation rate constants which varied by approximately 30% (0.047 ± 0.0039 min^{-1} at 200 μM to 0.068 ± 0.0048 min^{-1} at 700 μM), less than the 3.5-fold difference in chelator concentration, suggesting that much of the inactivation was independent of EDTA concentration. Such a case can arise when an enzyme loses a metal spontaneously and the metal is then trapped by a bystanding chelator, as has been demonstrated for angiotensin-converting enzyme (5). This mutant, though catalytically impaired, provided a setting in which to observe an *E. coli* allele coding for a deacetylase that undergoes inactivation by EDTA.

The *envA1*-encoded deacetylase which had been substantially inactivated by exposure to 20 μM EDTA for 2 h on ice, was reactivated by addition of 30 μM metal to the mixture in much the same manner as that described above for the wild-type *Pseudomonas* enzyme, albeit in a somewhat different rank order of competency (Table 4). However, these differences in effective activity restoration may well be a consequence of the mutant phenotype itself rather than being clearly reflective of the metal preference of the wild-type enzyme. From these combined results, we infer that the wild-type *E. coli* enzyme is also a metalloprotein that has a more stably bound metal than that of the EnvA1 mutant protein and that is less accessible to EDTA than the wild-type *P. aeruginosa* deacetylase.

TABLE 4. Inactivation and reactivation of UDP-3-*O*-acyl-GlcNAc deacetylase from several sources by divalent cations

Additive	Relative deacetylase activity of enzyme (%) from:			
	P. aeruginosa		*E. coli*	
	Wild-type cell extract	Purified enzyme[a]	Wild-type cell extract	*envA1* extract
Control (no additions)	100	100	100	100
EDTA (2 μM, no incubation)	1.5	<0.1	100	100
EDTA (20 μM, 2 h, 0°C)	—[b]	—	~100	11
Post-EDTA additions				
Chelex-water	1.1	<0.1	—	—
Zn(II)[c]	142	100	~100	30
Co(II)	83	—	—	68
Cu(II)	80	—	—	—
Mg(II)	1.7	<5	—	8

[a] Recombinant protein.
[b] —, not determined.
[c] Chloride salts supplied at 6 μM.

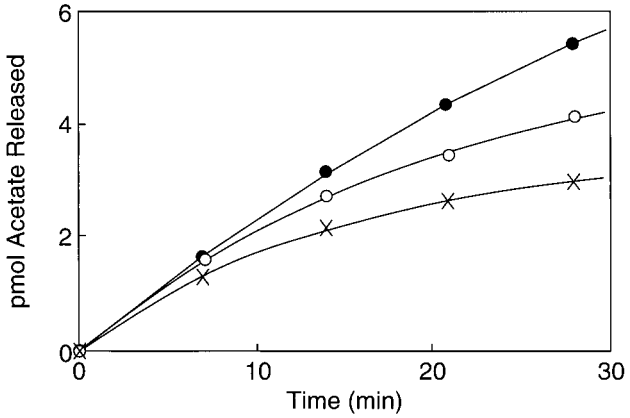

FIG. 10. Time-dependent inactivation by EDTA of deacetylase activity in extracts of an *E. coli envA1* mutant. Deacetylase assays were performed on membrane-free extracts prepared from *E. coli* LS620 harboring the *envA1* allele. Briefly, 0.62 mg of protein per ml was incubated on ice in complete assay cocktail minus substrate in the presence of several concentrations of EDTA. For each concentration of chelator, portions of the mixture were taken from a single reaction tube and assay was initiated by the addition of the missing substrate at 7, 14, 21 and 28 min after addition of chelator. Lines are theoretical fits to a first-order process. Data are for uninhibited samples (●) and samples inhibited by 20 (○) and 700 μM EDTA (×).

DISCUSSION

By probing genomic Southern blots of variably digested *P. aeruginosa* DNA at a range of stringencies with the *E. coli lpxC* gene, we were able to define conditions under which a single fragment gave a significant hybridization signal. Using these conditions, we were able to identify clones within a λgt11 insertion library that contained this gene. These positive clones harbored a 5.1-kb *Eco*RI fragment that was identical in size to the positive band appearing in the *Eco*RI-digested lanes of the original Southern genomic blots (data not shown). Initial DNA sequencing of the recovered fragment in pRL124LpxC revealed homologies to the *E. coli ddlB* gene and to the *lpxC* gene at each end of the fragment (Fig. 2). The spacing of these sequences was exactly that expected for the corresponding fragment from *E. coli* and indicated the presence of the complete *lpxC* gene. Further sequencing of a subclone revealed the reading frame as well as a sequence from the upstream *ftsZ* gene, conserved in context as in *E. coli* and *H. influenzae*.

The expression of soluble recombinant *P. aeruginosa* deacetylase in *E. coli* was most affected by reduced temperature rather than by medium composition. The activity of this deacetylase was readily assayed by using the substrate UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN[^{3}H]Ac. Presumably, this enzyme would encounter the *R*-3-hydroxydecanoyl derivative of UDP-GlcNAc in vivo. We have not investigated the relative activity of the purified *P. aeruginosa* deacetylase against these two substrates. However, the high level of activity of the protein in the standard assay is consistent with the observations of Williamson et al. that the deacetylases from wild-type crude extracts of several organisms do not appear to measure the substrate's acyl chain length (21).

The charcoal-based acetate release assay gives a faster determination of enzyme activity than previously described ^{32}P-based systems, allowing correspondingly greater experimental flexibility. We have used this simplified approach to demonstrate the inactivation of deacetylase activity in both crude extracts of wild-type *P. aeruginosa* and the purified recombinant enzyme by the metal chelator EDTA. This inhibition was subsequently relieved by the addition of excess metal. A heavy metal was required as opposed to a divalent cation. However, the data are insufficient to assign the metal a structural or catalytic role. This ready deactivation and then reactivation with any of several metals makes it impossible to know with certainty what metal is used for this enzyme in vivo, although zinc, for reasons of natural abundance and activity restoration capability, seems a likely candidate. The deacetylase primary sequence data from all currently available sources does not harbor any well-recognized metal-chelating motifs. We therefore assign the protein to the metalloamidase family as an apparently novel member.

In sharp contrast, the deacetylase from wild-type *E. coli* was not significantly susceptible to inhibition by EDTA. Yet, the *E. coli* enzyme does appear to harbor a metal, as shown by the increased sensitivity of the EnvA1 mutant protein to this chelator. It is tempting to speculate that the H19Y mutation harbored by this mutant has disrupted a metal ligand; this histidine is conserved among all deacetylases described to date. However, given the grossly reduced catalytic activity of this protein (24), it seems equally possible that more pleiotropic damage has been done in this allelic variant.

The dramatic difference in inactivation rates between the deacetylases from *E. coli* and *P. aeruginosa* suggests that these enzymes may have different K_ds for metal binding or perhaps do not chelate metal with the same ligation sphere. Alternatively, EDTA may be denied access to the active site of the *E. coli* protein by charge repulsions not seen in the *Pseudomonas* variant.

A TFASTA search of GenBank revealed a fourth putative member of the LpxC family in a DNA fragment from *N. gonorrhoeae* (Fig. 3). This reading frame was encoded in two out-of-frame segments that became contiguous after deletion of a single cytosine at bp 695 of accession number U58847. This cytosine may represent a sequencing error.

An alignment of these deacetylase protein sequences reveals the strong similarities among them. Curiously, the alignment shows a central region of maximal dissimilarity followed by a large block of identity. This may reflect either a two-domain structure for the deacetylase or a loop of great variability. Like *Haemophilus* and *Neisseria* proteins, the *Pseudomonas* protein harbors only one cysteine residue, but it is not conserved in sequence. On the basis of this somewhat cavalier usage of cysteine among these otherwise quite similar proteins, it seems unlikely that the metal carried by the deacetylase is anchored via a cysteinyl ligand. Other candidate ligands include histidine, of which five are completely conserved, and the acidic amino acids glutamate and aspartate, of which potentially 20 loci are conserved. Appropriate metal replacement derivatives combined with spectroscopy and site-directed mutagenesis should reveal these ligands.

Many deacetylases are known. Several appear to be metalloproteins, including N^8-acetylspermidine deacetylase from rat liver, an enzyme very effectively inhibited by metal-coordinating substrate analogs (12). An N^{α}-acetylhistidine deacetylase found in both trout (22) and tuna (14) is inhibited by chelators and is reactivated by the readdition of excess Co(II). These enzymes have not been cloned, and no direct comparisons can yet be made with the UDP-3-*O*-acyl-GlcNAc deacetylase in order to determine whether they are members of a broader family of metallodeacetylases.

The eukaryotic nuclear enzyme histone deacetylase has been shown to be inhibited by trichostatin A, a natural product possessing a hydroxamate moiety (23). Hydroxamic acid moieties are potent metal chelators, and the strong potency of this inhibitor (K_i = 3.4 nM) (23) suggests that this enzyme is also a metalloamidase. Intriguingly, histone deacetylase was recently cloned from human Jurkat cells (20), but no structural homologies to the bacterial lipid A deacetylase were readily apparent. If these enzymes share homologous metal signatures, then these features are dispersed among noise.

UDP-3-*O*-acyl-GlcNAc deacetylase is an enzyme that is essential to bacterial viability (3) and as such represents a likely target for the development of novel antibiotics. Onishi et al. (15) have described a set of hydrophobic hydroxamic acids that are inhibitors of UDP-3-*O*-acyl-GlcNAc deacetylase and possess antibacterial activity. These inhibitors are believed to bind to an active-site metal (15), a supposition consistent with results reported here. Small-molecule metalloprotease inhibitors of extraordinarily high potency have been described (10 to 27 fM against carboxypeptidase A) (13). An X-ray model of these proteins inactivated by the hydroxamate inhibitors would greatly assist in the development of inhibitors of increased potency. Such a detailed understanding of deacetylase structure may reveal new routes to the design and development of potentially new antibiotic substances of great potency.

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