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Clostridium perfringens Urease Genes Are Plasmid Borne

BRUNO DUPUY,[1]† GEORGES DAUBE,[2] MICHEL R. POPOFF,[3] AND STEWART T. COLE[1]*

Unité de Génétique Moléculaire Bactérienne[1] and Unité des Toxines Microbiennes,[3] Institut Pasteur, 75724 Paris Cédex 15, France, and Faculté de Médecine Vétérinaire, Université de Liège, B-4000 Liège, Belgium[2]

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Although many bacteria are ureolytic, and in some cases urease acts as a virulence factor, the urease phenotype has not been analyzed in the anaerobic pathogen *Clostridium perfringens*. In this study, ~2% of *C. perfringens* strains, representing the principal biotypes, were found to harbor the urease structural genes, *ureABC*, and these were localized on large plasmids that often encode, in addition, the lethal ε or ι toxins or the enterotoxin. This represents the first report of a plasmid-encoded urease in a gram-positive bacterium. The *C. perfringens* enzyme was highly similar to the ureases of other bacteria and cross-reacted with antibodies raised against the urease purified from *Helicobacter pylori*. Urease production was inhibited by urea and induced under growth conditions where the availability of nitrogen sources was limiting. To date, this form of regulation has been observed only for chromosomal *ureABC* genes.

Clostridium perfringens is a spore-forming gram-positive anaerobic pathogen commonly found in the lower intestinal tracts of humans and other mammals, as well as in soil and sewage. *C. perfringens* causes a variety of diseases ranging in severity from the frequently fatal gas gangrene to a mild but common form of food poisoning mediated by a potent enterotoxin, CPE (25, 37, 46). Clinical isolates are generally classified into five biotypes, A to E, by their production of the four lethal typing toxins, the alpha-, beta-, epsilon-, and iota-toxins (25, 38, 46). In addition to the typing toxins, a large variety of other toxins and hydrolytic enzymes, such as perfringolysin O, or theta-toxin, and collagenase, or kappa-toxin, (35, 36, 49), that are likely to play a significant role in pathogenesis, are produced by most strains of *C. perfringens.*

Genome mapping has proved a powerful tool (5) for studying genomic diversity and the variation in the toxin gene repertoire among the various *C. perfringens* biotypes. Contrary to what was first thought (46), it is now clear that the importance of plasmid-borne virulence genes to *C. perfringens* pathogenesis has been underestimated, as compelling evidence has been obtained recently for an extrachromosomal location for several known toxin genes (6, 31, 32). Among these are the *cpb*, *etx*, and *itxAB* genes encoding the lethal beta-, epsilon-, and iota-toxins, respectively, as well as the enterotoxin gene, *cpe* (9). Interestingly, in a minority of CPE^+ strains, the *cpe* gene is not localized on a plasmid but is found on the chromosome and the strains are responsible for food poisoning (9, 31).

Many bacteria produce urease (urea amidohydrolase; EC 3.5.1.5), a nickel-containing metalloenzyme that hydrolyzes urea to ammonia and carbamate (41). In most, but not all, cases the enzyme consists of three distinct subunits, UreA, UreB, and UreC, encoded by the *ureABC* gene cluster, which is often linked to a number of accessory genes, some of which are required for the biogenesis of urease cofactors (42). Expression of urease can be constitutive, inducible by urea, or controlled by nitrogen source availability, depending on the bacterium. Urease has been shown to act as a virulence factor in the urinary tract and in gastroduodenal infections due to *Proteus mirabilis* (30) and *Helicobacter pylori* (18), respectively.

During a recent genomic survey of *C. perfringens* (32), a gene equivalent to *ureC* of *H. pylori* was partially characterized (34). Although the *H. pylori* UreC protein is not a component of the holoenzyme and its relationship to urease is unclear (42), this finding raised the possibility that the enteropathogen *C. perfringens*, like *Clostridium beijerinckii*, *Clostridium inocuum*, *Clostridium sordellii*, and *Clostridium symbiosum*, may be ureolytic (41). This hypothesis was tested here by means of a PCR-assisted approach, which showed that some strains did indeed produce urease and that the corresponding genes were plasmid borne.

MATERIALS AND METHODS

Bacterial strains, vectors, and growth media. The *C. perfringens* strains used in this study were from the collections of the Institut Pasteur and the Université de Liège. The Cpe^+ type A strain NCTC10240 (Hobbs 13) was obtained from C. Duncan, Food Research Institute, University of Wisconsin, Madison, Wis. The principal properties (biotypes and origin) of all ureolytic *C. perfringens* strains are summarized in Table 1. The *C. perfringens* type A strain CPN50 also known as BP6K-N5 (4, 5), which does not make urease, was used as a urease-negative control strain throughout these studies. The *Escherichia coli* K12 strains DH5 (24) and JM101 (52) were used for plasmid preparations.

C. perfringens strains were grown anaerobically in TYG medium (3% bio-Trypcase, 2% yeast extract, 0.5% glucose, 0.1 thioglycolate [pH 7.4]) as described previously (21). For quantitative analysis of urease expression, organisms were grown in TYG medium or the minimal medium described by Sebald and Costilow (48) supplemented with 0.1% (wt/vol) urea. Sporulation of *C. perfringens* was assayed in Duncan-Strong sporulation medium (DSSM) with added raffinose as described previously (33, 39).

E. coli strains were grown in L broth (47). The plasmid vector pUC18 (52) was used for cloning in *E. coli* the structural genes of *C. perfringens* urease (pURE69), and selective pressure was maintained by adding ampicillin (100 μg/ml) to the medium.

Urease activity. Qualitative detection of urease activity was achieved by resuspending 10^9 bacteria in 1 ml of urea-indole medium (Diagnostic Pasteur) and incubating the suspension at 37°C. Release of ammonia due to urease activity raised the pH, inducing a color change from orange to red. Urease activity was measured by the Berthelet reaction (20) by using a modification of the procedure described by Cussac et al. (10). The quantity of ammonia liberated was determined from a standard curve correlating the absorbance at 625 nm to the ammonium concentration (from NH_4Cl). Urease activity was expressed as micromoles of urea hydrolyzed per minute per milligram of bacterial protein. The protein concentrations were determined by the Bradford assay (Sigma Chemicals), with bovine serum albumin as the standard.

Cloning procedure for urease structural genes. On the basis of published sequences of the urease structural genes of *P. mirabilis*, *H. pylori*, and *Canavalia ensiformis*, two oligonucleotide primers (ure3 [5′-GATATAGGAATAAAAGA TGG] and ure4 [5′-ACTTCTCCAACTCTTCCCAT]) corresponding to the highly conserved segments of the large subunit of urease (amino acids 85 to 91 and

* Corresponding author. Mailing address: Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cédex 15, France. Phone: (33) 1-45688446. Fax: (33) 1-45688953. E-mail: stcole@pasteur.fr.

† Present address: Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02111.

TABLE 1. Ureolytic strains of *C. perfringens* studied

Strain	Toxin type	Origin[a]	Major toxin(s)	Presence of *cpe*	IS element
41590A	A	Goat	Alpha	−	
88B236MF	A	Goat	Alpha	−	IS*1151*
42790-1R	A	Cow	Alpha	−	IS*1151*
G939	A	Cow	Alpha	−	IS*1151*
CPK2	A	ND	Alpha	−	IS*1151*
43457-CO5	A	Cow	Alpha	−	
43457-CO6	A	Cow	Alpha	−	
92E2743-C01	A	Cow	Alpha	−	
BARILOCHE	A	Sheep	Alpha	−	IS*1151*
46752-2	A	Fish	Alpha	−	IS*1151*
47019-C5	A	Cow	Alpha	−	
ATCC3626	B	ND	Alpha, beta, epsilon	−	IS*1151*
NCTC6121	B	ND	Alpha, beta, epsilon	−	IS*1151*
NCTC8533	B	ND	Alpha, beta, epsilon	−	IS*1151*
92E1897F	D	Deer	Alpha, epsilon	+	IS*1151*
CP76	D	Sheep	Alpha, epsilon	+	IS*1151*
9187	D	Sheep	Alpha, epsilon	−	IS*1151*
945P	D	Sheep	Alpha, epsilon	+	IS*1151*
NCTC8084	E	ND	Alpha, iota	+	IS*1151*
NCIB10748	E	ND	Alpha, iota	+	IS*1151*

[a] ND, not defined.

363 to 369, respectively; see Results and reference 34) were designed. These primers amplified an internal urease gene fragment in PCRs with DNA from *C. perfringens* CP76 as the template, which was then used as a probe in cloning experiments. *Hin*dIII digests of chromosomal DNA from *C. perfringens* CP76 were separated on an agarose gel, and fragments with sizes of approximately 2.2 kb were excised and eluted from the agarose with the GeneClean II kit (Bio 101, Inc., La Jolla, Calif.). This DNA was ligated into the desphosphorylated *Hin*dIII site of pUC18, and the ligation mixture was used to transform *E. coli* JM101 (52). Recombinant colonies were transferred to nitrocellulose filters (Hybond C; Amersham), and clones carrying the urease genes were identified by colony hybridization as described by Grunstein and Hogness (23). The nitrocellulose filters were prehybridized for 2 h at 65°C and then hybridized overnight at 65°C.

Nucleotide sequence determination and analysis. DNA sequencing was performed by the dideoxy chain termination method with the Sequenase T7 DNA polymerase (United States Biochemical Corp.) and double-stranded templates. The complete sequences of both strands of *ureABC* were determined by using subcloned fragments of pURE69 with the M13 universal primer, a reverse primer, and additional synthetic specific primers. Part of the sequence was obtained by inverse PCR (43) with circularized *Sca*I fragments with sizes of ~3.2 kb and appropriate primers. To avoid possible PCR-induced errors, the corresponding PCR fragments were sequenced directly. Sequences were compiled and analyzed as described previously (32), homology searches were performed by using the BLAST program (1, 2), and phylogenetic trees were constructed with PHYLIP (19) or CLUSTAL V (26). To determine the sequence of the *cpe* promoter region in strains CP76 and NCTC10240 we used total DNA and a pair of primers (OSM5 and OSM6 [39]), to amplify 500 bp upstream of the *cpe* gene by PCR.

Western blot and measurements of CPE protein in *C. perfringens* cells. Proteins were separated by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10%) and electroblotted onto nitrocellulose membranes. UreC and CPE on the filter were detected with anti-UreB from *H. pylori* (generously supplied by H. L. T. Mobley) and anti-CPE (kind gift of Per Einar Granum) antibodies in conjunction with PhoA-conjugated anti-rabbit immunoglobulin G antibodies. Quantification of CPE was carried out by using Vistra ECF Western blotting substrates and FluorImager systems (Molecular Dynamics).

Genomic DNA preparation, restriction enzyme digests, pulsed-field gel electrophoresis, and Southern blot analysis. All *C. perfringens* genomic DNAs were prepared in agarose plugs as described previously (5) and digested with *Apa*I (10 U) from Boehringer Mannheim or the intron-encoded endonuclease I-*Ceu*I (4 U) from New England Biolabs at 37°C for 3.5 h, as recently outlined (32).

Large restriction fragments were separated by field inversion gel electrophoresis as described previously (5). Gels were calibrated with *Saccharomyces cerevisiae* chromosomes (size range, 90 to 1,600 kb) and a mixture of λ concatemers and *Hin*dIII fragments (New England Biolabs) and processed for Southern blotting and hybridization analysis with Hybond C-extra filters (Amersham) as described previously (5, 6). The urease probe was the cloned *Hin*dIII fragment which carries the urease genes (*ureABC*), and all other probes (*cpe*, *etx*, and *itxA*) were generated by PCR with specific primers (12). The probe DNAs were labeled with [α^{32}P]dCTP by using the megaprime DNA labeling kit (Amersham).

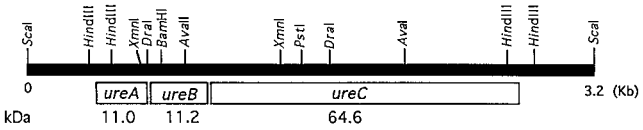

FIG. 1. Physical map of *C. perfringens* urease subunits gene cluster. The boxes, labeled *ureA*, *ureB*, and *ureC*, indicate the physical positions of each of the *ure* open reading frames. The number beneath each rectangle corresponds to the predicted molecular size for each polypeptide. Restriction endonuclease sites are indicated above the line.

Colony hybridization was performed on 2,661 *C. perfringens* isolates exactly as described previously (14).

Nucleotide sequence accession numbers. The DNA sequence of the *C. perfringens ureABC* genes has been deposited in the EMBL data library under the accession no. Y10356.

RESULTS

Identification of ureolytic *C. perfringens* strains. A number of *C. perfringens* strains from the laboratory collection were screened for the ability to produce urease by using whole cells in a qualitative colorimetric assay in which the pH change, resulting from ureolysis and ammonia accumulation, was detected with phenol red. Semiquantitative measurements indicated that all 20 ureolytic strains detected produced roughly similar levels of urease. Three type D strains (CP76, 91.87, and 945P) and two of biotype E strains (NCTC8084 and NCIB10748) were analyzed further (Table 1). In all cases, urease activity was detected in crude cell extracts and not in the culture supernatant, indicating that the enzyme was probably localized in the cytoplasm.

Cloning and sequencing of the *ureABC* operon. To clone the urease structural genes, advantage was taken of the highly conserved primary structure of the urease subunits from different organisms. A number of motifs that were perfectly conserved in the amino acid sequences of the large subunit of urease from *Canavalia ensiformis* (the jack bean plant), *H. pylori*, and *P. mirabilis* were identified (34) and used to design oligonucleotide primers suitable for PCR (see Materials and Methods). When DNA from the urease-producing *C. perfringens* strain CP76 was used as a template, a PCR fragment of the size expected was obtained while the urease-negative control strain, CPN50, yielded no product.

The fragment amplified in PCR was identified by partial DNA sequencing and then used as a probe in Southern blotting experiments to detect the corresponding gene. Single fragments with sizes of 2.2, 3.2, 4.2, 6.8, and 9 kb were detected after digestion with *Hin*dIII, *Sca*I, *Xba*I, *Hpa*I, and *Eco*RI, respectively, and the 2.2-kb *Hin*dIII fragment was subcloned into pUC18, creating pURE69, to facilitate further DNA sequence analysis. After completion of the sequence of pURE69, the 5′ end of *ureA* and the 3′ end of *ureC* were found to be missing. Consequently, an inverse PCR strategy was employed, and this resulted in a composite sequence of 2,689 bp encompassing the *ureABC* operon that has been deposited in the EMBL database under accession no Y10356. Inspection of the sequence revealed a typical operon structure, with short intergenic segments (17 and 13 bp), and this is depicted schematically in Fig. 1. The dG+dC content of the *ureABC* genes is 33%, significantly higher than that observed for other *C. perfringens* genes (~25%) although the codon usage is not abnormal (46).

In other bacteria, the *ureABC* genes are often accompanied by regulatory or accessory genes (42). For instance, in *Mycobacterium tuberculosis* and *P. mirabilis* these genes follow *ureC*

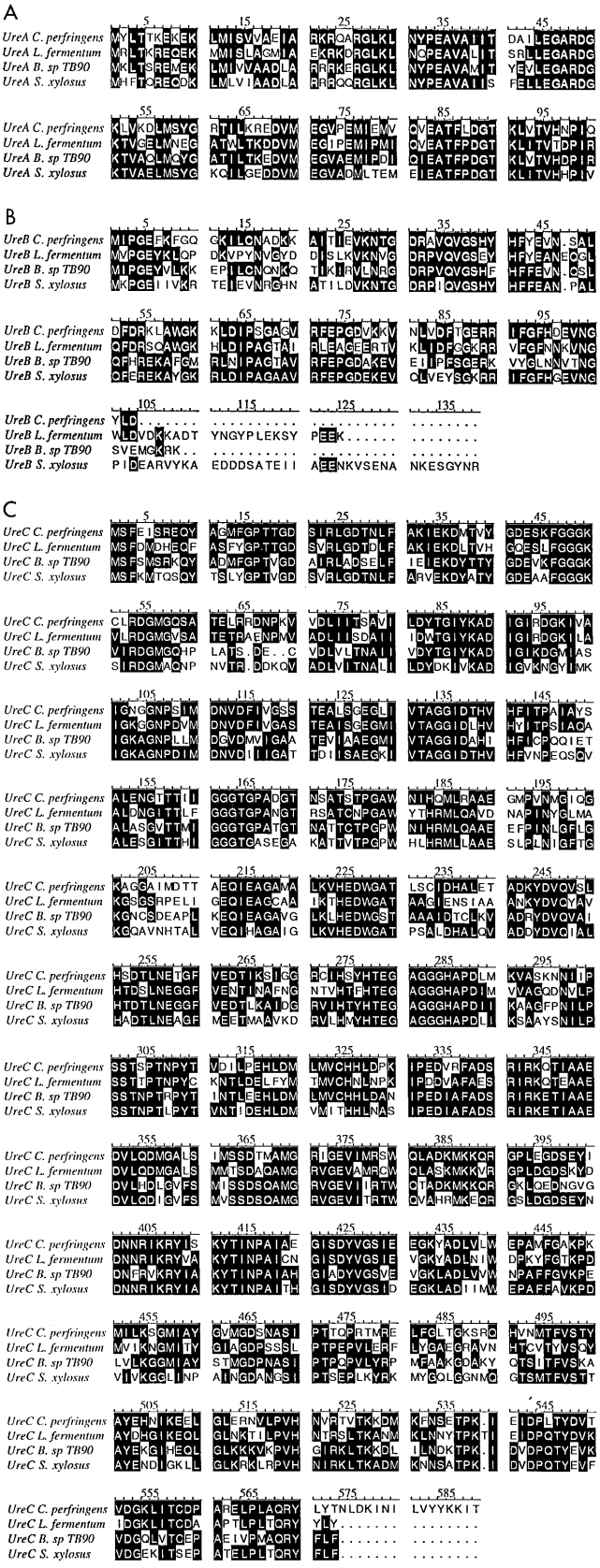

FIG. 2. Amino acid sequence similarities between *C. perfringens* urease subunits and those from *L. fermentum*, *Bacillus* sp. strain TB90, and *S. xylosus*. Identical amino acids are indicated by a black background. Dots indicate gaps inserted to optimize alignment. The residues His-139, His-141, His-224, His-251, His-277, His-325, Lys-222, and Asp-365 correspond to the key active site residues involved in nickel binding and catalysis (42).

by 1 and 27 bp, respectively (7, 42). No such genes were found to be closely linked to the *C. perfringens* operon (i.e., within 200 bp of the end of *ureC* or upstream of *ureA*), suggesting that *C. perfringens* may resemble *Rhizobium meliloti* where the accessory genes are separated from the urease structural genes (40).

Predicted properties of *C. perfringens* urease subunits. Computer-assisted interpretation of the sequence predicted that the proteins encoded by *ureA*, *ureB*, and *ureC* should contain 100, 102, and 588 amino acids, respectively. Furthermore, these polypeptides were highly related to those present in other ureases in terms of their sizes, amino acid contents, and primary structures, as can be seen from the alignments presented in Fig. 2. The highest identity scores were obtained with urease subunits from the gram-positive bacteria *Lactobacillus fermentum* (UreA, 65%; UreB, 57%; and UreC, 65%), *Bacillus* sp. strain TB90 (UreA, 70%; UreB, 51%; and UreC, 63%), and *Staphylococcus xylosus* (UreA, 62%; UreB, 59%; and UreC, 61%) although extensive similarity to those from *P. mirabilis*, *H. pylori*, *Klebsiella aerogenes*, and other gram-negative bacteria was also seen (data not shown). Given these remarkable levels of relatedness, and the conservation of key active site residues involved in nickel binding and catalysis, it is likely that the three-dimensional structure of *C. perfringens* urease will be very close to that of the *K. aerogenes* enzyme (29), as the primary structures of the subunits of these enzymes show ~57% identity.

To establish whether the *C. perfringens* enzyme belonged to a particular urease family, a phylogenetic tree was constructed by using the PHYLIP package (19) to determine the degree of relatedness (Fig. 3). Inspection of the unrooted tree revealed four main branches, with *C. perfringens* clustering with three other gram-positive organisms, *S. xylosus*, *L. fermentum* and *Streptococcus salivarius* (Fig. 3).

Immunodetection of urease subunits of *C. perfringens* CP76. To demonstrate convincingly that the *ureABC* operon present in *C. perfringens* CP76 was expressed and responsible for the urease activity observed, immunoblot experiments were performed. Antibodies raised against *H. pylori* urease were used to detect immunoreactive polypeptides, separated by SDS-polyacrylamide gel electrophoresis, in crude cell extracts and culture supernatants of *C. perfringens* CP76 (Ure^+) and CPN50 (Ure^-) and for positive control purposes in extracts of a clinical isolate of *H. pylori*. When antiserum directed against the denatured large subunit of *H. pylori* urease (UreB) (27) was used, a polypeptide that had the expected size of *C. perfringens* UreC, and was very close to the molecular size of *H. pylori* UreB, was clearly recognized (Fig. 4). The corresponding band was not detected in extracts of the Ure^- strain, *C. perfringens* CPN50. Western blotting experiments were also performed with antibodies raised against the small subunit of the *H. pylori* urease, UreA (equivalent to UreA and UreB of *C. perfringens*). Although this antiserum cross-reacted with several polypeptides, as described previously (27), two proteins with the expected sizes of UreA and UreB of *C. perfringens* (approximately 11 and 12 kDa, respectively; data not shown) were visualized in crude extracts of *C. perfringens* CP76. In further studies, no *C. perfringens* UreC subunit was found in the supernatant, confirming, as indicated by the urease activity assays, that *C. perfringens* urease is not secreted.

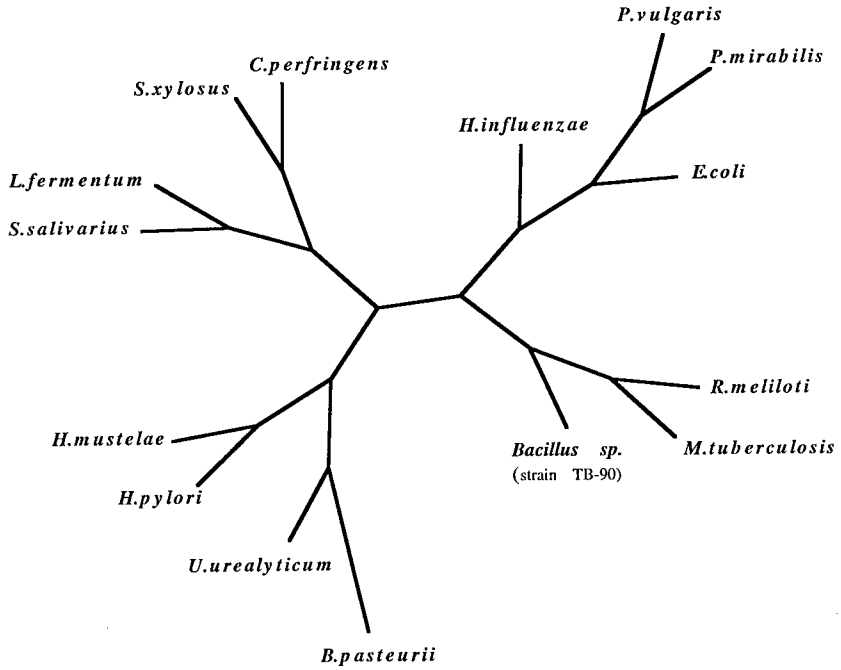

FIG. 3. Phylogenetic tree indicating the extent of relatedness of bacterial urease genes. The unrooted tree was constructed by the parsimony method with the DNAPARS routine of the PHYLIP package (19) to analyze segments of the *ureB* genes from the following bacteria (accession numbers are given in parentheses): *Bacillus* sp. strain TB-90 (D14439), positions 479 to 771; *Bacillus pasteurii* (X78411), 421 to 713; *C. perfringens* (Y10356), 1 to 293; *E. coli* (L03307), 1319 to 1617; *H. influenzae* (U32736), 4875 to 5167; *H. mustelae* (L33462), 280 to 571; *H. pylori* (M60398), 2974 to 3266; *L. fermentum* (D10605), 507 to 802; *M. tuberculosis* (L41141), 739 to 1035; *P. mirabilis* (M31834), 1598 to 1899; *R. meliloti* (S69145), 945 to 1237; *S. salivarius* (U35248), 733 to 1028; *Ureaplasma urealyticum* (X51315), 551 to 871; *Proteus vulgaris* (X51816), 722 to 1020; and *S. xylosus* (X74600), 886 to 1178. It should be noted that similar results were obtained with the CLUSTAL V program for phylogeny (26).

Distribution of urease genes among *C. perfringens* isolates. A panel of 2,661 human and veterinary isolates of *C. perfringens* was examined for the presence of the *ureABC* operon by means of colony hybridization with a *ureC*-specific probe (14). Fifty-three isolates (2%), mainly of veterinary origin, were found to contain the *ureC* gene and details of 20 of these, all of which were confirmed as being ureolytic, are presented in Table 1. Several of these ureolytic strains also harbored the *cpe*, *cpb*, *etx*, or *itxAB* toxin gene, and all members of this class (Ure^+ Tox^+) contained the insertion element, IS*1151* (13) compared to only 10% of the 2,661 strains tested.

Association with enterotoxemia. Retrospective examination of case records (14) revealed that urease production was more common (11%; 5 positive from a sample of 43 autopsies) among strains isolated at autopsy from cases of enterotoxemia among domesticated livestock. In most instances, inspection of the intestinal flora revealed the presence of both urease-positive and urease-negative strains of *C. perfringens*. The urease status of *C. perfringens* isolated from the large intestines, kidneys, livers, spleens, and peritoneums from five animals was determined by means of colony hybridization. Although mixed populations were found in the intestines, only urease-producing isolates were recovered from the organs.

Localization of the *ureABC* operon. Many genes for virulence factors are carried by large plasmids in *C. perfringens*, and these can be readily detected by comparing the hybridization profiles of pulsed-field gels on which undigested and I-*Ceu*I-cleaved DNA has been resolved, as I-*Ceu*I cuts only in the *rrn* operons found on the chromosome and, hence, does not affect the plasmid pattern (9, 31, 32). Genomic DNA prepared from all the urease producers (Table 1) and the control strain CPN50 was analyzed in this way and by digestion with the rare cutter *Apa*I. In all ureolytic strains examined, hybridization signals obtained with the *ureC* probe were unaffected by the action of I-*Ceu*I or *Apa*I and thus indicated a plasmid localization. Representative results obtained with five ureolytic strains, belonging to biotypes D and E, are presented in Fig. 5, in which, in some cases, plasmid DNA is clearly visible in untreated samples (Fig. 5A, lanes 2 to 4).

These five urease producers also harbor other plasmid-borne virulence genes such as *cpe*, *itxAB*, and *etx*. To determine whether these colocalized with the *ureABC* operon, additional hybridization experiments were performed. These revealed that in three cases (CP76, NCTC8084, and NCIB10748) the urease operon and the enterotoxin gene were probably on the same plasmids, whose sizes were estimated at 90, 130, and 130 kb, respectively, while in strain 945P, *cpe* and *ureABC* appear

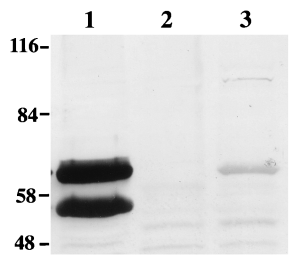

FIG. 4. Immunodetection of urease subunits. Proteins in crude cell extracts of *C. perfringens* CP76 and CPN50 grown in minimal medium were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and then detected with antibodies raised against the *H. pylori* UreB protein. Lanes: 1, *H. pylori*; 2, CPN50; 3, CP76. The positions of molecular weight markers (in thousands) are indicated on the left.

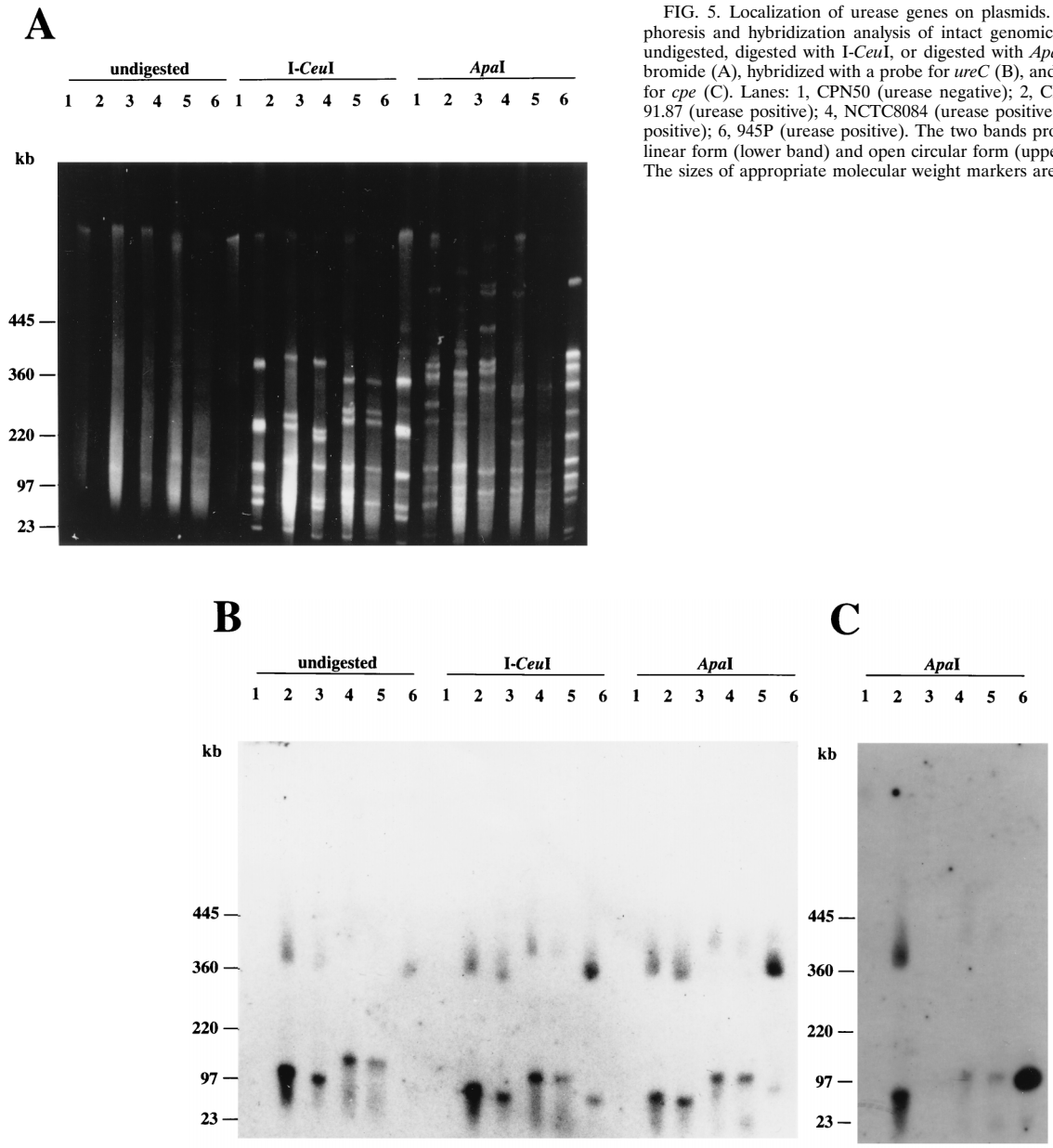

FIG. 5. Localization of urease genes on plasmids. Pulsed-field gel electrophoresis and hybridization analysis of intact genomic DNA which was either undigested, digested with I-*Ceu*I, or digested with *Apa*I stained with ethidium bromide (A), hybridized with a probe for *ureC* (B), and hybridized with a probe for *cpe* (C). Lanes: 1, CPN50 (urease negative); 2, CP76 (urease positive); 3, 91.87 (urease positive); 4, NCTC8084 (urease positive); 5, NCIB10748 (urease positive); 6, 945P (urease positive). The two bands probably correspond to the linear form (lower band) and open circular form (upper band) of the plasmids. The sizes of appropriate molecular weight markers are indicated on the left.

to be located on plasmids that were different but had similar sizes (~100 kb), as illustrated by the results obtained after *Apa*I digestion (Fig. 5B and C). In the type D and E strains examined, the same plasmid apparently carried the *ureABC* operon and the *etx* or *itxAB* gene, respectively (data not shown).

Regulation of urease production. To gain insight into possible control mechanisms governing expression of the *C. perfringens* urease genes, enzyme activity was measured for a series of strains, representing the four main biotypes, grown under a variety of conditions (Fig. 6). No significant ureolytic activity was detectable in any of the strains examined when rich medium was used, even when supplemented with urea. However, when minimal medium was employed, urease activity was strongly induced, to roughly similar levels, in all four ureolytic strains, although this effect was almost completely abolished by the addition of urea (Fig. 6). This result is consistent with expression of the *ureABC* operon being controlled by the availability of nitrogen sources.

Enterotoxin (CPE) and urease expression during sporulation in *C. perfringens* CP76 and NCTC10240. The finding that the urease operon and the *cpe* gene localized to the same plasmid in strain CP76 raised the possibility that urease synthesis, like enterotoxin (CPE) production, may be induced during sporulation, a process that may also result from nitrogen starvation. To test this possibility, the pattern of CPE and urease expression was monitored during the sporulation of *C. perfringens* CP76 and NCTC10240 (Ure^- Cpe^+) by growing cells in DSSM with added raffinose (see Materials and Methods) and directly measuring urease activity and the amount of CPE protein (Fig. 7). The efficiency of sporulation was high for strain NCTC10240 (95%) but, unfortunately, low for strain CP76 (>0.1%).

In strain CP76, the expression of urease activity occurred

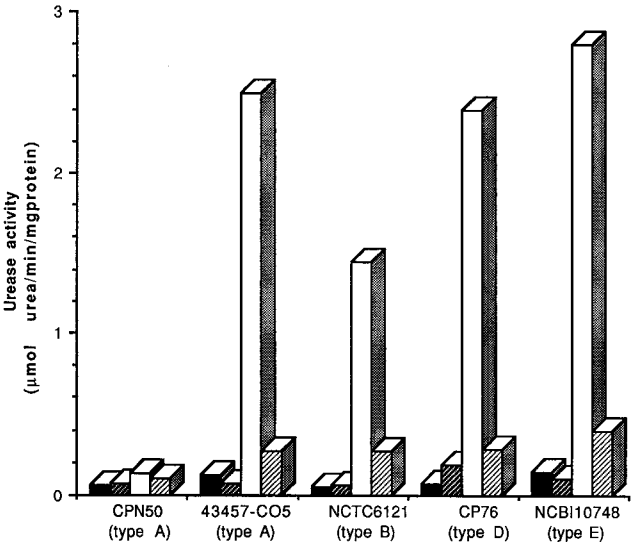

FIG. 6. Urease activity expressed by various *C. perfringens* strains grown under nitrogen-limiting conditions. Cultures were grown anaerobically at 37°C in TYG with (▨) or without urea (0.1% [wt/vol]) (■) or in minimal medium alone (□) or with urea (0.1% [wt/vol]) (▨), as described in Materials and Methods. The cells were harvested and washed in citrate-phosphate buffer (pH 7.0). Activities were determined as described in the text.

during the exponential phase and was maximal after 8 h of growth (Fig. 7B). Little CPE protein was detected, consistent with the very low level of sporulation observed for this strain and the requirement for sporulation to occur that *cpe* to be expressed. Under the same growth conditions, CPE protein was detected in strain NCTC10240. The induction of CPE occurred at the end of exponential growth phase (T_0) and increased throughout the stationary phase, but no urease activity was detectable (Fig. 7A).

To exclude the possibility that alterations to the *cpe* promoter region accounted for the lack of CPE production, the regulatory region from strain CP76 was amplified by PCR and its DNA sequence was determined. The sequence was identical to the corresponding region in strain NCTC10240 and belongs to the promoter type B, described by Melville et al. (39). Thus, the lack of *cpe* expression probably results from the sporulation defect of strain CP76. The combined data indicate that the enterotoxin and urease genes were not coregulated during the developmental cycle of *C. perfringens*.

DISCUSSION

The goal of this work was to establish whether *C. perfringens* strains were capable of producing urease, a potential virulence factor. It was established that roughly 2% of the 2,661 strains examined were ureolytic and that these harbored a classical *ureABC* operon, highly similar to that described in many other prokaryotes. The urease structural genes were located on a plasmid, and their expression was shown to be induced under growth conditions where nitrogen sources were scarce and repressed by urea (Fig. 6). The corresponding proteins were found to be immunologically related (Fig. 4) to the well-characterized urease from *H. pylori* (27, 42).

Although plasmid-borne urease genes have been described in *Providencia stuartii*, some strains of *E. coli* and a few *Salmonella* spp. (16, 17), this is the first report of a *ureABC* operon being located on a plasmid in a gram-positive bacterium. There is, however, a major difference in the regulatory pattern of urease gene expression in *C. perfringens* and those of the plasmid-borne genes of these gram-negative bacteria, in that in all five strains examined urease production was strongest in minimal medium and repressed by urea. This finding is diametrically opposed to the situation in the *Enterobacteriaceae*, mentioned above, in which transcription of the urease operon is positively controlled by the autoregulated *ureR* gene, which encodes an activator protein belonging to the AraC family (15).

In the enteric bacteria, urea acts as an inducer, whereas in *C. perfringens* it seems to be a corepressor or antiactivator. Instead, control of urease gene expression in *C. perfringens* appears to be exerted through the availability of nitrogen sources, as has been described for the chromosomal genes in *Klebsiella pneumoniae* (8) and *Bacillus subtilis* (3). In the latter organism, urease levels are induced by nitrogen limitation to an extent similar to those seen in *C. perfringens* (Fig. 6) (3). Given the relatedness between *B. subtilis* and *C. perfringens*, it is reasonable to suggest the existence of a similar control circuit in *C. perfringens*, and this can now be tested.

Urease production, like *cpe*-mediated enterotoxemia in *C. perfringens* (50, 51), is a relatively rare phenotype, and this is consistent with the fact that the genes are located extrachro-

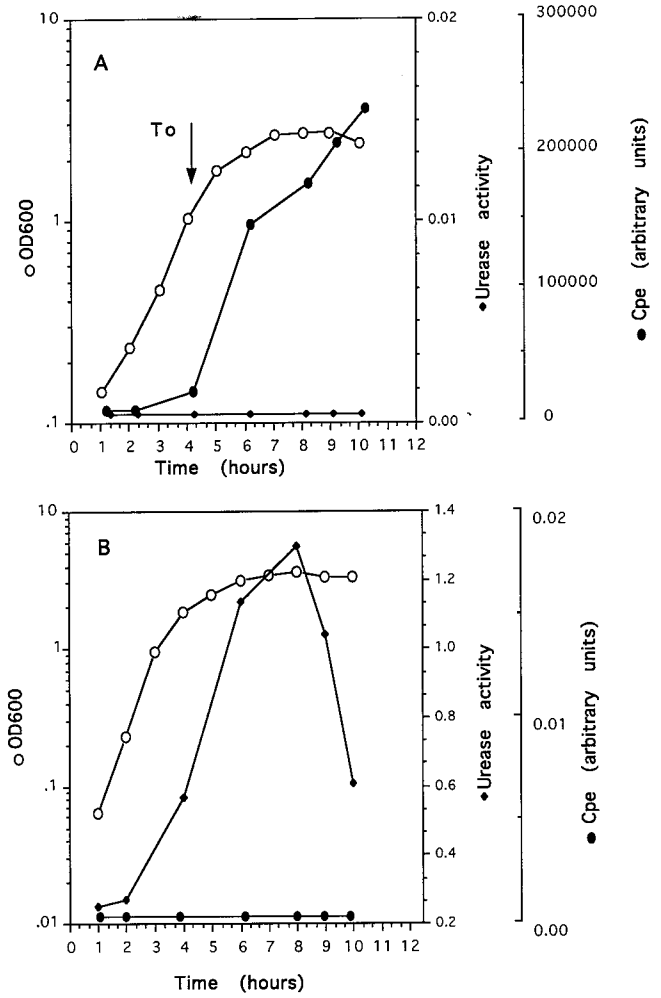

FIG. 7. Urease activity and CPE protein expression during the growth cycle of *C. perfringens* NCTC10240 (A) and CP76 (B) grown in DSSM. *T*0 indicates the end of the exponential growth phase. OD600, optical density at 600 nm.

mosomally on large plasmids. It is conceivable that the corresponding plasmids may have been transferred by conjugation or that the *ureABC* operon has been acquired recently from another ureolytic bacterium and then inserted into a resident plasmid. The dG+dC content of the urease gene cluster, which at 33% is significantly different from that of other plasmid-borne genes in *C. perfringens*, e.g., the *itxAB* genes (44) and the bacteriocinogenic plasmid, pIP404 (22), provides indirect evidence of horizontal transfer. The phylogenetic analysis indicates clearly that the *C. perfringens* urease genes are part of a gram-positive cluster (Fig. 3), and, for several reasons, it seems possible that they may have originated from *Clostridium sordellii*, an anaerobe occupying the same ecological niche as *C. perfringens*. Most strains of *C. sordellii* produce urease (45) although the dG+dC content of their genome is close to that of *C. perfringens*. It is noteworthy that all the ureolytic *C. perfringens* strains were of veterinary origin and that *C. sordellii* is a common pathogen in domesticated livestock.

The present study demonstrates that plasmids play a significant role in expanding the genetic repertoire of *C. perfringens* and that the organism might be acting as a recipient in interspecies exchanges. As can be seen from the data in Fig. 5, the plasmids carrying the *ureABC* genes differ in size and in some cases carry known virulence factor genes, such as *cpe*, *etx*, and *itxAB* (11, 28, 44). This raises the possibility that urease may also play a role in *C. perfringens* pathogenesis, as suggested by the preliminary analysis of isolates associated with veterinary enterotoxemia, or confer a selective advantage for growth in a given environment. The availability of the cloned *ureABC* operon will allow these hypotheses to be tested by reverse genetics.

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