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Helicobacter pylori EstV: Identification, Cloning, and Characterization of the First Lipase Isolated from an Epsilon-Proteobacterium[▽]

Cristian Ruiz,[1,2]* Serena Falcocchio,[1,2] F. I. Javier Pastor,[1] Luciano Saso,[2] and Pilar Diaz[1]*

Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain,[1] and Department of Human Physiology and Pharmacology "Vittorio Erspamer," University of Rome "La Sapienza," Rome, Italy[2]

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Bacterial lipases are attracting an enormous amount of attention due to their wide biotechnological applications and due to their roles as virulence factors in some bacteria. *Helicobacter pylori* is a significant and widespread pathogen which produces a lipase(s) and phospholipases that seem to play a role in mucus degradation and the release of proinflammatory and cytotoxic compounds. However, no *H. pylori* lipase(s) has been isolated and described previously. Therefore, a search for putative lipase-encoding genes was performed by comparing the amino acid sequences of 53 known lipolytic enzymes with the deduced proteome of *H. pylori*. As a result, we isolated, cloned, purified, and characterized EstV, a novel lipolytic enzyme encoded by open reading frame HP0739 of *H. pylori* 26695, and classified it in family V of the bacterial lipases. This enzyme has the properties of a small, cell-bound carboxylesterase (EC 3.1.1.1) that is active mostly with short-chain substrates and does not exhibit interfacial activation. EstV is stable and does not require additional cofactors, and the maximum activity occurs at 50°C and pH 10. This unique enzyme is the first lipase isolated from *H. pylori* that has been described, and it might contribute to ulcer development, as inhibition by two antiulcer substances (β-aescin and glycyrrhizic acid) suggests. EstV is also the first lipase from an epsilon-proteobacterium to be described. Furthermore, this enzyme is a new member of family V, probably the least-known family of bacterial lipases, and the first lipase of this family for which kinetic behavior, inhibition by natural substances, and other key biochemical features are reported.

Lipases are glycerol ester hydrolases that act on acylglycerols to liberate fatty acids and glycerol. Lipases can be divided into two main groups, (i) carboxylesterases (EC 3.1.1.1) and (ii) "true" lipases (EC 3.1.1.3), which differ in several biochemical features. Carboxylesterases hydrolyze short-chain substrates that are partially soluble in water, and they exhibit a typical Michaelis-Menten kinetic behavior. In contrast, "true" lipases have optimal activity with long-chain poorly water-soluble substrates that tend to form emulsions or micelles, and they usually exhibit "interfacial activation," a phenomenon consisting of a sharp increase in the enzyme activity as soon as the substrate forms an emulsion. This phenomenon is usually correlated with the presence of a "lid," a surface loop of the protein covering the active site and moving away in contact with a water-lipid interface (14, 17).

There is an increasing interest in bacterial lipases because they represent the most versatile and widely used enzymes in biotechnological applications and organic chemistry (the food industry, detergent formulation, synthesis of enantiopure compounds, etc) (16) and because of their role as virulence factors in some pathogenic bacteria (17). For these reasons, a large number of bacterial lipases have been isolated and characterized during the last few years. These enzymes have been grouped into eight families based on conserved sequence motifs and biological properties (3). Most bacterial lipase families are well established, and their members have been thoroughly studied. However, family V has not been well studied; *Acetobacter pasteurianus* Est2 is the only member of this family that has been isolated and cloned, and it has been only partially characterized (18, 19).

Helicobacter pylori is a gram-negative, spiral-shaped, flagellated, microaerophilic epsilon-proteobacterium that is found mainly in the antral and fundic segments of the stomach and in the duodenum of humans. Since it was isolated by Marshall and Warren in 1983 (23), there has been great interest in *H. pylori* because it colonizes about 60% of the world's population, causing gastritis or peptic ulcer disease, and because it is strongly associated with gastric carcinoma, gastric lymphoma, and several extragastroduodenal diseases. The mode of transmission of *H. pylori* is not well known, although it could be related to direct or fecal-oral transmission (12, 35).

Many virulence factors seem to be involved in the pathomechanism of *H. pylori* infection. These factors include various enzymatic activities (urease, lipase, phospholipase, protease, etc.) produced by all strains of *H. pylori* that have been described, the vacuolating cytotoxin (VacA), and the immunogenic protein encoded by *cagA* (cytotoxin-associated gene), which is localized in the *H. pylori* pathogenicity island together with other genes responsible for virulence, expression of proinflammatory cytokines, and expression of proteins for transport of the CagA protein to eukaryotic cells (35).

The existence of *H. pylori*-related lipase and phospholipase activities capable of degrading the lipids and phospholipids of gastric mucus was first reported by Slomiany and collaborators

* Corresponding author. Present address for Cristian Ruiz: Center for Adaptation Genetics and Drug Resistance and Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-0325. Fax: (617) 636-0458. E-mail: Cristian.Ruiz_Rueda@tufts.edu. Mailing address for Pilar Diaz: Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain. Phone: 34-93-4034627. Fax: 34-93-4034629. E-mail: pdiaz@ub.edu.
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(44, 45), who also described the inhibition of these activities by colloidal bismuth subcitrate and sofalcone, two antiulcer agents. Further studies revealed inhibition of these enzymatic activities by sucralfate, ranitidine bismuth citrate, and other antiulcer agents (29, 31, 32, 46, 47), suggesting that these enzymes are important in ulcer development. It is now thought that lipase and phospholipase activities produced by *H. pylori* are involved in hydrolyzing the lipids of gastric mucus, thus reducing its thickness and hydrophobicity. Weakening the mucus barrier favors *H. pylori* colonization of the host cell surface and makes the epithelium more accessible to gastric acid and pepsin. Moreover, lipase and (mainly) phospholipase activities seem to be related to disruption of the apical membrane of epithelial cells, to hemolysis, and to the generation of lysophospholipids and other cytotoxic and proinflammatory lipids detrimental for the mucous gel and epithelium integrity (5, 48, 51).

Nevertheless, there is still little information about *H. pylori* lipolytic enzymes despite the publication of the genome sequences of *H. pylori* strains 26695 (50) and J99 (1). The presence of carboxylesterase activity has been confirmed by nuclear magnetic resonance spectroscopy (24), although the number and biochemical properties of the lipase(s) produced by this bacterium are still not known. *H. pylori* also possesses several phospholipase activities (phospholipases A_1, A_2, and C) (30), although phospholipase A_2 is the only enzyme that acts on phospholipids whose gene (*pldA*) has been identified, cloned, and expressed (9).

H. pylori is thus a significant pathogen whose importance is unlikely to diminish in the foreseeable future. Therefore, extensive research is being carried out to understand the pathogenic mechanisms of this lipolytic bacterium and to develop methods to prevent infection or eradicate the organism. Here, we describe a search for the unknown lipase-encoding genes of *H. pylori*, as well as the cloning, characterization, and inhibition of carboxylesterase EstV. This novel enzyme is encoded by the HP0739 open reading frame (ORF) in *H. pylori* 26695 and is the first lipase identified in this pathogen. As far as we know, this unique enzyme is also the first lipase isolated from an epsilon-proteobacterium that has been described, as well as the first family V bacterial lipase whose kinetic behavior and other biochemical features have been characterized.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Virulent wild-type *H. pylori* strain 26695 (50) was kindly provided by R. Araujo. Fresh cultures of the strain grown on blood agar base supplemented with 5% lysed defibrinated horse blood, incubated for 4 days at 37°C in a microaerobic atmosphere, were provided each time. *Escherichia coli* strain DH5α was routinely cultured overnight at 37°C in Luria-Bertani broth or on Luria-Bertani agar plates and was used as the host strain for cloning and expression of lipase-encoding genes. Plasmid pUC19 was used as a cloning and expression vector.

Identification and sequence analysis of the HP0739 ORF of *H. pylori* 26695. The amino acid sequences of 53 previously described bacterial lipases (3) were compared using BLAST (2; http://www.ncbi.nlm.nih.gov/BLAST) to the complete deduced proteome of *H. pylori* 26695 (50). Proteins having amino acid regions similar to the consensus motifs of the different lipase families (3, 16) were selected and reanalyzed by performing a second BLAST analysis with all kinds of nonputative, previously described enzymes. Only the protein encoded by the HP0739 ORF exhibited higher levels of similarity to lipases than to other enzymes, which was confirmed by multiple-sequence alignment performed with the ClustalW Multalign software (49; http://www.ebi.ac.uk/clustalW).

The physicochemical parameters of the deduced protein encoded by HP0739 were analyzed at ExPASY (http://www.expasy.org), whereas an analysis of protein domains was performed using Prodom (42) and Pfam (4). Protein fold recognition using one- and three-dimensional sequence profiles coupled with secondary structure information was obtained from the 3D-PSSM web server (20). The signal peptide identification was performed with the SignalP V2.0 software (27), and an analysis of PEST sites was performed at http://www.at.embnet.org/toolbox/pestfind/. Predicted transmembrane regions were analyzed with the DAS-Transmembrane prediction server (7), and subcellular protein localization was predicted using PSORTb v.2.0 for bacterial sequences (13). The data obtained in these analyses confirmed the presence in the deduced protein encoded by HP0739 of the typical physicochemical and structural features of lipases; thus, the protein encoded by the HP0739 ORF was selected for further study.

DNA manipulation and cloning of the HP0739 ORF. Specific primers HPESTFW (5′-**GAA** TAA CAT *ATG* GCC AAA CGC AG-3′; NdeI site underlined, fragment of the Shine-Dalgarno region in bold type, and start codon in italics) and HPESTBW (5′-CGC TAA ATG GAT CCA A**CT** **A**AG AC-3′; BamHI site underlined and stop complementary codon in bold type) were designed for PCR amplification of the HP0739 ORF using *Pfu* polymerase (Stratagene, La Jolla, CA) and 29 cycles consisting of 30 s at 94°C, 20 s at 48°C, and 4 min at 72°C. Amplified DNA was purified with WIZARD columns (Promega) and ligated to SmaI-digested pUC19, and the resulting recombinant plasmid, pUC-EstV, was transformed into *E. coli* DH5α to obtain recombinant clone *E. coli* DH5α/pUC-EstV, using standard procedures (40).

Both DNA strands of purified PCR products and recombinant plasmids were sequenced as previously described (38) using a BigDye Terminator V3.1 cycle sequencing kit (Beckman-Coulter) and the CEQ8000 analytical system (Beckman-Coulter) available at the Serveis Científico Tècnics of the University of Barcelona.

Activity assays and protein analysis. The lipolytic activities of recombinant clones were detected by streaking the clones on CeNAN (ADSA Micro) agar plates supplemented with 1% (vol/vol) tributyrin, triolein, or olive oil, and 0.0002% (wt/vol) Rhodamine B (22), followed by incubation at 37°C for 16 to 48 h and detection of hydrolysis haloes on tributyrin plates or detection of pink or orange fluorescence with UV light irradiation on triolein and olive oil plates (22, 33). This method allowed detection of secreted and nonsecreted lipases, since enough nonsecreted lipase was released into the medium after the natural cell lysis that occurred in growing colonies. *E. coli* DH5α/pUC19 was used as a negative control. Lipase activity was confirmed by detection of 4-methylumbelliferone (MUF) release from MUF derivative substrates (8, 34), using cell extracts of *E. coli* DH5α/pUC-EstV, as well as cell extracts of *E. coli* DH5α/pUC as negative controls. Cell extracts were prepared in 50 mM phosphate buffer (pH 7) by sonication, as described previously (38).

The assays used to analyze the substrate preference of the HP0739-encoded enzyme (EstV), as well as its optimum temperature and pH, enzyme kinetics, and inhibition or activation by several agents, were routinely performed using purified EstV, whereas cell extracts of the recombinant clone *E. coli* DH5α/pUC-EstV and *E. coli* DH5α/pUC were used as positive and negative controls, respectively. Blanks that had the same composition as samples but lacked EstV were treated under the same conditions (temperature, pH, presence of inhibitors, etc.) as samples and used to subtract nonspecific substrate hydrolysis. All these assays were performed in triplicate, and each replicate was the result of an independent assay performed in duplicate. When needed, regression analyses were performed using regression curves with Rsquare coefficients greater than 0.99 obtained by using the Sigma-Plot 8.0 software. One unit of activity was defined as the amount of enzyme that released 1 μmol of *para*-nitrophenol (*p*-NP) or MUF per min under the assay conditions.

Substrate specificity assays were performed by measuring the release of *p*-NP from several *p*-NP derivative substrates, using a previously described colorimetric microassay (39). The assays were performed at pH 7 and at the optimum temperature of EstV at this pH (55°C). The optimum temperature of the enzyme was determined by using a range of 4 to 70°C and the same colorimetric microassay (39) performed at pH 7 with 1 mM *p*-NP butyrate as the substrate.

The optimum pH of EstV was determined by using a range of pH 3 to 12, essentially as previously described (33, 34). The assays were performed at the optimum temperature of EstV (55°C) using MUF butyrate as the substrate, which is very stable at extreme pH values. The buffers used were citrate buffer (pH 3 to 4), succinate-NaOH buffer (pH 4 to 6), phosphate buffer (pH 6 to 7.5), Tris-HCl buffer (pH 7.5 to 9.5), and glycine-NaOH buffer (pH 9 to 12), all prepared as described previously (33) and used at a final concentration of 100 mM. Briefly, EstV in 50 mM phosphate buffer (pH 7) (blanks contained only the buffer) was diluted 1:10 in a reaction premixture that was preincubated for 5 min at the assay temperature. The reaction mixtures (each buffer at a concentration

of 100 mM at the appropriate pH with 1 mM MUF butyrate, 0.6% [vol/vol] Triton X-100, 1.5 μg ml^{-1} of EstV) were incubated for 15 min at the assay temperature before the reaction was stopped with 400 μl of stop solution (30 μl of 20% [vol/vol] HCl plus 370 μl of distilled water). Samples were then incubated for 10 min at 22°C, and they were subsequently analyzed with a Cary Eclipse spectrofluorimeter (Varian) using an excitation wavelength of 323 nm and an emission wavelength of 448 nm. Activity was determined, after subtraction of the nonspecific hydrolysis observed with the corresponding blanks, by comparison of the fluorescence emission to the fluorescence emission data in MUF standard plots. Once the optimum pH was determined (pH 10), this assay was used to refine the optimum temperature of EstV at this pH and to confirm the optimum pH of EstV at the new optimum temperature found (50°C).

Enzyme kinetics were determined as previously described (33, 34) by using hyperbolic regression curves obtained under the optimal conditions for the enzyme and using MUF butyrate and MUF oleate at concentrations ranging from 0 to 1.5 mM.

Lipase inhibition or activation was analyzed by a previously described colorimetric microassay (39) performed at 37°C and pH 7, using 1 mM *p*-NP laurate as the substrate so that the results were comparable to the results obtained previously with other lipases (39). Lipase inhibition or activation was calculated by comparing the residual activity detected in the presence of the compound assayed with the residual activity of untreated samples. The concentrations that resulted in 16% lipase inhibition (IC_{16}) and IC_{50} were calculated from the curves for inhibition rate versus inhibitor concentration by regression analysis.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) electrophoresis was performed as previously described (8, 34). After protein separation, the gels were washed to remove the SDS and to allow protein renaturation by soaking them at room temperature with gentle mixing once in 2.5% (vol/vol) Triton X-100 for 30 min and twice in 50 mM phosphate buffer (pH 7) for 20 min. Lipase activity was detected by zymogram analysis performed by covering washed gels with 50 mM phosphate buffer (pH 7) containing 100 μM MUF butyrate or 400 μM MUF oleate (8, 34). After detection of lipase activity, proteins were stained with Coomassie brilliant blue R-250 for visualization of protein bands (8).

Purification of the HP0739 ORF product. The putative lipase encoded by the HP0739 ORF was purified by fast protein liquid chromatography (AKTA FPLC; Amersham Biosciences), using *E. coli* DH5α/pUC-EstV concentrated cell extracts prepared in 50 mM phosphate buffer (pH 7) by sonication as described previously (38) and a three-step gel filtration-ion exchange-gel filtration procedure. Briefly, 2-ml cleared cell extract samples were separated by gel filtration by loading them onto a Superdex 200 prep grade column (dextran-agarose matrix; 124 ml; mean particle size, 34 μm; Amersham Biosciences) equilibrated at a flow rate of 1 ml min^{-1} with a mobile phase consisting of 50 mM phosphate buffer (pH 7). Protein separation was monitored at 280 nm, and proteins were eluted in 2-ml fractions in which lipase activity was detected by a colorimetric microassay (39). The fractions exhibiting maximum lipase activity were recovered and concentrated fourfold before they were separated by ionic exchange using a Mono Q 5/50 GL column (polystyrene/divinyl benzene matrix; 5 ml; particle size, 10 μm; pH 2 to 12 stability; Amersham Biosciences). The column was equilibrated at a flow rate of 2 ml min^{-1} with a mobile phase consisting of 50 mM glycine-NaOH buffer (pH 10), and proteins were separated using a 0 to 1 M NaCl gradient. The eluted proteins were recovered in 1-ml fractions that were analyzed to detect lipase activity (39). Then 0.5-ml aliquots of the most active fractions were repurified by gel filtration after injection into a Superdex 200 10/300 GL column (dextran-agarose matrix; 24 ml; mean particle size, 13 μm; Amersham Biosciences) equilibrated at a flow rate of 0.5 ml min^{-1} with a mobile phase consisting of 50 mM phosphate buffer (pH 7). Proteins were eluted in 1-ml fractions, and the fractions containing HP0739-encoded carboxylesterase were detected by a colorimetric activity microassay (39) and analyzed by SDS-PAGE, followed by a zymogram analysis performed as described above (8, 34).

RESULTS

Identification of the HP0739 ORF from *H. pylori* 26695. The complete proteome of strain *H. pylori* 26695 was analyzed in order to find proteins exhibiting similarity to lipases. To do this, the amino acid sequences of 53 previously described bacterial lipases (3, 16) were compared by BLAST analysis with the putative proteins in the *H. pylori* 26695 proteome. Based on this analysis, the products of *H. pylori* ORFs HP0289, HP0346, HP0739, and HP0906, Omp26, and the previously described phospholipase A_1 PldA appeared to be the putative proteins with the highest levels of similarity to lipases. The amino acid sequences of these candidate proteins were then reanalyzed using BLAST to eliminate the proteins exhibiting higher levels of homology to other types of enzymes. Only the protein encoded by the HP0739 ORF exhibited a higher level of similarity to lipases than to other enzymes and thus was selected for further sequence analysis.

Sequence analysis of the HP0739 ORF: assignment of the protein to bacterial lipase family V. Analysis of the *H. pylori* 26695 HP0739 gene revealed an ORF consisting of 726 bp (from position 794021 to position 794746 inclusive), and the first nucleotide and the last 15 nucleotides overlapped the previous and subsequent ORFs. Two promoter sequences were found in the region upstream of the gene at positions 793809 to 793859 (score, 0.99) and at positions 793909 to 793958 (score, 1). A sequence having the typical features of a ribosome binding site (AAGAA), starting 10 bp upstream of the start codon, and several inverted repeats that could act as transcription terminators located 112 bp downstream of the stop codon were also found.

The amino acid sequence encoded by HP0739 (accession no. AAD07785), a deduced protein annotated by Tomb et al. (50) as a putative 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase related to *Pseudomonas putida* CE2010 dienoate hydrolase, exhibited the highest levels of identity (BLASTp) to the hypothetical protein Jhp0676 of *H. pylori* J99 (97% identity) and to other putative proteins of related strains, such as *Campylobacter jejuni*. The sequence encoded by HP0739 also exhibited 25 to 40% identity to several fragments of known and putative lipases belonging to family V (3), the family II-related *Mycoplasma hyopneumoniae* lipase (41), and other lipases. ClustalW analysis was performed to determine the exact levels of identity of the predicted protein encoded by HP0739 to the whole amino acid sequences of the most similar nonputative enzymes found in the BLASTp search, which revealed 13 to 21% identity to family V bacterial lipases, 16% identity to *M. hyopneumoniae* lipase, and 12% identity to the epoxide dienoate hydrolase from *P. putida* CE2010. Consequently, the protein encoded by HP0739 was preliminarily considered a lipase.

Multiple-sequence alignment of the predicted amino acid sequence encoded by HP0739 with family V lipases (3) revealed the presence of six blocks of similarity (Fig. 1) described as the sequence patterns conserved around the active site residues of family V lipases (3). The levels of amino acid identity of the HP0739-encoded protein with blocks 1 to 6 of family V conserved sequences were 44.4, 35.7, 58.3, 37.5, 30.7, and 13.3%, respectively. These results seem to indicate that the HP0739-encoded protein belongs to this family, since the levels of identity are quite significant except for block 6, the less conserved block in family V lipases. Furthermore, although there was some homology between EstV and the *M. hyopneumoniae* lipase related to family II (41), the GDSL motif or other conserved consensus patterns of family II lipases were not found in the HP0739-encoded protein, supporting the identification of this protein as a new member of bacterial lipase family V (3); this protein was designated EstV.

Further analysis of the EstV predicted amino acid sequence revealed that EstV was a 241-amino-acid protein with a relative molecular mass of 27,527 Da and a theoretical pI of 9.0,

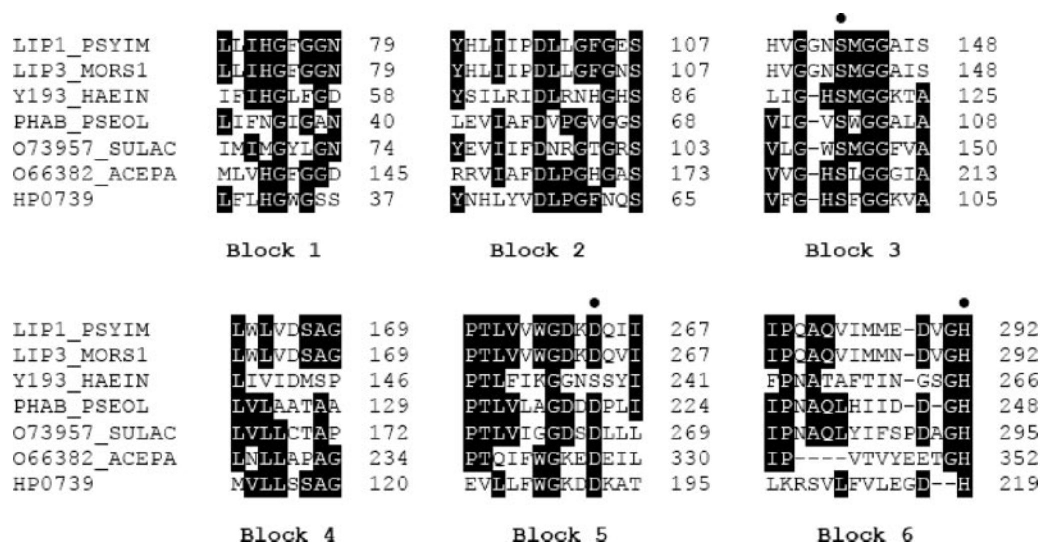

FIG. 1. Sequence pattern blocks conserved around the active site residues of family V lipolytic enzymes (3), including the predicted sequence of the HP0739-encoded enzyme. Dots indicate the amino acid residues belonging to the catalytic triad of lipases. LIP1_PSYIM, *Psychrobacter immobilis* (accession no. X67712); LIP3_MORS1, *Moraxella* sp. (X53869); Y193_HAEIN, *Haemophilus influenzae* (U32704); PHAB_PSEOL, *Pseudomonas oleovorans* (M58445); O73957_SULAC, *Sulfolobus acidocaldarius* (AF071233); O66382_ACEPA, *Acetobacter pasteurianus* (AB013096).

consistent with the relative molecular masses and pIs of several bacterial lipases and *H. pylori* phospholipase A_2 (3, 9, 16, 17, 38). No sequences with the features of a signal peptide were found, suggesting that the location of this protein is intracellular. EstV exhibited high predicted stability (>10 h in *E. coli*) and had no potential PEST sequences, which are thought to target eukaryotic proteins for phosphorylation and/or degradation. This analysis also confirmed that EstV has most of the typical features of lipases. In fact, the EstV sequence contained the typical pentapeptide Gly-His-Ser-Phe-Gly of lipases (17), as well as a high level of short nonpolar residues (35.7% of the total), as described previously for enzymes acting on hydrophobic substrates (11). Moreover, secondary structure prediction for EstV revealed the presence of eight β strands and seven α helices, consistent with the typical α/β fold of lipases (17). This analysis also showed that the conserved Gly-His-Ser-Phe-Gly pentapeptide containing the catalytic serine residue (Ser^{99}) formed a turn between strand β5 and the following α helix, corresponding to the so called "nucleophile elbow," which is present in all known lipases (17). Asp^{192} (located in a turn after strand β7) and His^{219} (located after β7) were identified as the two other members of the catalytic triad of EstV on the basis of their positions with respect to the prototypic α/β hydrolase fold (17) and on the basis of sequence alignments with other family V lipases (Fig. 1). Thus, EstV was definitively identified as a novel lipase belonging to family V of bacterial lipases.

In addition, protein fold recognition using one- and three-dimensional sequence profiles coupled with secondary structure information (20) suggested that EstV was a globular, compact, single-domain protein. However, a three-dimensional model of this enzyme could not be obtained due to its low level of similarity to previously crystallized lipases.

Cloning and purification of *H. pylori* EstV. Because EstV, the predicted protein encoded by the HP0739 ORF, was identified as a novel *H. pylori* 26695 lipase, we cloned and purified this enzyme so that biochemical and molecular characterization analyses could be performed. Amplification of the HP0739 ORF, including a few nucleotides in the regions upstream and downstream of the gene, was performed using primers HPESTFW and HPESTBW. The resulting 750-bp amplified DNA fragment was ligated to SmaI-digested pUC19 and cloned into *E. coli* DH5α. The recombinant clone obtained (*E. coli* DH5α/pUC-EstV) was analyzed on lipid-supplemented agar plates to detect the putative lipolytic activity of EstV. Clear hydrolysis zones were observed using tributyrin as a substrate (Fig. 2A), whereas no activity was found on plates

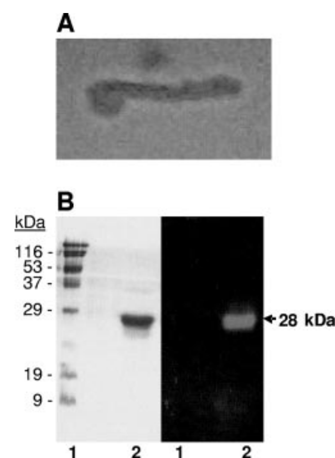

FIG. 2. Cloning and purification of EstV. (A) Hydrolysis halo produced by recombinant clone *E. coli* DH5α/pUC-EstV on agar plates supplemented with tributyrin. (B) SDS-PAGE gels containing purified EstV after Coomassie blue staining (left) and zymogram analysis using MUF butyrate as the substrate (right). Lanes 1, broad-range molecular weight protein marker; lanes 2, EstV purified fraction.

TABLE 1. Purification of EstV

Step	Total activity (mU)	Total protein (mg)	Sp act (mU mg protein^{-1})[a]	Purification (fold)	Yield (%)
Crude cell extract	44.0	14.2	3.1	1	100
Purified EstV fraction	8.6	1.3×10^{-2}	667.9	215	19.5

[a] The assays were performed at 50°C and pH 10, using 1 mM MUF butyrate as the substrate.

containing olive oil or triolein (not shown). Moreover, crude cell extracts of the recombinant clone exhibited activity with MUF butyrate and MUF oleate (1.7 ± 0.1 and $3.2 \times 10^{-2} \pm 0.2 \times 10^{-2}$ mU mg protein^{-1}, respectively).

EstV was purified almost to homogeneity by fast protein liquid chromatography of crude cell extracts of recombinant clone *E. coli* DH5α/pUC-EstV (Table 1). Purified EstV migrated as a band at ca. 28 kDa on SDS-PAGE gels and exhibited activity with MUF butyrate when these gels were analyzed by the zymogram method (Fig. 2B).

Enzyme characterization and inhibition assays. The lipase activity of purified EstV was tested with several *p*-NP and MUF derivatives (Fig. 3). EstV exhibited the typical behavior of carboxylesterases, showing a preference for short acyl chains. The highest level of activity (100%) was observed with *p*-NP acetate (537.3 ± 26.9 mU mg EstV^{-1} at 55°C and pH 7) and with MUF butyrate (667.9 ± 15.3 mU mg EstV^{-1} at 50°C and pH 10). EstV also efficiently hydrolyzed C_4 to C_5 *p*-NP derivatives (residual activity, >70%), whereas the activity decreased dramatically (residual activity, <20%) with *p*-NP or MUF derivatives with acyl chains consisting of eight or more carbon atoms (Fig. 3).

The effects of temperature and pH on the activity of EstV were also determined (Fig. 4). The enzyme had optimum temperatures of 55°C at pH 7 (using *p*-NP butyrate) and 50°C at pH 10 (using MUF butyrate). Since lipase activities with different buffers and substrates cannot be directly compared, 50°C and pH 10 were considered the optimal conditions for the enzyme, even though EstV exhibited higher activity at pH 6 (succinate-NaOH buffer) and pH 9.5 (Tris-HCl buffer). Moreover, EstV exhibited levels of activity higher than 60% at temperatures ranging from 34 to 60°C and moderate activity at pH 5 to 10.5. In addition, the enzyme was very stable, since it remained active for at least 30 days when it was stored at 4°C and pH 7 or 10.

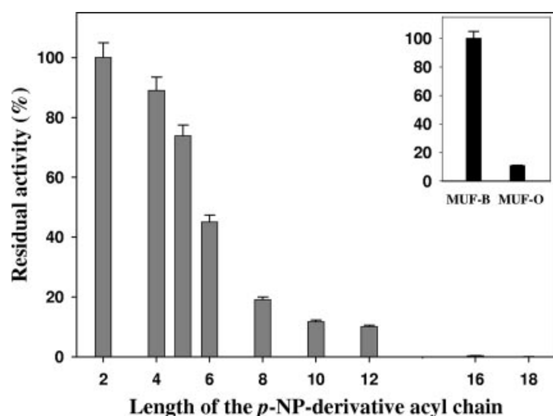

FIG. 3. Substrate profile of EstV: plot of relative activities based on the maximum activity (activity with *p*-NP acetate) versus length of the *p*-NP derivative acyl chain. (Inset) Activities with MUF butyrate (MUF-B) (C_4) (100%) and MUF oleate (MUF-O) (C_{18}). As described previously for carboxylesterases, the data obtained with the substrates analyzed revealed the preference of the enzyme for esters with short acyl chains.

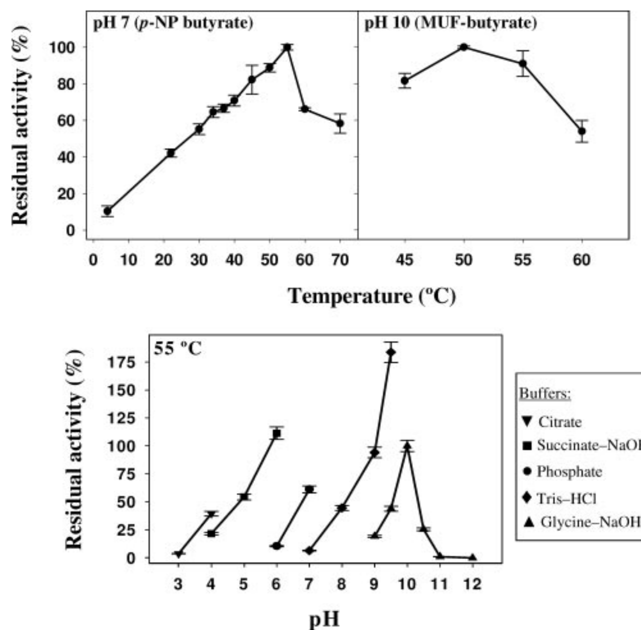

FIG. 4. Optimum temperature and optimum pH of EstV. The optimum temperatures are 55°C at pH 7 and 50°C at pH 10. Because the activities of the enzyme in different buffers and with different substrates cannot be directly compared, pH 10 is considered the optimum of EstV even though the enzyme activity is higher at pH 6 (succinate-NaOH buffer) and pH 9.5 (Tris-HCl buffer).

When the kinetic parameters of EstV were analyzed with MUF butyrate, the enzyme showed typical Michaelis-Menten behavior with no interfacial activation (not shown), as described previously for most carboxylesterases (17). The enzyme had calculated apparent V_{max} and K_m values of 695.3 ± 13 mU mg^{-1} and $5 \times 10^{-4} \pm 0.1 \times 10^{-4}$ M, respectively, a k_{cat} of 3.2 ± 0.1 s^{-1}, and a k_{cat}/K_m coefficient of $6.4 \times 10^3 \pm 0.1 \times 10^3$ s^{-1} M^{-1}.

The effects of different agents on the activity of EstV were also determined (Table 2). Of the cations analyzed, Ag^+ and Hg^{2+} at a concentration of 1 mM resulted in strong inhibition of EstV (residual activities, 5 and 49.5%, respectively). Significant inhibition was also observed with Cu^{2+}, Pb^{2+}, and Zn^{2+} (residual activities, 74 to 86%). In contrast, Ba^{2+}, Ca^{2+}, and Mg^{2+} activated EstV (residual activities, >130%). When these cations were assayed at a concentration of 10 mM, complete EstV inhibition was detected in the presence of Ag^+, whereas Cu^{2+}, Pb^{2+}, and Zn^{2+} also strongly inhibited the enzyme (residual activities, <50%). Addition of Co^{2+} and Fe^{2+} also resulted in moderate inhibition, whereas Ba^{2+}, Ca^{2+}, and Mg^{2+} activated EstV (residual activities, 127, 150, and 135% respectively) (Table 2).

TABLE 2. Effects of several agents on EstV

Agent	Residual activity (%)[a]	
	1 mM	10 mM
H_2O	100.0	100.0
$AgNO_3$	5.0	UD[b]
$BaCl_2$	130.0	127.3
$CaCl_2$	139.1	150.9
$CoCl_2$	98.0	77.3
$CuSO_4$	85.9	42.9
$FeCl_2$	111.7	83.0
$HgCl_2$	49.5	—[c]
$MgCl_2$	135.8	135.2
NaCl	102.9	118.6
NH_4Cl	107.6	101.0
$NiCl_2$	91.5	66.4
$Pb(CH_3COO)_2$	73.7	47.0
$ZnCl_2$	86.0	31.1
N-Acetylimidazole	103.9	99.2
p-Hydroxymercuribenzoic acid	115.9	135.0
PMSF	87.8	40.9
EDTA	86.7	64.0
SDS	11.4	7.2
Urea	56.7	32.1

[a] The standard deviations ranged from 2 to 10% of the mean values.
[b] UD, undetectable activity.
[c] —, $HgCl_2$ could not be assayed at a concentration of 10 mM by using this method.

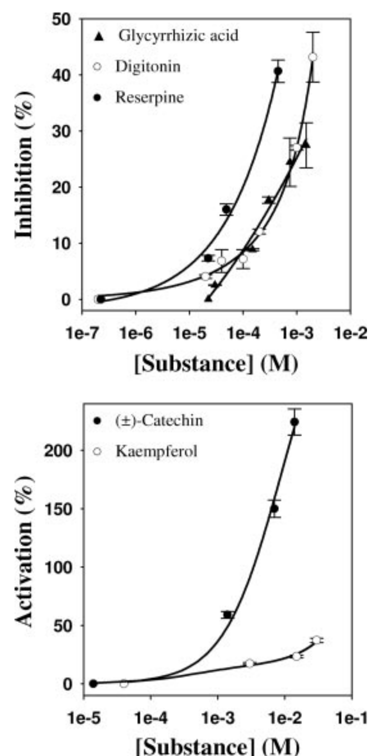

FIG. 5. Inhibition and activation of EstV by natural substances. Data for compounds exhibiting no activity with *H. pylori* EstV (digitonin, *Quillaja* saponin, and rescinnamine) are not shown.

The effects of the amino acid-modifying agents *N*-acetylimidazole (affecting tyrosine), *p*-hydroxymercuribenzoic acid (affecting cysteine), and phenylmethylsulfonyl fluoride (PMSF) (affecting serine) and the effects of EDTA, urea, and SDS were also tested (Table 2). PMSF, a typical inhibitor of lipases and other serine hydrolases, strongly inhibited EstV (residual activity with 10 mM PMSF, 41%), probably by covalent modification of the catalytic serine (21). In contrast, addition of *N*-acetylimidazole and *p*-hydroxymercuribenzoic acid caused no significant reduction in the enzyme activity, suggesting that serine, but not cysteine or tyrosine, is involved in the functional or structural domains of the enzyme. Of the other agents assayed, SDS resulted in the strongest inhibition at both 1 and 10 mM (residual activities, 11 and 7%, respectively), although the activity was partially recovered after SDS removal. Addition of urea also resulted in a high level of inhibition of EstV, probably due to its denaturing effects, whereas addition of EDTA resulted in less loss of activity (Table 2).

Inhibition of EstV by several plant secondary metabolites known for their antilipase activities was also tested (Fig. 5). EstV was strongly inhibited by reserpine (IC_{50}, 4.5×10^{-5} M), whereas addition of β-aescin and glycyrrhizic acid resulted in less inhibition of this enzyme (IC_{16}, 2.9 to 4.1×10^{-4} M). Interestingly, addition of (±)-catechin and kaempferol resulted in moderate activation (37.2%) and strong activation (224.3%) of EstV, respectively, when they were assayed at their maximum solubility concentrations (Fig. 5). In contrast, digitonin, *Quillaja* saponin, and rescinnamine had almost no effect on EstV activity (not shown).

DISCUSSION

Bacterial lipolytic enzymes are currently attracting an enormous amount of attention due to their wide biotechnological applications and due to their roles as virulence factors in some pathogenic bacteria (17), which has led to the isolation and characterization of a large number of bacterial lipases during the last few years. The lipases and phospholipases of pathogens such as *Propionibacterium acnes* (10, 15, 26) and *Staphylococcus* spp. (37) have been widely studied, and inhibition of these enzymes has been analyzed as a new potential pharmacological approach. In this work, we focused on the identification and characterization of *H. pylori* lipases because this bacterium is an important and widespread pathogen whose lipolytic activity with host lipids seems to favor bacterial colonization and development of ulcers by weakening the barrier properties of gastric mucus and probably also by producing inflammation, cytotoxicity, and cell lysis (5, 12, 35, 44, 45, 48, 51).

We searched for the unknown genes responsible for the lipase activity of this pathogen by examining the amino acid sequences of the lipases used by Arpigny and Jaeger (3) and Jaeger and Eggert (16) to define the different families and subfamilies of bacterial lipolytic enzymes and the amino acid sequences of the putative proteins in the *H. pylori* 26695 proteome. In this analysis, only the putative protein encoded by the HP0739 ORF exhibited higher levels of similarity to lipases than to other enzymes, although it was putatively annotated as a 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (50) related to *P. putida* dienoate hydrolase, an enzyme involved in the degradation of aromatic compounds such as toluene (28). However, it is known that family V lipases exhibit significant amino acid sequence similarity to various bacterial nonlipolytic enzymes, such as epoxide hydrolases, dehalogenases, and halo-

peroxidases, which also possess the typical α/β hydrolase fold and the catalytic triad of lipases (3). Thus, we decided to continue our computational study of the HP0739 ORF, which revealed that the deduced HP0739-encoded protein was a small, globular, single-domain protein with a pI of 9.0 and was probably located in the cytoplasm. This study also showed that the HP0739-encoded protein belongs to the serine hydrolase group and has common features of basic lipases (11, 17), as well as the conserved sequence motifs of family V bacterial lipases in its deduced amino acid sequence (Fig. 1), suggesting that the protein could be grouped in this family.

The HP0739-encoded protein was cloned and purified in order to confirm its lipolytic activity and to perform a biochemical analysis of the enzyme. The enzyme exhibited the typical behavior of carboxylesterases; it was active on tributyrin (C_4) but not on triolein (C_{18}) or olive oil and showed a marked preference for short-chain (C_2 to C_6) *p*-NP and MUF derivatives (Fig. 2 and 3). Analysis of the kinetic parameters of the enzyme with MUF butyrate revealed Michaelis-Menten behavior with no interfacial activation, a behavior typical of carboxylesterases (17). Thus, on the basis of sequence similarity, the presence of carboxylesterase activity, and the kinetic properties, the protein that was isolated was identified as a new carboxylesterase (EC 3.1.1.1) belonging to bacterial lipase family V and was designated EstV.

Comparison of the biochemical properties of EstV with those of other lipases is difficult since very little information about family V lipases is available. For this family, only carboxylesterase Est2 of *A. pasteurianus* has been isolated, cloned, and partially characterized previously (18, 19), while the other members of family V are four putative carboxylesterases and a polyhydroxyalkanoate depolymerase (3). Moreover, no information concerning Est2 kinetic behavior or Est2 activity with medium- and long-chain *p*-NP derivatives is available, since these biochemical features are described here for the first time for this family. However, based on the activities with other lipids, EstV seems to have a wider substrate range than Est2, because no activity with triacylglycerols longer than tripropionin (C_3) or with lipid derivatives with acyl chains longer than C_4 has been reported for Est2 (18).

When its other biochemical properties were examined, EstV was found to be a stable enzyme with maximum activity at 50°C and pH 10 and to also exhibit high levels of activity at temperatures ranging from 34°C to 60°C and at pH 5 to 10.5 (Fig. 4). Such optimal conditions make EstV very interesting for biotechnological applications but seem surprising for an enzyme from an acid-tolerant neutrophilic bacterium adapted to a host whose temperature is 37°C. Nevertheless, we should consider several aspects of *H. pylori* enzymes and lipases in general. Similar optimal conditions (maximum activity at 50 to 60°C and pH 8 to 9) have been found for other *H. pylori* enzymes, such as phospholipase C (52), and although the reasons are unclear, this fact could be related to the high intracellular pH of this bacterium when it grows in neutral conditions (internal pH of 8.1 at pH 7.4 [53]). Furthermore, the optimum temperature of EstV is consistent with the optimum temperatures reported for *A. pasteurianus* Est2 (18) and many other carboxylesterases (33, 36, 38), whereas the optimum pH of EstV is similar to those reported for several carboxylesterases and "true" lipases (11, 36). In fact, it is known that the optimal conditions for lipolytic enzymes usually are quite different from the optimal growth conditions for the bacteria that produce them. For the pH, the reason for the differences seems to be the fact that lipases exhibit optimum activity when the active site is slightly negatively charged, which is usually correlated with the electrostatic signature of the enzyme (11). Thus, an optimum pH of 10 for EstV is not so surprising taking into account the fact that this protein has a pI of 9.

The analysis of the effects of different agents on the activity of EstV revealed significant inhibition by Ag^+, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, PMSF, SDS, urea, and EDTA and activation by Ba^{2+}, Ca^{2+}, and Mg^2. In general, the effects of the cations and agents that were analyzed are in agreement with the general effects of these compounds on other lipases (14). However, within family V, there are some differences between EstV and Est2 from *A. pasteurianus*. Both of these enzymes, which can be compared only with respect to the effects of three compounds, Hg^{2+}, PMSF, and EDTA, are inhibited by PMSF, whereas Hg^{2+} and EDTA cause significant inhibition of EstV activity but not Est2 activity (18). The effects of several plant secondary metabolites known for their antilipase activities were also tested, which revealed that EstV was inhibited by β-aescin, glycyrrhizic acid, and reserpine (Fig. 5). Interestingly, glycyrrhizic acid is also an inhibitor of *P. acnes* lipase (10), one of the main virulence factors of this bacterium. Moreover, both β-aescin and glycyrrhizic have known antiulcer effects and are important constituents of herbal drugs used in the therapy of ulcers and other lipase-related diseases, such as acne (6, 15, 25, 43), which suggests that the therapeutic effectiveness of these substances might be also related to their antilipase activities. Of the other substances analyzed, only (±)-catechin and kaempferol had an effect on the enzyme. Both of these flavonoids activated EstV, which indicates that their antiulcerogenic activities are not related to EstV activity but are related to other effects as inhibitors of *H. pylori* growth (6) and as gastroprotective and anti-inflammatory agents (6).

Like the physiological roles of most lipases, including Est2 from *A. pasteurianus*, the physiological role of *H. pylori* EstV is unknown. Several facts suggest that EstV lipase might contribute to *H. pylori* virulence: (i) previous results have indicated that an *H. pylori* lipase(s) may have a role in ulcer development because lipase activity of this pathogen is capable of degrading the lipids of gastric mucus (31, 32, 44, 45, 46, 47), and EstV is the only enzyme with lipase activity that has been identified in this bacterium; and (ii) *H. pylori* lipase activity can be inhibited by antiulcer agents (32, 44, 45, 46, 47), and EstV is inhibited by the antiulcer substances β-aescin and glycyrrhizic acid. On the contrary, other aspects suggest that EstV might not be related to *H. pylori* virulence: (i) EstV is activated by the antiulcer substances (±)-catechin and kaempferol, but it is also possible that an increase in EstV activity, which was very substantial with (±)-catechin, is detrimental for *H. pylori*, perhaps by causing hydrolysis of the bacterium's own lipids; and (ii) EstV seems to be intracellular, which would make its access to host lipids difficult; however, this enzyme could be involved in degradation of diacylglycerols and other lipids taken up by the bacterium after their release due to the activity with host lipids of *H. pylori* extracellular phospholipases or host hydrolases produced during early inflammation and, thus, could be an important member of an enzymatic chain responsible for deg-

radation and assimilation of host lipids by *H. pylori*. In any case, further experiments are necessary to elucidate the biological role(s) of EstV. Such experiments, including knockout and complementation of the *estV* gene and subsequent analysis of the effects on *H. pylori* colonization and ulcer development under different conditions, have been possible only with *H. pylori* phospholipase A_2 because no genes responsible for lipase activity have been identified previously in this pathogen. Therefore, we intend to encourage such research in order to examine the biological significance of EstV and its potential as a new pharmacological target. The fact that EstV is intracellular does not reduce this potential since, although in research on inhibition of lipases as a new approach for the therapy of lipase-related infectious diseases workers have focused on secreted lipases (10, 15, 37), it is known that many intracellular enzymes are excellent pharmacological targets (inhibition of DNA gyrase by fluoroquinolones, inhibition of RNA polymerase by rifampin, inhibition of dihydrofolate reductase by trimethoprim, etc. [54]).

The isolation, cloning, and characterization of the EstV carboxylesterase of *H. pylori* have very great significance for different reasons. On the one hand, this is the first time that a lipase of this pathogen has been cloned and characterized, which provides new opportunities for more research concerning the physiological and pathogenic significance of lipase activity in *H. pylori*. Furthermore, as far as we know, EstV is also the first lipase of an epsilon-proteobacterium that has been described, and thus our findings increase our knowledge about the biochemical and physiological traits of the epsilon-proteobacterial group, which is probably the least-known proteobacterial group. On the other hand, EstV is a new member of family V, probably the least-known family of bacterial lipases. Characterization of this enzyme was a very important step for increasing our knowledge about this family, since here we provide for the first time information about the activity with substrates with C_2 to C_{18} acyl chains, the kinetic behavior, and inhibition by several agents and natural substances for an enzyme belonging to this family of lipases, which could contribute to the development of biotechnological applications for this interesting and unexploited group of enzymes.

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