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The Putative Arthritogenic Cationic 19-Kilodalton Antigen of *Yersinia enterocolitica* Is a Urease β-Subunit

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The gene coding for a putative 19-kDa arthritogenic antigen of *Yersinia enterocolitica* O:3 (A. K. H. Mertz et al., J. Clin. Invest. 87:632–642, 1991) was cloned and sequenced after amplification of part of the gene by the polymerase chain reaction using degenerate primers, inferred from the amino acid sequence. The deduced amino acid sequence of the antigen showed similarity to small subunits of ureases from several different organisms, including the jack bean urease. Screening of a genomic library of *Y. enterocolitica* O:3 with a 19-kDa-antigen-specific DNA probe allowed recombinant clones containing the entire urease operon to be obtained. These clones expressed urease activity in *Escherichia coli*.

Yersinia enterocolitica serotype O:3 strains cause infections accompanied by enterocolitis and mesenterial lymphadenitis (26). Patients with certain HLA types, especially those positive for HLA-B27, quite often develop postinfectious symptoms, such as reactive arthritis, erythema nodosum, and uveitis (26). Despite intensive efforts in many laboratories to identify the bacterial arthritogenic factor(s), only minimal progress has been made. A putative bacterial arthritogen is lipopolysaccharide, which has been found in synovial fluid cells of affected joints (9). Another putative arthritogen is a cationic 19-kDa antigen derived from *Y. enterocolitica* O:3, which when injected intra-articularly into preimmunized rats triggered joint inflammation (16). In this study, we further characterized the 19-kDa antigen and identified it as a small β-subunit of urease.

MATERIALS AND METHODS

Bacterial strains and plasmids. A *Y. enterocolitica* serotype O:3 strain isolated from a case of enteritis (Freiburg strain 10543) was used as a source for purifying the 19-kDa antigen (16). *Y. enterocolitica* O:3 strain 6471/76-c (23) was used for the construction of the genomic library (1). The cloning vector in the library was pBR322 (5). Recombinant plasmids isolated from this library are shown in Fig. 2. Bacteriophage M13mp18 (17) was used as a vector in single-stranded sequencing. *Escherichia coli* C600 (2) and JM103 (17) were used as host strains for the genomic library and M13mp18, respectively.

Construction of the genomic library. The genomic library has been described earlier (1). Briefly, the genomic DNA of 6471/76-c was partially digested with restriction endonuclease *Sau*3AI, and fragments larger than 6 kb were isolated by preparative electrophoresis. These fragments were ligated to plasmid pBR322, which was opened with restriction endonuclease *Bam*HI.

PCR. Polymerase chain reaction (PCR) was performed by using degenerate oligonucleotide combinations Pr5 and Pr3 (see Results). Amplification was carried out in a reaction mixture containing 20 ng of *Y. enterocolitica* genomic DNA, 300 pmol of each oligonucleotide combination, 200 μM each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Boehringer GmbH, Mannheim, Germany) in 10 mM Tris-HCl–1.5 mM $MgCl_2$–50 mM KCl plus 1 mg of gelatin per ml in a reaction volume of 100 μl at pH 8.3 at 20°C. Amplification for 30 cycles was carried out under the following conditions: denaturation at 94°C for 45 s, annealing at 35°C for 1 min, and extension at 72°C for 2 min.

Purification of the 19-kDa antigen. Purification of the 19-kDa antigen has been described in detail earlier (16). *Y. enterocolitica* O:3 strain 10543 was grown in brain heart infusion medium at 30°C. Fifty grams of washed cells was disrupted in a Braun homogenizer, and after DNase treatment, the ribosomal pellet was obtained by differential centrifugation. This pellet was acid extracted, and the soluble fraction was dialyzed to neutrality. Further purification was performed on a fast protein liquid chromatography system (Pharmacia-LKB, Uppsala, Sweden) using a mono-S cation exchanger and a sodium chloride salt gradient (0.15 to 2.0 M); a highly purified fraction eluted at 0.3 M NaCl in low quantities was employed in this study.

Amino-terminal sequence determination of the 19-kDa antigen and its tryptic peptides. Two hundred micrograms of protein was digested with 4 μg of sequencing-grade trypsin for 4 h at 37°C in 300 μl of phosphate-buffered saline (PBS) buffer, adjusted to pH 8.0. The fragments were separated on a reversed-phase high-pressure liquid chromatography (HPLC) column (PEP-S; Pharmacia) by 0.1% trifluoroacetic acid-acetonitrile gradient elution as described earlier (22). Peptides were sequenced in a pulsed-liquid gas-phase sequencer with on-line identification of the amino acid derivatives (models 477A and 120A; Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. The N-terminal sequence on 60 μg of the whole protein was determined in the same way.

SDS-PAGE and immunoblotting. A total cell lysate of bacteria was prepared by sonicating about 200 mg of bacteria (wet weight) in 1 ml of PBS containing a protease inhibitor. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (3) by using a 4% stacking gel and a 12.6% separating gel. The

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TABLE 1. Amino acid sequences of proteolytic fragments and of the amino terminus of the intact protein

Peptide	Sequence[a]	Positions in deduced sequence[b]
NH_2 terminus	STKTNSTKAT SEKTDSLKTN RGTKSSAGYS EQNTPLGGxI LAD	2–44 (C)
Peak 7	AAAYGK + GFK[c]	88–93, 159–161
Peak 8	TDSLK	15–19
Peak 12	ALEFDR	82–87
Peak 15	LNISSTTAIR	95–104
Peak 20	NTGDRPIQVG SxF	62–74 (H)
Peak 27	FEPGDET	105–111
Peak 31	QTLYGFNNLV $DGx_1TGx_2GVVP\ Nx_3x_4R$	122–145 (W, E, S, E)
Peak 32	SSAGYSEQNT PLGGxILADT PITFN	26–50 (C)

[a] x, an unidentified amino acid residue.
[b] See Fig. 4. Amino acids represented by "x" under "Sequence" are identified in parentheses, as deduced from the DNA sequence.
[c] Peak 7 was interpreted in the first analysis as AFAYGK, but reanalysis after availability of the nucleotide sequencing data revealed that this peak contained the two peptides AAAYGK and GFK.

separated bands were transferred to an Immobilon membrane (Millipore, Molsheim, France) by electrotransfer. The membrane was blocked with 10% skim milk in PBS and incubated first with rabbit antiserum to the 19-kDa antigen diluted 1:5,000 (16) and, after washing, with peroxidase-conjugated goat anti-rabbit antibodies (Dianova) at a 1:4,000 dilution. Finally, the bound peroxidase was detected by incubating the blot in substrate solution containing diaminobenzidine (Sigma, St. Louis, Mo.).

Antiserum to the 19-kDa polypeptide was raised in rabbits by repeated immunization with purified antigen (see above) in Freund's complete adjuvant; a panel of mouse monoclonal antibodies was also produced. Titer and specificity of the antibodies were tested by Ouchterlony's diffusion test and in immunoblot. In the latter case, SDS-PAGE gels were run under both native and reducing conditions; this switched the antigen from a 38-kDa thiol-linked dimer to the 19-kDa monomeric form, providing a further specificity control. The antibodies obtained could be absorbed with the antigenic fraction (16).

DNA techniques. Isolation of DNA, restriction digestions, colony hybridizations, labelling of probes, and autoradiography, etc., were performed by using standard techniques (3). DNA sequencing was performed on single- and double-stranded templates by the dideoxy chain termination method using the Sequenase version 2.0 kit (United States Biochemicals), as recommended by the supplier. Primers for sequencing and for PCR were synthesized by using the PCR-Mate DNA synthesizer (Applied Biosystems).

Computer analysis. The DNA sequences were handled by the GENEUS (11) and Genetics Computer Group (University of Wisconsin) (7) program packages. Data bank searches for homologous polypeptides were performed by the TFASTA program (15). The amino acid sequences were aligned by using the GAP and LINEUP programs, and the consensus sequence was generated by the PRETTY program.

Nucleotide sequence accession number. The nucleotide sequence of the urease operon genes *yeuA*, *yeuB*, and *yeuC* of *Y. enterocolitica* O:3 has been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number Z18865.

RESULTS

Amino acid sequence determination for the 19-kDa protein and its tryptic fragments. The 19-kDa protein was sequenced from its N terminus to the 43rd residue. Tryptic peptides generated from the whole protein were separated by reversed-phase HPLC and subjected to Edman degradation. Table 1 shows the sequences obtained and a comparison with the DNA-deduced primary structure (see below). These results agreed completely.

PCR cloning. On the basis of the determined amino acid sequence of the amino terminus and the peak 31 fragment, two degenerate oligonucleotide combinations were constructed. Degenerate oligonucleotide Pr5 corresponds to amino acids 2 to 10 of the amino-terminal sequence, and Pr3 corresponds to the amino acids present in the peak 31 fragment (Table 1). The codon usage in the published sequences of *Y. enterocolitica* was used as a guideline in selecting the bases for wobble positions. Taking into account the 19-kDa size of the intact protein (150 to 180 amino acids), the anticipated length of the amplified PCR product could be at most 500 bp (in case the peak 31 fragment is located close to the carboxy terminus of the protein). Accordingly, if the peak 31 fragment is located closer to the amino terminus of the protein, the PCR product would be shorter. Thus, any PCR product shorter than 500 bp could be considered representative. Chromosomal DNA of *Y. enterocolitica* O:3 6471/76-c was used as a template, and upon amplification under conditions given in Materials and Methods, several fragments of different sizes, among which a fragment of about 400 bp was dominant, were obtained. This fragment was purified from a preparative agarose gel and cloned into M13mp18, and its DNA sequence was determined (Fig. 1). One of the reading frames was open throughout the length of the fragment and some of the sequenced peptides as well as the amino-terminal amino acid sequence could be identified from this open reading frame (Fig. 1 and Table 1).

The 400-bp fragment was used as a probe to screen the genomic library of *Y. enterocolitica* O:3 previously cloned into pBR322 (1). Nine positive clones were identified. The recombinant plasmids of the clones were shown to carry six different overlapping inserts of *Y. enterocolitica* DNA, ranging in size between 3 and 13.5 kb. The restriction maps of these plasmids are shown in Fig. 2.

Nucleotide sequence. The recombinant plasmid of one of the recovered clones, p19kd-107, was used as a template to determine the DNA sequence outside the sequenced 400-bp PCR-amplified fragment. The whole sequence is shown in Fig. 1.

It is interesting that the degenerate primers, constructed to generate the 400-bp fragment by PCR, had significant homology to the actual sequence only in their 3′ ends and that

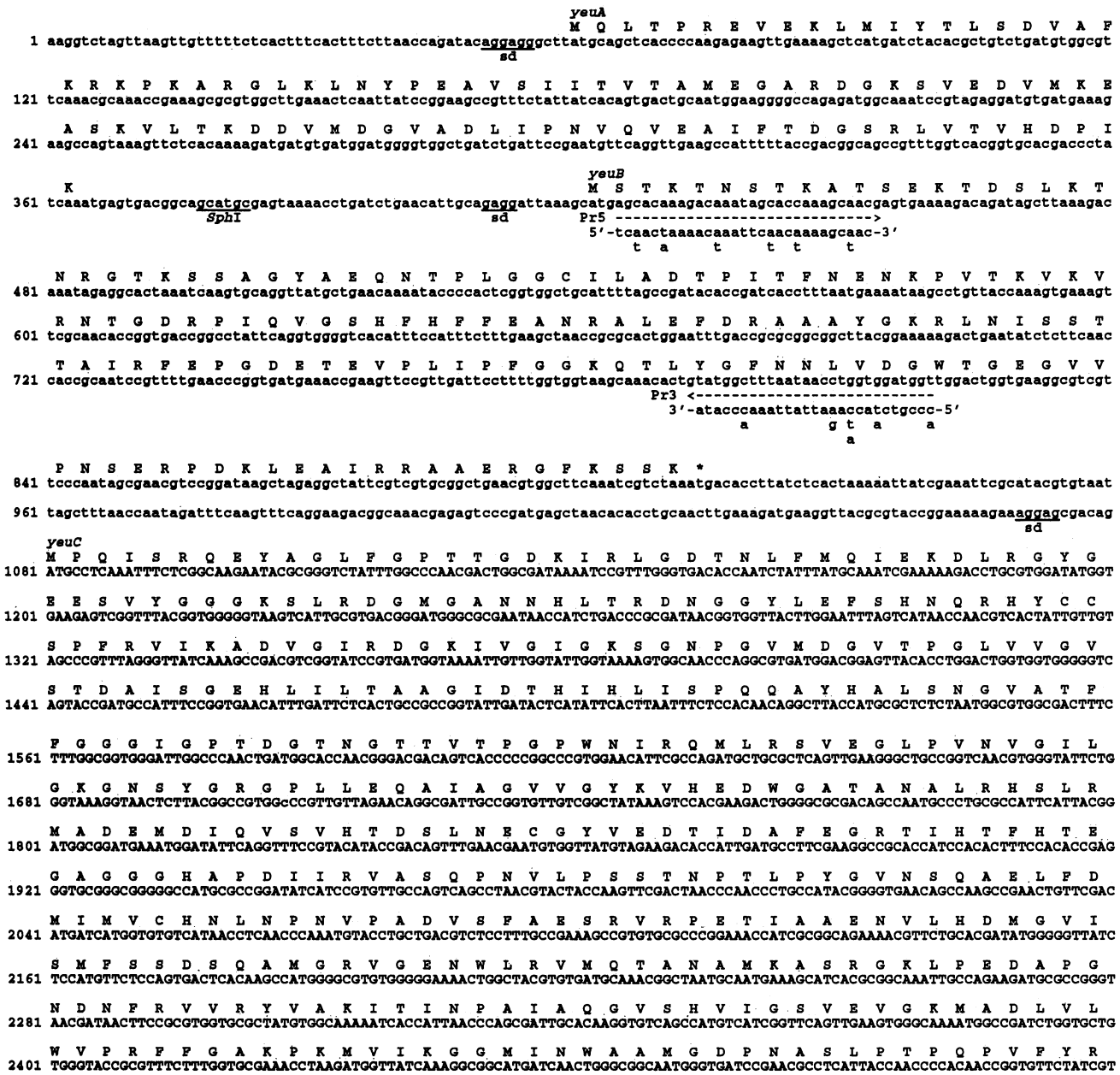

FIG. 1. Nucleotide sequence of the urease operon genes *yeuA*, *yeuB*, and *yeuC* of *Y. enterocolitica* O:3. The positions and sequences of the degenerate primer combinations, Pr3 and Pr5, deduced from amino acid sequences, are indicated. Note that none of the degenerate primer combinations perfectly matched the genomic sequence. The sequences of the oligonucleotide pair incorporated into the cloned and sequenced PCR product (see text) are given closest to the genomic sequence. Amino acid sequences of the open reading frames are given by the one-letter code; stop codons are indicated (*). The ribosomal binding sequences (sd) and *Sph*I and *Cla*I restriction sites are also indicated.

identity in a sequence as short as 8 nucleotides had been sufficient for initiation of amplification.

An open reading frame which could code for a 164-amino-acid polypeptide was identified (Fig. 1). All the sequenced peptide fragments shown in Table 1 could be tracked to this open reading frame. The calculated size of the polypeptide was 17.9 kDa, with a high pI of 10.2.

A second open reading frame was identified upstream of

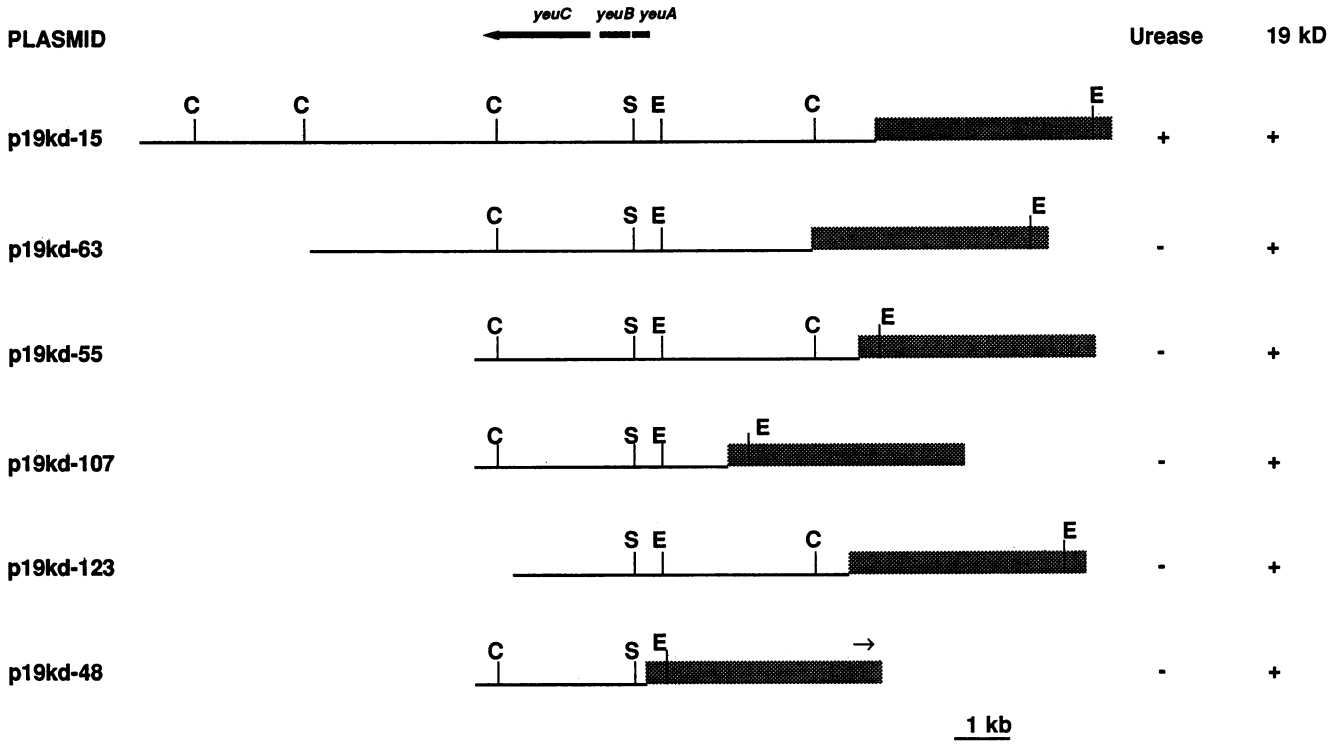

FIG. 2. Recombinant-DNA plasmids positive for the 19-kDa antigen probe. pBR322 DNA in the plasmids is shown as a shaded box, and the *Y. enterocolitica* DNA is shown as a solid line. Restriction enzymes: E, *Eco*RI; H, *Hin*dIII; S, *Sph*I; C, *Cla*I. Urease expression and the 19-kDa antigen expression of the clones carrying these plasmids are shown on the right. Only the *Eco*RI site of pBR322 is shown to indicate its orientation. The arrow above the p19kd-48 map indicates the position and the 5'-to-3' direction of the pBR322-specific primer used to determine the direction and location of the open reading frames shown by a thick line and arrow at the top.

the one described above. This frame had a coding capacity for 102 amino acids. The calculated size of the polypeptide was 11.3 kDa, with a pI of 5.95. An *Sph*I restriction site was found between the open reading frames. The *Sph*I site was mapped in plasmid p19kd-48 as shown in Fig. 2. In PCR analysis, using p19kd-48 as a template and Pr6 or Pr7 (positions 746 to 763 and 493 to 473, respectively, in Fig. 1) together with a pBR322-specific primer (arrow above the p19kd-48 map in Fig. 2), a 2.3-kb amplified product was obtained with the Pr6-containing PCR mixture while nothing was amplified in the Pr7-containing PCR mixture (data not shown). On the basis of this information, we could map the open reading frames and their directions as shown in Fig. 2.

A third open reading frame was identified downstream of the 19-kDa open reading frame. This open reading frame had a coding capacity for 573 amino acids. The calculated size of the polypeptide was 61.5 kDa, with a pI of 6.33.

The recombinant plasmids were analyzed by immunoblotting with antiserum specific for the 19-kDa antigen (Fig. 3). All of the plasmids expressed a 19-kDa antigen, immunologically identical to the *Y. enterocolitica* 19-kDa antigen (Fig. 3, lanes 1 to 3 and 5), as revealed by using polyclonal rabbit and monoclonal mouse antibodies to the 19-kDa polypeptide. The host strain of the recombinant plasmids, *E. coli* C600, was negative (Fig. 3, lane 4).

The 19-kDa protein is a urease subunit. The amino acid sequences of the three open reading frames, deduced from the nucleotide sequence, were compared with the GenEMBL nucleotide data base by using the TFASTA program of the Genetics Computer Group program package (7, 15). Several high scores were obtained. All of these turned out to be subunits of ureases (4, 6, 13, 14, 19–21, 24, 25). Alignments of the complete amino acid sequences of the 19-kDa antigen and the polypeptides of the upstream and downstream open reading frames with the urease subunits are shown in Fig. 4.

These findings led us to search for urease activity in the clones identified from the *Y. enterocolitica* O:3 genomic library. The clone carrying the largest plasmid, p19kd-15, was clearly positive for urease activity, as tested by using Christensen's urea agar (Fig. 2). On the basis of these results, the structural gene of the 19-kDa antigen was designated *yeuB*, the upstream gene was designated *yeuA*, and the downstream gene was designated *yeuC* (for *Y. enterocolitica* urease).

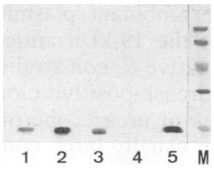

FIG. 3. Expression of the 19-kDa antigen by *Y. enterocolitica* O:3 strain 10543 and *E. coli* strains. Bacterial samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection was performed with a rabbit anti-19-kDa-antigen antiserum and total cell lysates as antigens. Lane 1, *E. coli* C600/p19kd-48; lane 2, *E. coli* C600/p19kd-107; lane 3, *E. coli* C600/p19kd-15; lane 4, *E. coli* C600; lane 5, *Y. enterocolitica* 10543; lane M, molecular weight markers.

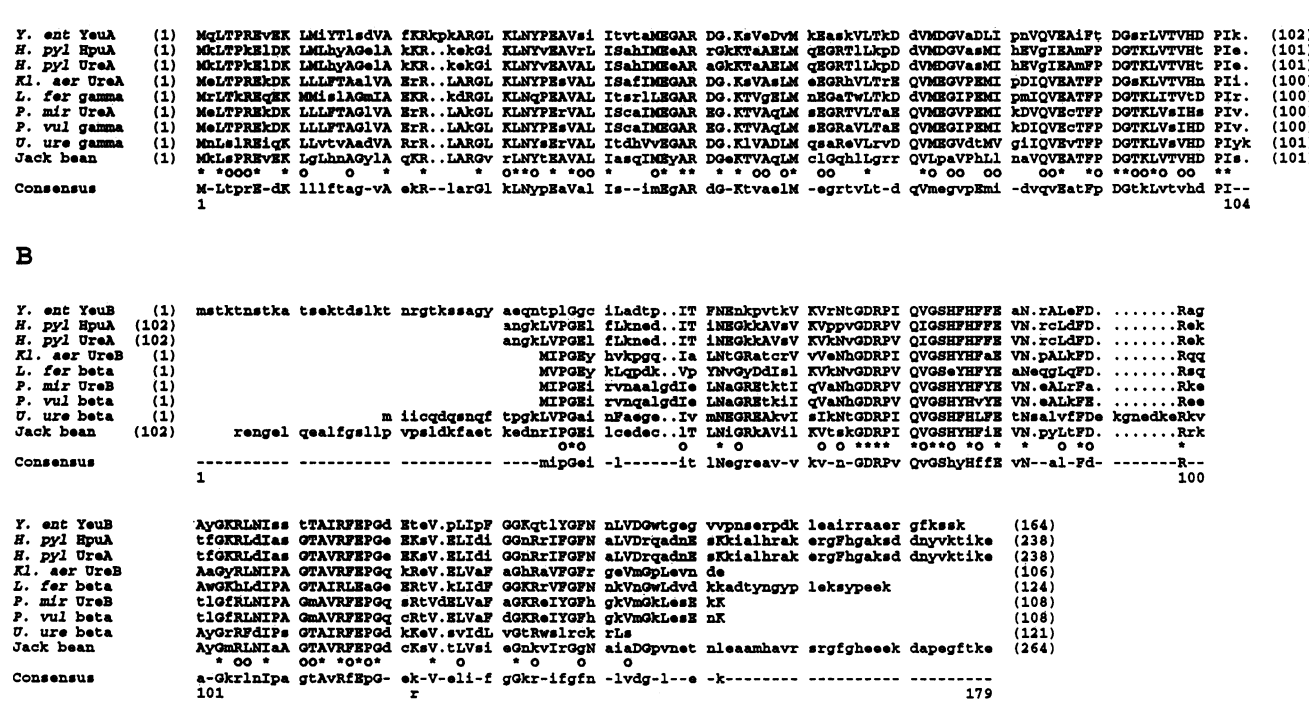

FIG. 4. Alignment of the *Y. enterocolitica* O:3 urease γ- (A), β- (B), and α- (C) subunit amino acid sequences with the corresponding subunit sequences of different organisms. The polypeptides were aligned by using the GAP program, such that all sequences were "gapped" against each other to obtain maximal fitting. The gapped sequences were then lined up, and the consensus peptide was created by the PRETTY program. A consensus was created whenever four or more sequences had the same or similar amino acids at a particular position. Positions at which the consensus threshold was not reached are shown by dashes. In the sequences, amino acids identical or similar to those in the consensus are in uppercase letters; others are lowercase. Positions occupied by the same amino acid in all sequences are indicated by uppercase letters in the consensus sequence and by asterisks above the letters. Positions occupied by the same amino acid in seven of the nine sequences are marked by circles above the consensus sequence. Gaps are shown by dots. Amino acid sequences are as follows: small and large subunits of two *Helicobacter pylori* (*H. pyl*) ureases (HpuB, HpuA, and UreA; accession [acc.] numbers for the nucleotide sequences, M60398, X57132, and X17079), *Klebsiella aerogenes* (*Kl. aer*) urease α-, β-, and γ-subunits (UreC, UreB, and UreA; acc. no. M36068), *Lactobacillus fermentum* (*L. fer*) urease α-, β-, and γ-subunits (acc. no. D10605), *Proteus mirabilis* (*P. mir*) urease α-, β-, and γ-subunits (UreA, UreB, and UreA; acc. no. M31834), *Proteus vulgaris* (*P. vul*) urease α-, β-, and γ-subunits (acc. no. X51816), *Ureaplasma urealyticum* (*U. ure*) urease α-, β-, and γ-subunits (acc. no. X51315), and *Canavalia ensiformis* (jack bean) urease (acc. no. M65260). The first and last positions of each sequence used in the comparison are indicated in parentheses. Note that the *H. pylori* small subunit and the jack bean urease sequences continue from panel A to panel B; the latter also continues to panel C.

DISCUSSION

This article describes the molecular cloning, sequencing, and identification of a cationic 19-kDa antigen isolated from *Y. enterocolitica*. The antigen was identified as a β-subunit of the *Y. enterocolitica* urease. Two lines of evidence support this conclusion. First, the amino acid sequence similarities to other urease small subunits are convincing (Fig. 4). Second, recombinant plasmids carrying and expressing the gene of the 19-kDa antigen conferred urease activity to urease-negative *E. coli* strains. Likewise, the size of the insert in the urease-positive clones is in accordance with the known sizes of urease operons of other bacteria. Bacterial ureases are usually large composite structures of two or three different subunits, designated α-, β-, and γ-subunits (4, 14, 19). In addition to the genes for the subunits, the urease operons contain genes coding for accessory and regulatory proteins (13, 14, 20). The accessory proteins are required to assemble the holoenzyme, which needs nickel ions for activity (20). Thus, the sizes of the urease operons are usually between 5 and 10 kb. In this study, the urease activity was expressed only by the recombinant plasmids containing the largest *Y. enterocolitica* DNA insert, i.e., 13.4 kb (p19kd-15), but not by p19kd-63, which contained a 9.2-kb insert. On the basis of the data obtained in this study and assuming that *yeuA* is the first gene in the operon, the minimal size of the operon would be over 6 kb (Fig. 2). No apparent promoter motifs were detected upstream of the *yeuA* gene. Most probably, expression of the 19-kDa antigen by an *E. coli* strain carrying plasmid p19kd-48 was made possible by the tetracycline resistance gene promoter of pBR322. Further work will be needed to elucidate the structure of the *Y. enterocolitica* urease operon.

Comparison of the amino acid sequences of the *Y. enterocolitica* urease subunits with other corresponding sequences revealed that the γ-subunit is highly conserved. Identities ranged between 51 and 60%, and similarities ranged between 67 and 80%. In addition, the lengths of the polypeptides are constant, ranging between 100 and 102 amino acid residues. The β-subunit seems to be less conserved, with the lengths of the polypeptide chains ranging between 106 and 164 amino acid residues. Likewise, the identities and similarities be-

C

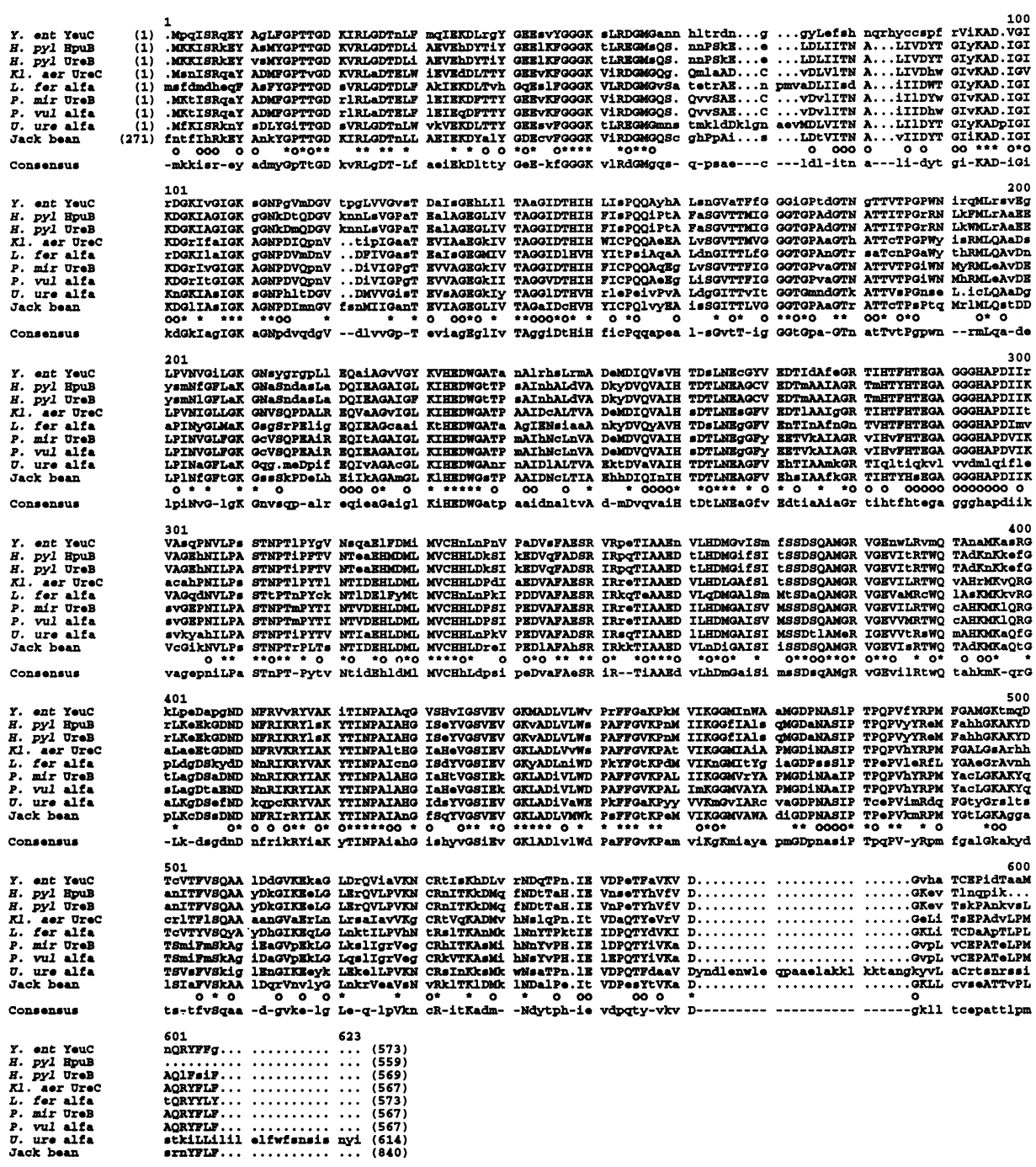

FIG. 4—*Continued.*

tween the sequences range between 33 and 52% and between 51 and 67%, respectively. Comparison of the amino acid sequences of the α-subunit (Fig. 4C) suggests that it is also highly conserved. The length is about 570 amino acid residues, and the identities and similarities between the sequences range between 48 and 57% and between 68 and 75%, respectively.

The regularity of occurrence of lysine residues in the N terminus of the 19-kDa antigen (positions 4, 9, 17, 19, and 25, etc.) is a striking structural feature, reminiscent of many DNA-binding proteins. It explains the polypeptide's high affinity for polyanionic molecules, like nucleic acids, and accounts for its occurrence in the ribosomal pellet as well as for the marked tendency for aggregation with other proteins

(16). Under the conditions employed for preparation, the polypeptide was obtained as a thiol-linked dimer (a single cysteine residue at position 40 was found), usually complexed with other molecules (16). The cellular distribution of the urease or the 19-kDa antigen was not examined; it is likely to be cytoplasmic, as this appears to be the case with all other urease-positive enteric bacteria studied to date (18). The antigen may be released from living cells and, in addition, be set free following cellular degradation.

In humans, arthritis following *Y. enterocolitica* O:3 infection is closely linked with the HLA-B27 antigen. No homologies between the B27 sequence and the 19-kDa antigen were seen. However, we found a panel of nine possible nonapeptides with the HLA-B27-specific anchor residue arginine at position P2. Of these, nonapeptide RRAA ERGFK, the nine residues in positions 153 to 161, should be ranked at the top because of the large consensus in sequence pattern to the peptide motifs fitting to the binding cleft of the HLA-B27 molecule (12, 15b). On the basis of structural studies, this nonapeptide would bind excellently to HLA-B27 (15a). Thus, the 19-KDa antigen could function as a source of an arthritogenic or immunogenic peptide to be presented to cytotoxic T lymphocytes. The relevance of this finding to *Y. enterocolitica*-induced reactive arthritis remains to be elucidated.

The β-subunit of the *Y. enterocolitica* O:3 urease, the 19-kDa cationic antigen, described here, attracted our attention because it has arthritogenic potential in rats (16). Antibody to the 19-kDa peptide is found in humans (16) and may prove to be a useful marker of *Yersinia* infection. As occurs with some other cationic antigens, intra-articular challenge of preimmunized rats induces a chronic destructive joint lesion (8, 16, 28); however, it is the first bacterial product reported to have this ability. Whether the β-subunit, as an antigen, plays a role in the induction of human and experimental *Yersinia*-reactive arthritides (10, 26, 27) remains to be determined.

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