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CDP-Alcohol Hydrolase, a Very Efficient Activity of the 5′-Nucleotidase/UDP-Sugar Hydrolase Encoded by the *ushA* Gene of *Yersinia intermedia* and *Escherichia coli*▽†

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Nucleoside 5′-diphosphate-X hydrolases are interesting enzymes to study due to their varied activities and structure-function relationships and the roles they play in the disposal, assimilation, and modulation of the effects of their substrates. Few of these enzymes with a preference for CDP-alcohols are known. In *Yersinia intermedia* suspensions prepared from cultures on Columbia agar with 5% sheep blood, we found a CDP-alcohol hydrolase liberated to Triton X-100-containing medium. Growth at 25°C was deemed optimum in terms of the enzyme-activity yield. The purified enzyme also displayed 5′-nucleotidase, UDP-sugar hydrolase, and dinucleoside-polyphosphate hydrolase activities. It was identified as the protein product ($UshA_{Yi}$) of the *Y. intermedia ushA* gene ($ushA_{Yi}$) by its peptide mass fingerprint and by PCR cloning and expression to yield active enzyme. All those activities, except CDP-alcohol hydrolase, have been shown to be the properties of UshA of *Escherichia coli* ($UshA_{Ec}$). Therefore, $UshA_{Ec}$ was expressed from an appropriate plasmid and tested for CDP-alcohol hydrolase activity. $UshA_{Ec}$ and $UshA_{Yi}$ behaved similarly. Besides being the first study of a UshA enzyme in the genus *Yersinia*, this work adds CDP-alcohol hydrolase to the spectrum of UshA activities and offers a novel perspective on these proteins, which are viewed here for the first time as highly efficient enzymes with k_{cat}/K_m ratios near the theoretical maximum level of catalytic activities. The results are discussed in the light of the known structures of $UshA_{Ec}$ conformers and the respective homology models constructed for $UshA_{Yi}$, and also in relation to possible biological functions. Interestingly, every *Yersinia* species with a sequenced genome contains an intact *ushA* gene, except *Y. pestis*, which in all its sequenced biovars contains a *ushA* gene inactivated by frameshift mutations.

Nucleoside 5′-diphosphate-X (NDP-X) hydrolases with different specificities for the nucleoside (N) and X moieties of the NDP-X substrates are widespread among all the kingdoms of life. They possess interesting properties by virtue of their varied activities and relationships between structure and function and the roles they play in the disposal, assimilation, or modulation of the effects of their substrates. Many NDP-X hydrolases belong to the Nudix protein superfamily, characterized by a conserved amino acid motif and by their reputation for being housecleaning enzymes (5, 28). However, there are also non-Nudix NDP-X hydrolases, including mammalian enzymes, like nucleotide pyrophosphatases/phosphodiesterases (6), dinucleoside triphosphatase (42), and ADP-ribose/CDP-alcohol pyrophosphatase (ADPRibase-Mn) (10, 11), or, relevant to this work, the bacterial 5′-nucleotidase/UDP-sugar hydrolase (UshA) (see below).

The *Escherichia coli* 5′-nucleotidase/UDP-sugar hydrolase ($UshA_{Ec}$) is a periplasmic metallophosphoesterase expressed from the *ushA* gene as an immature precursor which, upon export to the periplasm, undergoes maturation by proteolytic cleavage of a signal sequence (7, 8, 13). The substrate specificity of $UshA_{Ec}$ is complex, because it hydrolyzes many nucleotides and NDP-X compounds, but not without discrimination (Fig. 1A to C), so that different types of activities have been defined: 5′-nucleotidase (including apyrase-like activity), UDP-sugar hydrolase, and dinucleoside polyphosphate hydrolase activities (18, 20, 32, 38). Extensive structural studies of mature $UshA_{Ec}$ have been performed, including solving the structures of several enzyme conformers in complex with substrate (ATP), substrate-analog inhibitor (α,β-methylene-ADP), or products (adenosine and phosphate) (24–26, 39, 40). However, the structural basis of its substrate specificity is still unsolved.

Few NDP-X hydrolases with a preference for CDP-X substrates are known. Besides the above-mentioned ADPRibase-Mn, there are two bacterial Nudix enzymes: the bifunctional YZGD from *Paenibacillus thioaminolyticus*, which is both a pyridoxal phosphatase and an NDP-X hydrolase with some resemblance to the specificity of ADPRibase-Mn (46), and a CDP-choline hydrolase identified in a survey of the Nudix hydrolases present in the *Bacillus subtilis* genome (48).

Here, starting from a CDP-alcohol hydrolase activity recovered from bacterial suspensions of *Yersinia intermedia*, we found that the UshA ortholog in this species ($UshA_{Yi}$), and $UshA_{Ec}$ itself, hydrolyzes CDP-alcohols (Fig. 1D) with very high k_{cat}/K_m ratios. Besides being the first study of a UshA

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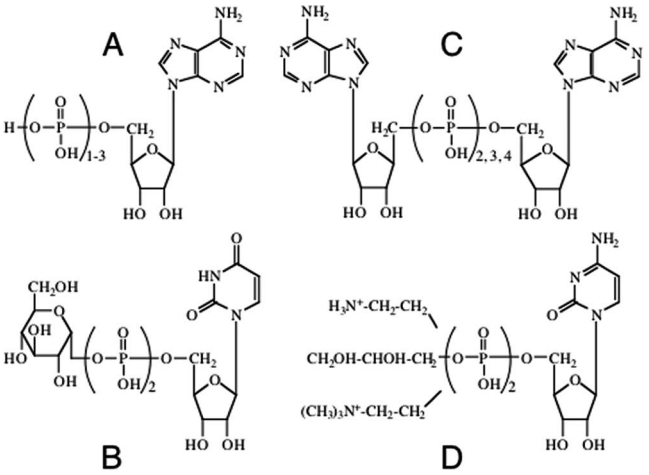

FIG. 1. Major groups of substrates for the activities of $UshA_{Ec}$ and $UshA_{Yi}$ enzymes. The structures shown are typical substrates hydrolyzed by the known 5′-nucleotidase and apyrase-like (AMP, ADP, and ATP are shown) (A), UDP-sugar hydrolase (UDP-glucose is shown) (B), or dinucleoside polyphosphate hydrolase (Ap_2A, Ap_3A, and Ap_4A are shown) (C) activities and by the novel CDP-alcohol hydrolase activity (CDP-ethanolamine, CDP-glycerol, and CDP-choline are shown) (D) reported in this work.

enzyme in the genus *Yersinia*, this work makes the unexpected addition of CDP-alcohol hydrolase to the spectrum of UshA activities and offers a novel perspective on these proteins, which appear to be highly efficient enzymes acting near catalytic perfection.

MATERIALS AND METHODS

Bacteria, media, and other products for bacterial cultures. The isolation of the *Y. intermedia* strain BA-1 was accidental, since it was obtained as a contaminant of chromatographic fractions of rat lung supernatant. *Y. intermedia* colonies were originally isolated from cultures in Columbia agar with 5% sheep blood (COS). They were positively identified with MicroScan Combo Gram Negative 1S panels from Siemens (Madrid, Spain) and ID 32 GN galleries from bioMérieux (Madrid, Spain). The strain is deposited in the Colección Española de Cultivos Tipo (Burjassot, Valencia, Spain) with accession no. CECT 7230. For this work, we used glycerol cultures kept frozen at −80°C in our laboratory. *E. coli* BL21 and BL21(DE3) were purchased as transformation-competent, Gold-type preparations from Stratagene (Cultek, Madrid, Spain).

Precast COS plates were from bioMérieux. Liquid Luria-Bertani (LB) medium contained 10 g/liter Bacto tryptone, 5 g/liter yeast extract (both from Difco, Francisco Soria Melguizo, Madrid, Spain), and 5 g/liter NaCl. For solid LB medium, the liquid medium was supplemented with 15 g/liter Bacto agar (from Difco). For culture of plasmid-transformed *E. coli* cells, 0.1 g/liter ampicillin (from Sigma-Aldrich Química, Madrid, Spain) was added to LB media. For induction experiments, isopropylthiogalactoside (IPTG) (from Roche, Barcelona, Spain) was used at the concentrations indicated.

Chemicals and biochemicals. All the inorganic salts and acids, EDTA, and sodium dodecyl sulfate (SDS) were from Merck. Triton X-100, all the nucleotidic compounds, and other enzyme substrates tested were from Sigma-Aldrich. Tris was from Roche. Auxiliary enzymes for assays of the activities or the reaction products were from Sigma-Aldrich (glycerol kinase and lactate dehydrogenase) or from Roche (alkaline phosphatase, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, hexokinase, phosphoglucomutase, pyrophosphatase, and pyruvate kinase).

Products for molecular biology. Enzymes used for cloning included NotI and SalI (Roche), T4 DNA ligase (New England Biolabs, Izasa, Barcelona, Spain), and *Pfu* ultra-high-fidelity DNA polymerase (Stratagene). Plasmid pGEX-6P-3 was purchased from GE Healthcare (Barcelona, Spain), and pLM-2, containing the *E. coli ushA* gene ($ushA_{Ec}$), was prepared as described by McMillen et al. (29).

Purification of endogenous CDP-alcohol hydrolase of *Y. intermedia*. Bacterial cells from glycerol cultures kept at −80°C were seeded on 10-cm COS plates and grown for 36 h at 25°C. The cells were scraped from the surfaces of four plates with a stainless steel spatula, resuspended in 24 ml of buffer tMg (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$), sedimented by centrifugation at 10,000 × *g* (25°C), and resuspended in buffer tMg supplemented with 5 mg/ml Triton X-100. After standing for 30 min at 25°C, the cells were sedimented by centrifugation for 10 min at 10,000 × *g* at 4°C. The supernatant of this centrifugation constituted step 1 of CDP-alcohol hydrolase purification, and it was chromatographed in a Sephacryl S-200 column (83 cm by 2.4 cm; GE Healthcare) equilibrated and eluted with buffer tMg supplemented with 0.1 M KCl at a flow rate of 17 ml/h (purification step 2). Fractions of 4.5 ml were collected and assayed for CDP-ethanolamine hydrolase activity. Those corresponding to the single activity peak recovered at an elution volume of ~240 ml were pooled; dialyzed for 24 h against 1 liter of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ (with one buffer change); and applied to a Q-Sepharose column (25 cm by 1.6 cm; GE Healthcare) equilibrated in dialysis buffer. After application of the sample, the column was washed at 25 ml/h with 150 ml of the same buffer, followed by a linear 200-ml gradient of 0 to 400 mM KCl in the same buffer. The enzyme was recovered as a single peak of CDP-alcohol hydrolase activity centered around the gradient fraction with ~160 mM KCl. This was the preparation used for enzyme characterization (step 3) (see Table S1 in the supplemental material). However, for peptide mass fingerprinting (PMF) of protein bands potentially associated with CDP-alcohol hydrolase activity, an additional purification step 4 was performed. The pooled fractions of the step 3 peak were diluted 1:1 in 5 mM $MgCl_2$ and applied to a Bio-Gel HTP column (25 cm by 1.6 cm; Bio-Rad) equilibrated in 5 mM $MgCl_2$. The unbound protein was washed with the same salt solution, and the enzyme was eluted with a 100-ml linear gradient of 0 to 400 mM sodium phosphate, pH 7. Due to the high phosphate concentration, the chromatographic profile of CDP-ethanolamine hydrolase could not be determined using the P_i liberation assay (see below). Instead, the enzyme was assayed by measuring the formation of the yellow product 4-nitrophenol using bis-*p*-nitrophenylphosphate as the substrate. The active fractions were pooled, dialyzed twice against 350 volumes of buffer tMg, assayed for CDP-ethanolamine hydrolase activity, and immediately submitted to SDS-polyacrylamide gel electrophoresis (PAGE) and then processed for PMF analysis (Servicio de Proteómica, UAM, Madrid, Spain).

PCR cloning of the *Y. intermedia* ortholog of the *ushA* gene. Genomic DNA was isolated from *Y. intermedia* CECT 7230 using the DNA Isolation Kit for Cells and Tissues according to the manufacturer's instructions (Roche). The *ushA* gene ortholog open reading frame (ORF) was amplified with primers accorded to the sequence that in the genome of *Y. intermedia* ATCC 29909 codes for the hypothetical protein ZP_00834762 (the candidate pinpointed by the PMF of the endogenous CDP-alcohol hydrolase of *Y. intermedia*). The forward primer, GG ATAGTCGACATGCGTTTTTCATTACCGACC, contained a SalI site (underlined) followed by the first seven ORF codons; the reverse primer, GTCAGGC GGCCGCTTACTTATAAACAATCTC, contained a NotI site (underlined) followed by the reverse complement of the last six codons, including the stop codon. A product of the expected size (1,653 bp) was obtained, cut with SalI and NotI, and inserted into the corresponding sites of the pGEX-6P-3 plasmid to obtain plasmid pGEX-6P-3-Yi-*ushA*. Both strands of the insert were sequenced in the Servicio de Secuenciación Automática (IIB, CSIC-UAM, Madrid, Spain).

Expression and purification of recombinant UshA proteins from *Y. intermedia* and *E. coli*. To express the product of the *Y. intermedia ushA* gene, liquid LB cultures of BL21 cells transformed with pGEX-6P-3-Yi-*ushA* were grown overnight at 37°C. Five milliliters of the overnight culture was inoculated in 100 ml of fresh medium and cultured at 28°C until the A_{600} reached 0.8; then, IPTG (0.5 mM) was added and incubation continued for 2 h. The postsonication supernatant obtained in 10 ml of 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50 mM KCl containing one dissolved tablet of protease inhibitor cocktail (Complete Mini; Roche) was concentrated to 2.7 ml by ultrafiltration. For purification, this preparation was fractionated by gel filtration in a Sephadex G-75 column (135 cm by 0.9 cm; GE Healthcare) equilibrated and eluted with buffer tMg with 50 mM KCl at a flow rate of 2 ml/h. Fractions (1 ml) were collected and assayed for CDP-ethanolamine hydrolase and 5′-nucleotidase activities. A single peak with both activities was collected at a V_e of ~58 ml. The active fractions were pooled, dialyzed for 24 h against 100 volumes of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and chromatographed in a Q-Sepharose column as described for the endogenous CDP-alcohol hydrolase of *Y. intermedia* (see above).

To overexpress the product of the *E. coli ushA* gene, liquid LB cultures of BL21(DE3) cells transformed with pLM-2 were processed as described above, except that 1 mM IPTG was used for induction and, rather than lysing the bacteria by sonication, the periplasm was obtained (29) and used for enzyme

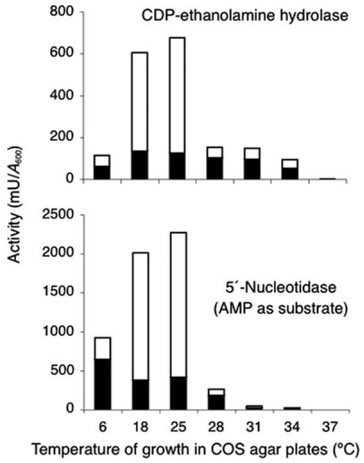

FIG. 2. Recovery of CDP-ethanolamine hydrolase and 5′-nucleotidase activities from *Y. intermedia* suspensions. CECT 7230 cells were collected from the surfaces of four COS plates and processed as described in Materials and Methods for the purification of endogenous CDP-alcohol hydrolase, except that (i) COS cultures were grown at different temperatures and (ii) after centrifugation to obtain the step 1 supernatant, the cell precipitates were resuspended in 10 ml of buffer tMg with Triton X-100 and were lysed by sonication. Enzyme activities were assayed in the supernatants (white portions of the bars) and in the lysed precipitates (black portions of the bars). The activities are expressed relative to the numbers of cells in the suspensions as estimated by A_{600} measurements.

purification as described for the endogenous CDP-alcohol hydrolase of *Y. intermedia* up to step 3 (see above).

Enzyme activity assays. Unless otherwise indicated, phosphohydrolytic activities were determined at 37°C by discontinuous assay of P_i liberation, either directly from substrates with a terminal phosphate (chain) or coupled to alkaline phosphatase for substrates without terminal phosphates. The standard reaction mixtures (50 to 400 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, an amount of enzyme sample within the linearity range, 0.5 mM substrate, and, when needed, 9 units/ml alkaline phosphatase. The incubations were stopped by the addition of one of the P_i reagents indicated below.

Assays of enzyme reaction products. P_i was assayed colorimetrically with a dodecyl sulfate-ascorbate-molybdate reagent prepared in two different versions depending on the sensitivity required (35): either 0.7 ml of the standard reagent was added to 50- to 100-μl samples or 0.2 ml of a concentrated reagent was added to 0.4-ml samples.

The amount of glycerol was assayed spectrophotometrically with glycerol kinase coupled to phosphoenolpyruvate kinase and lactate dehydrogenase (14), L-glycerol-3-phosphate with glycerol-3-phosphate dehydrogenase (30), glucose with hexokinase coupled to glucose-6-phosphate dehydrogenase (3), and glucose-1-phosphate with phosphoglucomutase coupled to glucose-6-phosphate dehydrogenase (4).

Nucleosides and nucleotides were assayed by ion pair reverse-phase high-performance liquid chromatography in a Hypersil-ODS column (15 cm by 0.4 cm) protected by a guard column of the same material (1 cm by 0.4 cm) and connected to an Agilent HP 1100 chromatograph. Samples (20 μl) were manually injected, and the elutions were performed at a flow rate of 0.5 ml/min: a 5-min isocratic elution with 1 mM sodium phosphate, pH 7, 20 mM tetrabutylammoniun bromide, 10% (vol/vol) methanol, followed by a 20-min gradient elution with 1 to 100 mM sodium phosphate at constant tetrabutylammoniun bromide and methanol levels. Chromatograms were recorded at 250 nm. Product peaks were identified by their retention times, compared with authentic standards, and integrated. Since in every reaction studied, the substrate and nucleotidic product had equivalent molar extinction coefficients, the product peak areas were converted to molar amounts using the area of the substrate peak as an internal standard.

Nucleotide sequence accession number. The sequence of the 1,653-nucleotide insert ORF of plasmid pGEX-6P-3-Yi-*ushA* was deposited in GenBank (accession no. EF472003).

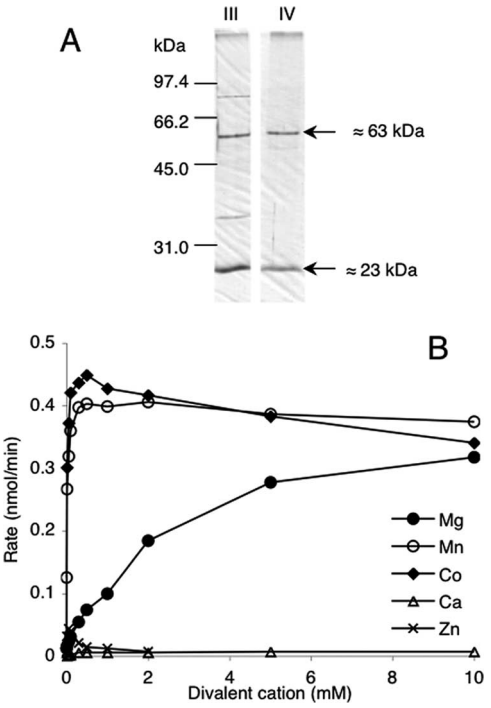

FIG. 3. Endogenous CDP-alcohol hydrolase from *Y. intermedia*. (A) SDS-PAGE analysis of partially purified preparations. The lanes show analyses of purification steps 3 (III) and 4 (IV) shown in Table S1 in the supplemental material. The gels were stained with silver. The arrows on the right mark the protein bands analyzed by PMF. The band identified as belonging to $UshA_{Yi}$ is the ~63-kDa band. (B) Activation by divalent cations. For this experiment, the enzyme (purification step 3 [see Table S1 in the supplemental material]) was dialyzed against 250 volumes of 20 mM Tris-HCl, pH 7.5, for 20 h, with one buffer change. The initial rates of CDP-ethanolamine hydrolysis were measured in 0.1-ml reaction mixtures using the standard assay, except that different divalent chloride salts were used at the indicated concentrations and bovine serum albumin was omitted. Equal amounts of dialyzed enzyme were used for the assay with each divalent cation.

RESULTS

Purification and characterization of an endogenous CDP-alcohol hydrolase of *Y. intermedia* and identification of the ortholog of *ushA* as a candidate for cloning. Strain CECT 7230 of *Y. intermedia* was isolated in the laboratory as a contaminant of a partially purified rat lung fraction in which studies of CDP-alcohol hydrolases were in progress. From those experiments, it became clear that *Y. intermedia* produced a CDP-ethanolamine hydrolase. This activity appeared in the extracellular medium of pure *Y. intermedia* suspensions prepared with bacteria collected from the surfaces of COS plates and suspended in a buffer containing Triton X-100. The amount of enzyme in that medium was strongly dependent on the temperature at which the COS cultures were grown. Maximum activity was obtained from cultures grown at 25°C, while the activity declined significantly when the bacteria were grown at 28°C or higher (Fig. 2). The enzyme was purified 160-fold from *Y. intermedia* suspensions prepared from bacteria grown at 25°C (see Table S1 in the supplemental material). The last purification step contained two protein bands of ~63 kDa and ~23 kDa (Fig. 3A).

TABLE 1. Substrate specificities of the endogenous CDP-alcohol hydrolase of *Y. intermedia* ($UshA_{Yi}$) and overexpressed $UshA_{Ec}$

Substrate	Activity[a]	
	$UshA_{Yi}$[b]	$UshA_{Ec}$[c]
AMP	302	169
ADP	146–291[d]	153–306[d]
ATP	118–353[d]	101–303[d]
CMP	213	139
CDP	105–210[d]	139–278[d]
GMP	388	
UMP	147	
L-Glycerol-3-phosphate	<1	<1
D-Glucose-1-phosphate	<1	<1
Phosphoethanolamine	<1	<1
Phosphocholine	<1	<1
2′-AMP	<1	<1
CDP-glycerol	237	137
CDP-ethanolamine	100	100
CDP-choline	28	64
CDP-glucose	4	1
UDP-galactose	55	24
UDP-*N*-acetylglucosamine	40	48
UDP-glucose	38	20
UDP-glucuronate	36	16
UDP-*N*-acetylgalactosamine	36	18
UDP-galacturonate	10	3
ADP-glucose	<1	<1
dTDP-glucose	3	1
GDP-glucose	<1	<1
GDP-mannose	<1	<1
ADP-ribose	33	5
Ap_2A	3	2
Ap_3A	12	2
Ap_4A	<1	<1
Ap_5A	<1	<1
NAD^+	<1	2
NADH	<1	1
FAD	<1	2
bis-*p*-Nitrophenylphosphate	4	1
Glycerophosphocholine	2	<1
Glycerophosphoethanolamine	<1	<1
4-Nitrophenylphosphocholine	<1	<1
4-Nitrophenyl-dTMP	<1	<1
2′,3′-cyclic AMP	<1	1
3′,5′-cyclic AMP	<1	<1

[a] Percentage of that measured with CDP-ethanolamine.
[b] Assayed with enzyme from purification step 3 (see Table S1 in the supplemental material).
[c] Overexpressed activity in lysate supernatants of *E. coli* BL21 cells transformed with the cloned *ushA* gene of *E. coli* (plasmid pLM-2) (29).
[d] Assuming that the number of P_i moles formed per mole of substrate split was 1 to 3 (ATP) or 1 to 2 (ADP and CDP).

A study of enzyme specificity showed, besides CDP-alcohol hydrolase activity on CDP-glycerol, CDP-ethanolamine, and CDP-choline (but not CDP-glucose), a set of activities typical of $UshA_{Ec}$: 5′-nucleotidase, UDP-sugar hydrolase, and dinucleoside polyphosphate hydrolase (Table 1). All were measured with Mg^{2+} as the activating cation, but the enzyme was also activated by Co^{2+} or Mn^{2+}, but not by Ca^{2+} (Fig. 3B). $UshA_{Ec}$ is known to be activated by all these cations, including Ca^{2+} (20, 29, 32, 38).

The study of the reactions with CDP-alcohols and UDP-glucose revealed in both cases the same product pattern (see Table S2 in the supplemental material). The nucleotide CMP or UMP was not detected either as a final or an intermediate product. Instead, the nucleoside cytidine or uridine was apparently formed directly from the substrate, together with an equimolar amount of P_i. In addition, the hydrolysis of UDP-glucose gave an equimolar amount of D-glucose-1-phosphate, and the hydrolysis of CDP-glycerol yielded 0.5 mol of L-glycerol-3-phosphate per mol of cytidine and P_i (since CDP-glycerol is a racemic mixture, the formation of another 0.5 mol of D-glycerol-3-phosphate, which does not react in the enzymatic assay used, can be assumed). Glycerol and D-glucose were not direct reaction products, but when the reaction was coupled to alkaline phosphatase, they were detected at the rate of 1 mol per mol of nucleoside, together with 2 mol of P_i. These results correspond to the following general reaction: NDP-X + $2H_2O$ $\rightarrow$ N + P_i + P-X, where N is the nucleoside cytidine or uridine and P-X is the corresponding phosphoalcohol or phosphosugar (phosphoethanolamine, phosphocholine, DL-glycerol-3-phosphate, or D-glucose-1-phosphate). This reaction is identical to the hydrolysis of UDP-glucose by $UshA_{Ec}$: the phosphoanhydride and the nucleoside 5′-ester linkages are hydrolyzed without detectable signs of nucleoside monophosphate (NMP) as an intermediate, possibly because it is hydrolyzed as soon as it is formed without leaving the active center (20).

The native molecular weight of the CDP-alcohol hydrolase, estimated by gel filtration chromatography, was about 52,000 (not shown). This left unclear whether the enzyme is a monomer of the ~63-kDa band or an oligomer of the ~23-kDa band seen by SDS-PAGE (Fig. 3A). In this respect, it is relevant that the molecular weight of mature $UshA_{Ec}$, calculated from its amino acid sequence, is 58,100 (8), but during gel filtration chromatography, it behaves as a 52-kDa protein (38).

To investigate the molecular identity of the ~63-kDa and ~23-kDa protein bands, their tryptic PMFs were obtained and used to run Mascot searches (http://www.matrixscience.com) against the NCBInr database. The best hits corresponded to hypothetical proteins conceptually translated from *Yersinia* genomic DNA sequences. The ~23-kDa protein was related with low significance to *Yersinia enterocolitica* and *Y. intermedia* superoxide dismutases. The ~63-kDa protein pinpointed, with a strong score, a *Y. intermedia* (strain ATCC 29909) hypothetical protein (GenBank accession no. ZP_00834762) that showed 75% amino acid identity with $UshA_{Ec}$.

PCR cloning and expression of the *Y. intermedia* CECT 7230 *ushA* gene ($ushA_{Yi}$) and biochemical characterization of its recombinant product. The evidence supported the notion that CDP-alcohol hydrolase was likely an activity of $UshA_{Yi}$. Therefore, we cloned $ushA_{Yi}$ by PCR, constructed plasmid pGEX-6P-3-Yi-*ushA*, and sequenced its 1,653-nucleotide insert ORF. With respect to the genome of *Y. intermedia* ATCC 29909, it showed four variations: C412T, T1056C, G1257A, and G1490A, with only the last changing the encoded amino acid (Ser497Asn).

The expression of plasmid pGEX-6P-3-Yi-*ushA* was expected to yield an 87.8-kDa GST-$UshA_{Yi}$ fusion protein with affinity for glutathione-Sepharose. SDS-PAGE analysis of the lysate supernatants of transformed *E. coli* cells showed three bands of about 80 kDa, 60 kDa, and 30 kDa responsive to induction with IPTG (Fig. 4A). Of these, only the ~30-kDa band was also induced in control cells transformed with vector pGEX-6P-3, where the expression of the GST protein alone was expected. The same supernatants showed the IPTG-induced overexpression of a set of Mg^{2+}-dependent enzyme activities: CDP-alcohol hydrolase, 5′-nucleotidase, UDP-sugar

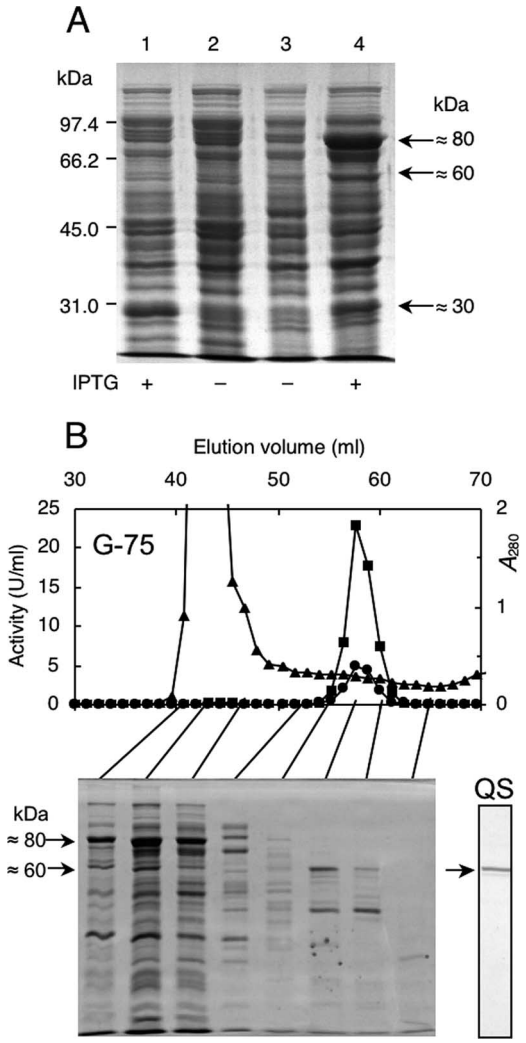

FIG. 4. Expression and purification of the recombinant $UshA_{Yi}$ protein. (A) Proteins induced by IPTG in *E. coli* cells transformed with plasmid pGEX-6P-3-Yi-*ushA*. The cells transformed with the empty vector pGEX-6P-3 (lanes 1 and 2) or with pGEX-6P-3-Yi-*ushA* (lanes 3 and 4), either with (+) or without (−) IPTG induction, were lysed and centrifuged. Shown is the SDS-PAGE analysis (Coomassie blue staining) of 12-μl (lanes 1 and 2) or 8-μl (lanes 3 and 4) samples of lysate supernatants. The arrows point to the three proteins induced by IPTG, two of which are specific to cells transformed with the *ushA* gene of *Y. intermedia*. The ~80-kDa band may correspond to the 87.8-kDa GST-$UshA_{Yi}$ fusion protein. The ~60-kDa band may correspond to the $UshA_{Yi}$ protein, either including (60.3 kDa) or not including (58.0 kDa) the predicted signal sequence (see Fig. S1 in the supplemental material). (B) Copurification of the activities overexpressed from pGEX-6P-3-Yi-*ushA* with an ~60-kDa protein band but not with the GST-$UshA_{Yi}$ fusion protein. Shown are (G-75) the chromatographic profiles of purification step 2, including enzyme activities (■, 5′-nucleotidase on AMP; ●, CDP-ethanolamine hydrolase), A_{280} (▲), and SDS-PAGE analyses of selected fractions as indicated and (QS) the analysis of the enzymatically active fractions after purification step 3 (see Table S3 in the supplemental material). The arrows mark the gel positions of the ~80-kDa band (putative GST-$UshA_{Yi}$ fusion protein) and the ~60-kDa band. The PMF of the latter demonstrated unambiguously the presence of the $UshA_{Yi}$ protein (not shown).

TABLE 2. Hydrolytic activities overexpressed in *E. coli* cells transformed with $ushA_{Yi}$

Substrate	Activity (U/mg)		
	pGEX-6P-3	pGEX-6P-3-Yi-*ushA*	Overexpressed[a]
AMP	0.08	18.81	18.73 (427)
UDP-glucose	0.08	2.15	2.07 (47)
CDP-glycerol	0.09	7.04	6.95 (158)
CDP-ethanolamine	0.06	4.45	4.39 (100)
CDP-choline	0.09	2.1	2.01 (46)
CDP-glucose	0.11	0.26	0.15 (3.4)
ADP-ribose	0.02	1.0	0.98 (22.3)
Ap_3A	0.01	0.56	0.55 (12.5)
2′-AMP	0.07	0.09	0.02 (0.5)
2′,3′-cyclic AMP	0.15	0.13	−0.02 (−0.5)
3′,5′-cyclic AMP	0.09	0.09	0.00 (0.0)

[a] Percentages are in parentheses.

hydrolase, and dinucleoside polyphosphate hydrolase (Table 2), in agreement with the specificity of the endogenous enzyme of *Y. intermedia* (Table 1). Despite several attempts, neither the ~80-kDa nor the ~60-kDa protein band nor the IPTG-induced enzyme activities were adsorbed onto glutathione-Sepharose. In addition, when the lysate supernatant of *E. coli* cells transformed with $ushA_{Yi}$ and induced with IPTG was submitted to gel filtration chromatography, very little 5′-nucleotidase or CDP-alcohol hydrolase activity coeluted with the ~80-kDa band. Instead, they coeluted with the ~60-kDa protein, which by its size could correspond to the $UshA_{Yi}$ moiety separated from GST (Fig. 4B). To test this point, and to eliminate significant interference by the $UshA_{Ec}$ protein of the expression host, the PMF of the 60-kDa protein band was obtained (not shown). It confirmed unambiguously the presence of $UshA_{Yi}$ without signs of $UshA_{Ec}$-specific peptides (for a possible explanation for the recovery of $UshA_{Yi}$ separated from GST upon expression of plasmid pGEX-6P-3-Yi-*ushA*, see below). Recombinant $UshA_{Yi}$ was then purified to near homogeneity (Fig. 4B, lane QS) by an additional ion-exchange chromatography step (the purification results are summarized in Table S3 in the supplemental material). This purified preparation displayed the same substrate specificity (not shown) displayed by the IPTG-induced set of activities in *E. coli* cells transformed with pGEX-6P-3-Yi-*ushA* (Table 2).

Presence of a signal sequence in $UshA_{Yi}$. To ascertain whether $UshA_{Yi}$ contains a periplasmic signal sequence like that of $UshA_{Ec}$ (7, 8, 13), both proteins were submitted to informatics analysis. First, four different programs available online (19, 27, 31, 50) predicted a periplasmic location both for $UshA_{Yi}$ and $UshA_{Ec}$, in agreement with experimental data for $UshA_{Ec}$. Second, $UshA_{Yi}$ and $UshA_{Ec}$ were subjected to predictive analysis for the presence of a signal sequence with the SignalP program (15). For $UshA_{Ec}$, the predictions indicated a signal sequence with a peptidase site between amino acids 25 and 26, in agreement with experimental evidence (7, 8, 13). For $UshA_{Yi}$, SignalP also pointed to a strong signal sequence with a peptidase site between either amino acids 23 and 24 or 25 and 26. The signal sequences at the N ends of $UshA_{Ec}$ and $UshA_{Yi}$ are indicated in Fig. S1 in the supplemental material.

Tryptic fingerprinting can provide evidence of the presence or absence of signal peptides. In the case of $UshA_{Yi}$, the

TABLE 3. Kinetic parameters and catalytic efficiencies of $UshA_{Yi}$ and $UshA_{Ec}$

Substrate	From this work[a]						From others
	k_{cat} (s^{-1})		K_m (μM)		k_{cat}/K_m (M^{-1} s^{-1})		k_{cat}/K_m (M^{-1} s^{-1})
	$UshA_{Yi}$	$UshA_{Ec}$	$UshA_{Yi}$	$UshA_{Ec}$	$UshA_{Yi}$	$UshA_{Ec}$	$UshA_{Ec}$
AMP	735	372	9.6	1.8	7.7×10^7	2.1×10^8	1.0×10^7[b] 5.4×10^7[c]
CDP-glycerol	399	324	11.6	2.9	3.4×10^7	1.1×10^8	
CDP-ethanolamine	330	261	8.1	1.7	4.1×10^7	1.5×10^8	
CDP-choline	172	231	10.3	2.4	1.7×10^7	9.6×10^7	
UDP-glucose	97	71	9.6	10.4	1.0×10^7	6.8×10^6	1.0×10^7[d]
ADP-ribose	72		5.8		1.2×10^7		
Ap_3A							1.0×10^7[e]

[a] $UshA_{Yi}$ was the recombinant enzyme of *Y. intermedia* expressed in *E. coli* and purified (see Table S3 in the supplemental material). $UshA_{Ec}$ was the overexpressed enzyme of *E. coli* BL-21 cells transformed with plasmid pLM-2; the enzyme was purified by the same procedure as for $UshA_{Yi}$, and a nearly homogeneous preparation was obtained (not shown). The standard enzyme assay was used, with a concentrated P_i reagent to measure initial rates at different substrate concentrations. The data were adjusted to the Michaelis-Menten equation (12).

[b] Calculated as follows, with Co^{2+} as the activating cation: $k_{cat} = 750\ s^{-1}$ and $K_m = 74$ μM (39).

[c] Calculated as follows, with Co^{2+}-Ca^{2+} as the activating cation: $k_{cat} = 2{,}440\ s^{-1}$ and $K_m = 45$ μM (38).

[d] Calculated as follows, with Mn^{2+} as the activating cation: $k_{cat} = 504\ s^{-1}$ and $K_m = 45$ μM (38).

[e] Calculated as follows, with Mn^{2+} as the activating cation: $k_{cat} = 360\ s^{-1}$ and $K_m = 35$ μM (38).

expected tryptic peptide formed by amino acids 3 to 28, with a calculated mass of 2,679.237 Da, is diagnostic of the presence of the signal sequence. Interestingly, experimental data for the endogenous and the recombinant proteins differed in this respect: the PMF of endogenous $UshA_{Yi}$ included the diagnostic peptide, while recombinant $UshA_{Yi}$ did not (not shown). It was concluded that the endogenous protein corresponded, at least in part, to a $UshA_{Yi}$ precursor. This can be explained by the liberation of immature $UshA_{Yi}$ from *Y. intermedia* in the cell suspensions prepared in the presence of Triton X-100. On the other hand, the recombinant protein appeared as the mature form of $UshA_{Yi}$, indicating that the signal peptidase of *E. coli* acted on recombinant $UshA_{Yi}$. Two pathways could account for the unexpected expression of mature $UshA_{Yi}$ from a plasmid encoding a fusion of GST with immature $UshA_{Yi}$ (pGEX-6P-3-Yi-*ushA*): either the translated fusion protein was processed directly by the *E. coli* signal peptidase, or part of the mRNA transcribed from plasmid pGEX-6P-3-Yi-*ushA* was translated from the immature $UshA_{Yi}$ initiation codon and the immature protein was processed by the signal peptidase.

CDP-alcohol hydrolase activity of $UshA_{Ec}$. CDP-alcohol hydrolase activity has never been reported for a UshA enzyme. To elucidate whether it is a peculiarity of $UshA_{Yi}$ or a more general feature, $ushA_{Ec}$ was expressed in *E. coli* from plasmid pLM-2 (29). In addition to the known 5′-nucleotidase, UDP-sugar hydrolase, and dinucleoside polyphosphate hydrolase activities, CDP-alcohol hydrolase was strongly overexpressed (Table 1).

The catalytic efficiencies of $UshA_{Ec}$ and $UshA_{Yi}$ activities, including CDP-alcohol hydrolase, are near the limits of enzymatic perfection. Since the catalytic efficiency ratio, k_{cat}/K_m, cannot be greater than the diffusion-controlled enzyme and substrate association rate constant, the upper limit for this ratio is 10^8 to 10^9 M^{-1} s^{-1}. Values of $\geq 10^7$ M^{-1} s^{-1} are typical of so-called "perfect" enzymes, which are limited by the diffusion rather than the chemical steps of their mechanisms (17). To our knowledge, there are two studies reporting k_{cat} and K_m values for some $UshA_{Ec}$ substrates, but they have not been explicitly used to derive k_{cat}/K_m ratios (38, 39). In the present work, the saturation kinetics of $UshA_{Ec}$ and $UshA_{Yi}$ were studied for a selection of substrates with Mg^{2+} as the activating cation (Table 3). Considering the k_{cat}/K_m ratios, the 5′-nucleotidase activity on AMP was the most efficient of those studied, followed very closely by CDP-alcohol hydrolase, and then by UDP-glucose and ADP-ribose hydrolase activities. The 5′-nucleotidase and CDP-alcohol hydrolase activities of $UshA_{Ec}$ displayed k_{cat}/K_m ratios of $\geq 10^8$ M^{-1} s^{-1}, while UDP-glucose hydrolase displayed a 10-fold-lower value, similar to those calculated for the Mn^{2+}-dependent UDP-glucose and diadenosine tnphosphate (Ap_3A) hydrolase activities from published saturation parameters (38, 39). A similar picture was found for $UshA_{Yi}$, except that the k_{cat}/K_m values for 5′-nucleotidase and CDP-alcohol hydrolase activities were somewhat lower than those for $UshA_{Ec}$, but still higher than 10^7 M^{-1} s^{-1}.

Theoretical analysis of $UshA_{Yi}$ structure in comparison to that of $UshA_{Ec}$. The structures of several $UshA_{Ec}$ conformers have been solved by X-ray diffraction and revealed a monomeric protein with two domains linked by an α-helix. The N-terminal domain displayed the structural features typical of the metallodependent phosphatase superfamily, and it also contained the catalytic site. However, the substrate first binds far away from this site, in the C-terminal domain of the so-called "open" conformer. This inactive conformer is converted to the "closed" active conformer by a hinge-bending C-domain rotation of 96° that brings the bound substrate close to the catalytic center (24–26). To evaluate whether $UshA_{Yi}$ could adopt structures similar to those of $UshA_{Ec}$ and work by similar mechanisms, we adopted a theoretical approach. The $UshA_{Yi}$ protein (accession no. ABO69612) has the same length as the immature precursor of $UshA_{Ec}$ (accession no. NP_415013). They align with 75% sequence identity and 86% similarity, including identity (see Fig. S1 in the supplemental material). Therefore, theoretical structures for $UshA_{Yi}$ were obtained by automatic homology modeling on the SWISS-MODEL server (41), using as models the X-ray structures of the two $UshA_{Ec}$ conformers that represent the extreme stages

of the 96° rotation. The homology models of $UshA_{Yi}$ showed the two-domain organization, the structural features of metallophosphoesterases in the N-terminal domain, and the possibility of adopting open and closed conformations, similarly to $UshA_{Ec}$ (see Fig. S2 in the supplemental material). In fact, comparing $UshA_{Yi}$ with $UshA_{Ec}$ structures, a full sequence and spatial conservation of the amino acids of the metallophosphoesterase signatures, the substrate-binding sites, and the catalytic centers was observed (see Fig. S1 and S3 in the supplemental material).

DISCUSSION

Major advances in regard to UshA enzymes. In this study, a UshA protein was cloned, expressed, and characterized for the first time from bacteria of the genus *Yersinia*. This led to the finding of two properties shared by $UshA_{Yi}$ and $UshA_{Ec}$ that had remained unnoticed in this type of enzyme. One is the possession of CDP-alcohol hydrolase activity, not to a minor degree, but to the extent of being the second highest activity of the enzyme, not much lower than the 5′-nucleotidase and higher than the UDP-sugar hydrolase activity that lends its name to the *ushA* gene and its encoded protein. In retrospect, the reason why the high CDP-alcohol hydrolase activity of $UshA_{Ec}$ remained hidden may have been the low activity toward CDP-glucose compared to UDP-glucose, a feature that, after its early description (20), may have discouraged testing CDP-alcohols as substrates. The other novel feature found in this work for UshA enzymes is a catalytic efficiency (k_{cat}/K_m) so high that it allows their inclusion in the category of "perfect" enzymes. This is particularly noteworthy considering that the catalytic cycle of the enzyme involves a 96° rotation of its C domain forward and back (see Fig. S2 in the supplemental material). It seems a considerable feat for this large motion, together with the chemical steps, to occur at a rate near that of the diffusion-controlled encounter of the enzyme and substrate in solution.

The specificity patterns of UshA activities may depend on an interplay of substrate interactions with different protein domains. The phosphohydrolytic behavior of UshA enzymes is rather complex, because although they hydrolyze a broad spectrum of phosphorylated compounds, they can be sorted into groups that depict an apparently multifunctional enzyme: 5′-nucleotidase (including apyrase-like activity), CDP-alcohol hydrolase, UDP-sugar hydrolase, and dinucleoside polyphosphate hydrolase. The apparent multifunctionality arises from the fact that, considering NDP-X as the prototypical substrate of the enzyme, those activities differ in their abilities to discriminate either among N groups or among X groups. The picture is not complete, because only a limited set of N/X combinations is available to the experimenter in the form of NDP-X compounds. However, the rather extensive list tested (Table 1) (18, 20, 32, 38) allows some conclusions to be drawn. The UDP-sugar hydrolase activity discriminates strongly for U against the other nitrogen bases (A, G, C, and T) so that UDP-glucose, UDP-galactose, UDP-*N*-acetylglucosamine, and UDP-*N*-acetylgalactosamine are hydrolyzed at similar rates, while ADP-glucose, GDP-glucose, CDP-glucose, and dTDP-glucose are not or are only marginally hydrolyzed. In contrast, the 5′-nucleotidase/apyrase activity or activities show little discriminatory ability among nitrogen bases: all the NMP, NDP, and nucleoside triphosphate substrates are hydrolyzed at relatively similar rates. The CDP-alcohol hydrolase activity reported here adds further complexity to the UshA specifity pattern. Namely, the strong discrimination for uracil versus cytosine in the N moiety of the substrate, observed when glucose occupies the X position, is apparently overridden when glycerol of ethanolamine substitutes for glucose, as CDP-glycerol and CDP-ethanolamine are very good substrates, hydrolyzed with higher efficiencies than UDP-glucose.

The influence that the X group of the substrate exerts on the enzyme's ability to discriminate among N groups (or vice versa) can be accounted for by interactions of the NMP and P-X moieties of the substrate with different parts of the active site that are located in different protein domains. The structures of UshA complexes with the substrate ATP (see Fig. S2 in the supplemental material) (inactive conformer), the substrate-analog inhibitor α,β-methylene-ADP (see Fig. S2 in the supplemental material) (active conformer), or the reaction products adenosine and P_i (not shown) (inactive conformer) show that the NMP moiety binds to a C-domain pocket fitting AMP through several contacts (see Fig. S3A in the supplemental material). On the other hand, the β and γ phosphate groups of ATP (i.e., its P-X moiety) do not make any contact with the open UshA conformer and protrude freely toward the solvent (see Fig. S2 in the supplemental material), while the β phosphonate of α,β-methylene-ADP, the nearest equivalent to a substrate studied in complex with UshA, makes contacts with the N domain of the closed conformer (see Fig. S2 and S3A in the supplemental material). Assuming that the NMP and P-X moieties of UDP-sugars and CDP-alcohols interact with the C and N domains of the closed conformer, we suggest that the specificity pattern of UshA enzymes results from a subtle interplay between NMP and P-X interactions, which might have consequences for the positioning of the scissile bonds of different compounds with respect to catalytic groups. The details of such interplay remain to be studied.

The biological roles of UshA enzymes. Because of its periplasmic location, $UshA_{Ec}$ has a role in the utilization of extracellular nucleotides. *E. coli* mutants isolated by a deficiency in 5′-nucleotidase activity are unable to take up and use AMP as a carbon source, which requires periplasmic conversion of AMP to adenosine (1, 49). Very recently, *ushA* knockouts of *E. coli* have been shown to be unable to grow on AMP as the only carbon source and are significantly delayed or impaired in their growth on other purine nucleotides. Growth on nonadenine purine nucleotides is made possible by an acid phosphatase encoded by the *aphA* gene, which at neutral pH acts as an efficient 5′ nucleotidase of purine nucleotides, except AMP. Double knockouts in *ushA* and *aphA* do not grow on nucleotides (22).

In the gram-positive *Corynebacterium glutamicum*, but not in *E. coli*, the *ushA* gene is part of the phosphate starvation regulon, increasing its expression under conditions of phosphate deprivation (21), and its encoded UshA protein, with 5′-nucleotidase and UDP-sugar hydrolase activities, is required for growth on AMP or UDP-glucose as sole sources of phosphorus (36).

The resemblance to $UshA_{Ec}$, both structurally and enzymatically, indicates that $UshA_{Yi}$ may play a role similar to that of

$UshA_{Ec}$ in regard to extracellular nucleotides. Besides *Y. intermedia*, with the exception of *Yersinia pestis*, all the *Yersinia* species for which sequenced genomes are available (*Y. enterocolitica*, *Yersinia pseudotuberculosis*, *Yersinia mollaretii*, *Yersinia berkovierii*, and *Yersinia frederiksenii*) contain genes coding for closely related UshA proteins (85% identity and 92% similarity, including identity) with conserved amino acids at all the positions of the substrate binding site and the dimetallic center (BlastP and alignment results not shown). The exceptionality of *Y. pestis* comes from two frameshift mutations: a single-nucleotide insertion between 252 and 253 and the deletion of nucleotide 967 in the *ushA* genes of the seven sequenced biovar genomes of the species (reference 34; nucleotide Blast and alignment results not shown). Most likely, the frameshifts prevent the expression of a functional UshA protein in *Y. pestis* (33). In addition, BlastN and TBlastN searches of the genomes of *Yersinia* species indicated they lack genes orthologous to *aphA*. Therefore, it is possible that wild-type *Y. pestis* is unable to use nucleotides as a carbon source, similar to the *ushA* and *aphA* double knockouts of *E. coli* (see above).

The following factors seem to correlate the *ushA* genes of the genus *Yersinia* with the polysaccharidic O-antigen gene cluster: (i) the cluster contains genes related to the metabolism of NDP sugars and the biosynthesis of O antigen; (ii) *ushA* genes are located downstream and near the O-antigen clusters of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serovar O:8 (16, 34, 43, 45); (iii) the lack of expression of O antigen in *Y. pestis* has been attributed to frameshift mutations in a few genes of the O-antigen cluster, and as noted above, this includes the nearby *ushA* (34, 44); (iv) the expression of the O-antigen cluster of *Y. enterocolitica* O:8 is markedly higher at near-ambient temperature than at 37°C (2), a behavior very similar to $UshA_{Yi}$ enzyme activities in our experiments (Fig. 2). These correlations make us wonder about a hypothetical role of *Yersinia* UshA proteins in the modulation of UDP sugars as glycosidic precursors.

Concerning the novel CDP-alcohol hydrolase activities of $UshA_{Ec}$ and $UshA_{Yi}$ on CDP-glycerol, CDP-ethanolamine, and CDP-choline, one would consider whether their very high catalytic efficiencies (Table 3) could be related to some of the known physiological roles of these compounds. (i) CDP-glycerol is the precursor from which gram-positive bacteria synthesize polyglycerolphosphate, one of the major forms of cell wall teichoic acid (47). (ii) CDP-ethanolamine and CDP-choline are well known as key intermediates of mammalian pathways for the synthesis of phosphatidylethanolamine and phosphatidylcholine; although its operation in bacteria is far from general, there is at least one well-documented case of use of the CDP-choline pathway by *Treponema denticola* (23). (iii) CDP-choline is the putative precursor for the "decoration" of cell surface lipopolysaccharides or (lipo)teichoic acids with phosphocholine (9, 37). Any of these CDP-alcohol-dependent processes could be affected by UshA enzymes with CDP-alcohol hydrolase activity, like $UshA_{Ec}$ and $UshA_{Yi}$. To our knowledge, there is no evidence for CDP-alcohol use by *Yersinia* or *E. coli*; therefore, we ran TBlastN searches, using the amino acid sequence of $UshA_{Ec}$ as the probe, against the sequenced genomes of *T. denticola* and other bacterial species selected for their putative abilities to use either CDP-glycerol to synthesize teichoic acid or CDP-choline to decorate their surfaces with phosphocholine. In this way, we found nearly 30 bacterial species potentially using a CDP-alcohol for one of the above-mentioned purposes and containing a possible UshA ortholog ($10^{-20} >$ E value $\geq 10^{-256}$) in their genomes (not shown).

Finally, the CDP-alcohol hydrolase activities of UshA proteins in the periplasmic space could act on CDP-alcohols from the infected host. In cases of intracellular infection, the CDP-alcohol hydrolase could disrupt the normal functioning of phospholipid biosynthetic routes by the hydrolysis of CDP-ethanolamine and CDP-choline.

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