Molecular analyses of three virulence genes candidates clusters, characteristic of urease-positive thermophilic *Campylobacter* (UPTC), one taxon within the *Campylobacter lari* species

麻布大学大学院環境保健学研究科

環境保健科学専攻遺伝子生物学

DE0902　中西　滋之

Molecular analyses of three virulence genes candidates clusters, characteristic of urease-positive thermophilic *Campylobacter* (UPTC), one taxon within the *Campylobacter lari* species

DE0902 Nakanishi Shigeyuki

Laboratory of Molecular Biology
The PhD Course of the Graduate School of
Environmental Health Sciences

Campylobacter lari 種の１つのタキソンである urease-positive thermophilic *Campylobacter* (UPTC) に特徴的な３つの病原性遺伝子候補クラスターの分子解析

環境保健学研究科　環境保健科学専攻

博士後期課程

遺伝子生物学分野

DE0902　中西滋之

Contents

Summary

The genus *Campylobacter* is a gram-negative spiral and rod bacterial organism and a pathogen of human and animals, and causes campylobacteriosis as zoonosis. *Campylobacter* has been the most common cause of food poisoning in Japan and the United Kingdom for more than these ten years. *Campyobacter jejuni*, *C. coli* and *C. lari* are known as pathogens for the campylo-bacteriosis. However, no animal models were established to clarify the mechanisms of campylobacteriosis, and any pathogenic factors still remain unclear.

In 1985, urease-positive thermophilic *Campylobacter* (UPTC) organisms were isolated in England and classified into biovar or variant of *C. lari*. Urease-negative (UN) *C. lari* and UPTC, two major taxa in the *C. lari* species, were isolated from the natural environment and clinical specimens all over the world infrequently. Although more than 100 clinical isolates of *C. lari* are identified as the author is aware, only four UPTC

clinical isolates are reported. Hence, an association of UPTC with any human diseases still remains unclear.

Therefore, in this study, molecular identification and characterization of virulence genes candidates in *C. lari* were performed and comparative analyses of those were also carried out between UN*C. lari* and UPTC, major two taxa of *C .lari*. Cytolethal distending toxin (*cdt*), *P450* involved in an oxidative reaction and UPTC urease were chosen for molecular and comparative analyses of these operon and their adjacent loci. Construction of the recombinant full-length and several deletion variants of urease gene operon from the UPTC CF89-12 isolate and their expressions of these recombinants were carried out in *Escherichia coli* cells.

In the present study, firstly, molecular and comparative analyses of the full-length cytolethal distending toxin (*cdt*) gene operon and its adjacent genetic loci [2.7-9.4 kilo base pairs (kbp) in length] were carried out with 12 UPTC isolates

by using several PCR primer pairs. Three putative open reading frames (ORFs) for *cdtA*, *cdtB* and *cdtC*, two putative promoters and a hypothetically intrinsic ρ-independent transcription terminator were identified within all the operons of the 12 UPTC isolates examined. Although the number of amino acid residues slightly varied within the putative *cdtA* and *cdtC* ORFs among the isolates, those in the *cdtB* were identical among all the UPTC isolates, as well as the six UNC. *lari* examined previously. Regarding the *cdt* genes in UPTC CF89-12, each ORF commenced with an ATG start codon and terminated with a TAG stop codon for *cdtA* and *cdtB* and a TAA for *cdtC*. Start and stop codons of the three ORFs for the other 11 UPTC isolates were also identified. Two putative promoter structures, consisting of sequences at the -35-like (TTAATA) and -10-like (TATTAA) regions, as well as the start codon (ATG), were identified for the transcriptional promoter, immediately upstream of the *cdtA* gene in all the 12 isolates. Although the genetic heterogeneity of the *cdtB* gene

locus occurred in all 28 *C. lari* isolates (n = 16 UNC. *lari;* n = 12 UPTC) examined, DNase I -specific all nine amino acid residues were completely conserved in all their *cdtB* genes. Variable genes insertions with heterogeneous order and combinations occurred between *cdtC* and *lpxB* genes in all the UPTC organisms examined.

Then, in the present study, two sets of PCR primers are constructed to clone the cytochrome *P450* structural gene including putative promoter and terminator structures and its adjacent genetic loci in *Campylobacter lari* isolates. The putative ORFs of the *P450* genes from 11 *C. lari* isolates (*n*=5 for UNC. *lari; n*=6 UPTC) examined consisted of 1,365 or 1,371 bases (455 or 457 amino acid residues), differing from those of the other thermophilic campylobacters [1,359 (453) for *C. jejuni* and *C. upsaliensis;* 1,368 (456) for *C. coli*]. Each of the putative ORFs from all the 11 isolates examined was also shown to carry start and stop codons and ribosome binding sites.

Two putative promoter structures, consisting of sequences at the -35-like and -10-like regions were also identified upstream of the ORFs, respectively. A single copy of the *P450* gene in the genome DNA was identified with UNC. *lari* JCM2530^{T} and UPTC CF89-12, respectively, based on Southern blot hybridization analysis. In addition, when reverse transcription (RT) - PCR analyses were carried out, the transcription of the *P450* structural gene in *C. lari* organisms *in vivo* was confirmed. The transcription initiation site for the gene was also determined by a primer extension analysis. High nucleotide sequence identities (95.2-98.8%) of the full-length *P450* structural genes were shown with each of the 12 *C. lari* isolates including *C. lari* RM2100 strain. In addition, UNC. *lari* and UPTC organisms showed similar findings with the neighbour-joining method, based on the nucleotide sequence information of the *P450* structural gene.

Finally, we constructed *in vitro* recombinant full-length

urease gene operon molecule from the UPTC CF89-12 isolate and expressed the recombinant urease gene in *E. coli* cells. We also constructed *in vitro* several deletion recombinant variants of urease subunits genes and expressed and characterized those in *E. coli* cells. A positive urease reaction with the log-phase cultured *E. coli* JM109 cells transformed with pGEM-T vector carrying the recombinant full-length UPTC urease gene operon was detected ~~both~~ with the isopropyl-β-D- thiogalactopyranoside (IPTG). In addition, among several large deletion recombinant variants of UPTC urease subunits genes, *ureA*-, *ureB*-, *ureE*-, *ureF*-, *ureG*-, and *ureH*- deficient, only *ureE*-deletion variant showed a positive urease reaction, acceleratedly (15 folds). In addition, a complete *ureE* deletion (100%) recombinant variant was also identified to show a positive reaction of urease, acceleratedly (18 folds). These urease positive reactions were depend on the recombinant *E. coli* cells cultured in L Broth medium containing the $NiCl_2$ (750 μM).

In addition, urease subunits A and B were immunologically identified by western blot analysis with polyclonal rabbit anti-urease α (A) and β (B) raised against *Helicobacter pylori*. These results suggest that no UreE subunit require for the UPTC urease activity and UreE may work negatively in the physiological condition.

Thus, in the present study, three pathogenic genes candidates for *C. lari* were molecular identified, and characterized. When these pathogenic genes candidates were compared between *C. lari* organisms including UNC. *lari* and UPTC and *C. jejuni* organisms, 75.9-77.2% similarities of *P450* amino acid sequences were shown to each other. However, 52.1-60.9% similarities of *cdtA* amino acid sequences, 66.4-68.7% of *cdtB* and 50.8-67.2% of *cdtC* were shown. Thus, relatively genetic heterogeneities in *cdtA* and *cdtC* were identified. This may be consistent with the results of the CDT effect on HeLa cells described from our laboratory, most recently.

In conclusion, *cdt* and *P450* genes as virulence genes candidates were analyzed in detail in molecular level and appeared many new knowledge. In addition, several deletion recombinant variants of urease subunits gene were constructed and especially, a new function of UreE subunit appeared to be demonstrated.

要約

カンピロバクター属細菌はグラム陰性らせん状桿菌で、人及び動物に感染し下痢症などを引き起こす。そしてこれは人獣共通感染症であるカンピロバクター症の起因菌である。カンピロバクター属細菌による食中毒は細菌性食中毒ではイギリスや日本でここ数年1位を占めており、ヒトのカンピロバクター症の起因菌として *Campylobacter jejuni*、*Campylobacter coli*、*Campylobacter lari* 等が知られている。しかしこれらカンピロバクター症の発症のメカニズムを解明するための動物実験系は未だ確立されておらず、更にその病原因子も未確定のままであるのが現状である。

urease-positive thermophilic *Campylobacter* (UPTC)は1985年にEnglandで初めて分離され、*C. lari* のbiovar又はvariantとされている。*C. lari* の2つの代表的なtaxonであるurease-negative (UN) *C. lari* とUPTCは共に自然環境や臨床材料から分離され、臨床由来株は *C. lari* 全体では少ないながら世界的に分離が報告されているが、UPTCはわずか4株のみであり、更にUPTCとヒトカンピロバクター症の相関は未確定のままである。この様に、UN*C. lari* 及びUPTCのヒト臨床分離株が少なくそれ故にカンピロバクターの感染過程のそれぞれのプロセスに関わる病原性因子候補をUN*C. lari* 及びUPTCで解析し、*C. jejuni* のそれらと比較分子解析することは重要かつ有効であると考えられる。その様な研究の進展の中で「*C. lari* が高温性カンピロバクターに起因するカンピロバクター症研究の有効な比較対照細菌種であり得る」との仮説、更にUPTCのurease遺伝子の役割についてもその答えが得られものと考えた。

そこで本研究では、*C. lari* の病原性因子候補の分子実体をUN*C. lari* とUPTCを代表的な2つのtaxonとする *C. lari* 株間で比較解析することをによってこの問題にアプローチしようと考えた。具体的には細胞膨張化致死毒素 cytolethal distending toxin (*cdt*) 及び生体内酸化反応を触媒するP450、そしてUPTCに特徴的なureaseを対象としてUPTCのこれらオペロンの全長と隣接する遺伝子座の比較分子解析、更にUPTCのウレアーゼ遺伝子オペロン組換え体分子及び欠失変異体の作成とその大腸菌内での発現解析を行った。

まず、UPTC 12株の *cdt* 遺伝子オペロンの全長と隣接する遺伝子座[2.7-9.4 kilo

base pairs (kbp)]の比較分子解析を複数の *in silico* にデザインしたPCR用プライマー対を用いて行った。推定される3つの *cdtA*、*cdtB* 及び *cdtC* の open reading frame (ORF)と、推定される2つのプロモーター及び1つの内在的 ρ 非依存性転写終結因子が供試したUPTCの全12株で初めて同定された。*cdtA* と *cdtC* のORFのアミノ酸残基数にはわずかな差異が認められたが、*cdtB* のORFでは全てのUPTC株でそのアミノ酸残基数が同一であり、またこれは以前調べたUN*C. lari* 6株の結果と同じであった。UPTC CF89-12株では3つの *cdt* 遺伝子は全て開始コドンがATGであり、終止コドンは *cdtA* と *cdtB* ではTAG、*cdtC* ではTAAであった。他のUPTC 11株の3つの *cdt* 遺伝子の開始と終止コドン、-35様領域 (TTAATA) と-10様領域 (TATTAA) の配列からなる2つの推定される転写のプロモーター構造も同定された。一方、本研究で用いたUPTC 12株を含む全 *C. lari* 28株 (UN*C. lari* 16株、UPTC 12株) で *cdtB* 遺伝子座位の遺伝的多型性が認められたが、DNase I に特徴的な9アミノ酸残基全て[E [metal ion-binding residue (MBR)] - E [catalytic residue (CR)] - R [DNA contact residue (DCR)] - H (CR) - D (MIBR) - N (DCR) - D (CR) - D (MIBR) - H (CR)]が *cdtB* 遺伝子中で完全に保存されていた。このことは3つのサブユニットからなる *cdt* のうちで細胞膨化致死作用の機能を有する *cdtB* 因子が *C. lari* 株間で広く保存されている事を示唆している。更に興味あることに、本研究で解析した全てのUPTC株で *cdtC* と *lpxB* 遺伝子の間には、その組み合わせが多様な遺伝子の挿入が認められた。しかし、UN*C. lari* 株ではその様な挿入は認められなかった。

次に、*C. lari* の推定されるプロモーター及びターミネーターを含むシトクローム *P450* の構造遺伝子と隣接する遺伝子座を分子クローニングするための2組のPCRプライマー対を設計した。*C. lari* 11株 (UN*C. lari* 5株、UPTC 6株) の1,365又は1,371塩基 (455又は457アミノ酸残基) からなる *P450* 遺伝子の推定されるORFは他の高温性 *Campylobacter* のもの[*C. jejuni* と *C. upsaliensis* は1,359 (453アミノ酸残基)、*C. coli* は1,368 (456アミノ酸残基)]と差異が認められた。更に、11株の *P450* 遺伝子それぞれの開始、終止コドンとリボソーム結合部位が同定され、-35様領域 (TTAATA) と-10様領域 (TATTAA) の配列からなる推定される2つのプロモーター構造もORFの上流に同定された。また、*P450* 遺

伝子がUN*C. lari* JCM2530^T とUPTC CF89-12のゲノム中で単一コピー存在することがサザンブロットハイブリダイゼーション解析により確認された。更に、*C. lari*種細胞内の *in vivo* での *P450*構造遺伝子の転写がreverse transcription (RT)-PCR解析により確認され、転写開始部位もprimer extension法で決定された。加えて、*P450*構造遺伝子全長での高い塩基配列の相同性（95.2-98.8%)が*C. lari* 12株で示され、*P450*構造遺伝子の配列情報に基づいてneighbour-joining法により作成された系統樹ではUN*C. lari* とUPTCが類似しており識別困難であることが明らかとなった。

次いで、*in vitro* でUPTC CF89-12株のウレアーゼ遺伝子オペロン全長の組換え体分子を作成し、その組換え体ウレアーゼ遺伝子を大腸菌細胞内で発現させた。また、6つのウレアーゼサブユニット遺伝子（*ureA*、*B*、*E*、*F*、*G*及び*H*）をそれぞれ欠失させた組換え変異体を作成し、これらを大腸菌細胞内で発現させてその性状を解析した。組換え体ウレアーゼ遺伝子全長のオペロン分子を持つ大腸菌JM109を培養し、対数増殖期でisopropyl-β-D-thiogalactopyranoside (IPTG)により発現を誘導したところ、誘導した細胞及び誘導していない細胞で共にウレアーゼ反応は陽性であった。更に、ウレアーゼサブユニット遺伝子欠失組換え変異体（*ureA*-、*B*-、*E*-、*F*-、*G*及び*H*-それぞれの欠失変異体）を作成し、同様の解析を行ったところ、*ureE*-欠失(63%欠失)変異体のみがウレアーゼ活性の亢進(15倍）を示し、他ではウレアーゼ活性は認められなかった。また、完全な*ureE*欠失(100%欠失)変異体も同様にウレアーゼ活性を亢進(18倍）した。更に、これらのウレアーゼ活性はいずれも大腸菌培地中での$NiCl_2$（750 μM)の存在に依存していた。また、組換え体ウレアーゼサブユニットAとBはpolyclonal抗*Helicobacter pylori*ウレアーゼα、β抗体を用いたウエスタンブロットでそれぞれ陽性反応を示した。この様な結果から*ureE*はUPTCのウレアーゼ活性には必須ではなく、生理的条件下では抑制的に働いているサブユニットである可能性が示唆された。

これらの病原性遺伝子候補をUPTCとUN*C. lari*を含む*C. lari*と*C. jejuni*株間で比較すると、*P450*では75.9-77.2%の類似性を示した。一方、*cdt*遺伝子オペロンに関しては、*cdtA*では52.1-60.9%、*cdtB*では66.4-68.7% そして*cdtC*で

は 50.8-67.2%の類似性を示し、*cdtA* および *cdtC* における遺伝的多様性が認められた。このことは、*C. lari* において *cdt* の働きが株間で異なる可能性を示唆している。

以上の様に本研究においては、まず *Campylobacter* の病原性遺伝子候補として *cdt* 及び *P450* 遺伝子に着目し、その詳細な分子レベルでの解析を行い多くの新知見を得た。更に *Campylobacter* では一般的ではない urease 遺伝子の欠失組換え変異体の作成に成功し、更にサブユニット UreE の新しい機能を明らかにした。

Construction, expression and characterization of recombinant molecules of the urease gene operon from a urease-positive thermophilic *Campylobacter* (UPTC) isolate

S. Nakanishi, T. Nakajima, A. Tazumi, K Matsubara[1], J. E. Moore, [2, 3] B. C. Millar[2] and M. Matsuda

Laboratory of Molecular Biology, Graduate School of Environmental Health Sciences, Azabu University, Fuchinobe 1-17-71, Chuo-ku Sagamihara 252-5201, [1]Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Kanagawa-machi, 920-1191, Japan; [2]Department of Bacteriology, Northern Ireland Public Health Laboratory, Belfast City Hospital, Belfast BT9 7AD, [3]School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA, N Ireland, UK

*Address for correspondence and reprints:
Prof Motoo Matsuda
Laboratory of Molecular Biology, Graduate School of Environmental Health Sciences,
Azabu University, 1-17-71, Fuchinobe, Chuo-ku Sagamihara 252-5201, Japan
Tel, 81-42-769-1942 Fax, 81-42-754-7661
e-mail, matsuda@azabu-u.ac.jp

Abstract

Recombinant molecule of full-length urease gene operon was constructed *in vitro* from the Japanese urease-positive thermophilic *Campylobacter* (UPTC) CF89-12 isolate and expressed in *Escherichia coli* cells. Several large deletion recombinant variants of urease subunits genes were also constructed and expressed in *E. coli* cells. A positive urease reaction with the log-phase cultured *E. coli* JM109 cells in the $NiCl_2$ containing medium transformed with pGEM-T vector carrying the recombinant molecule of the full-length operon was detected with isopropyl-β-D-thiogalactoside. Among the several deletion recombinant variants, each *ureA*-, *ureB*-, *ureE*-, *ureF*-, *ureG*- and *ureH*- large deficient, only *ureE*- large deletion variant (63% deficient) showed a positive urease reaction (approximately 15-fold). In addition, a *ureE*-complete deletion recombinant variant (100% deficient) constructed also showed a positive reaction of urease (approximately 18-fold). In addition, recombinant urease subunits, A and B, were immunologically identified by western blot analysis with anti-urease α (A) and β (B) raised against *Helicobacter pylori*.

Keywords: construction, expression and characterization, recombinant urease genes, recombinant variants, urease-positive thermophilic *Campylobacter*

Introduction

Campylobacter lari was first recognized as a nalidixic acid-resistant thermophilic *Campylobacter* (Skirrow and Benjamin 1980). In 1985, an atypical and unusual organism of urease-positive and nalidixic acid-sensitive thermophilic *Campylobacter* (UPTC) was isolated from the natural environment in England (Bolton et al. 1985). Thereafter, the characterization of UPTC as a variant or biovar of *C. lari* has been described (Mégraud et al. 1988; Owen et al. 1988). After the original description of UPTC had appeared, isolates of UPTC have been reported in France (Mégraud et al. 1988; Bézian et al. 1990), Northern Ireland (Wilson and Moore 1996; Kaneko et al. 1999; Moore et al. 2002; Matsuda et al. 2003), The Netherlands (Endtz et al. 1997), England (Fitzgerald et al. 1998), Japan (Matsuda et al. 1996; Matsuda et al. 2002), and more recently, Sweden (Waldenström et al. 2007).

Many species of bacteria produce urease (urea amidohydrolase; EC3.5.1.5), a nickel-containing metalloenzyme that hydrolyzes urea to ammonia and carbamate (Mobley and Hausinger 1989). This organism, UPTC, is an atypical taxon with genus *Campylobacter* which produces urease (Matsuda and Moore 2004), as well as *C. sputorum* biovar paraureolyticus (On et al. 1998). Most recently, a reclassification of *Bacteroides ureolyticus* as *Campylobacter ureolyticus* com. nov., the third taxon of urease-producing *Campylobacter*, was also described (Vandamme et al. 2010; Bullman et al. 2011).

We have already demonstrated cloning, sequencing and characterization of a urease gene operon consisting of two putative promoter structures, at the -35- and -10- like regions,

six closely spaced and putative open reading frames (ORFs) of two structural (*ureA* and *ureB*) and four accessory (*ureE*, *ureF*, *ureG* and *ureH*) genes, probable ribosome-binding sites (RBSs) for each ORFs and a putative ρ-independent transcriptional terminator region from a genomic DNA library constructed with the Japanese UPTC CF89-12 isolate cells (Kakinuma et al. 2007). This urease gene operon was approximately 5.1-kilo base pairs (kbp) in length and showed high nucleotide sequence identities to those of some *Helicobacter* organisms (Kakinuma et al 2007). In addition, most recently, we have also described molecular analysis and characterization of a urease gene operon from *C. sputorum* biovar paraureolyticus (Kakinuma et al. 2011).

However, we have never attempted to construct *in vitro* approximately 5.1-kbp recombinant DNA molecule which can express catalytically active urease enzyme in *Escherichia coli* by amplifying the urease gene operon from the UPTC CF89-12 isolate until now.

Therefore, the aim of the present study was to construct *in vitro* recombinant molecule of the UPTC CF89-12 full-length urease gene operon and to express the recombinant urease molecule in *E. coli* cells. Then, we also aimed to construct *in vitro* several deletion recombinant variants of urease subunits genes and to express and characterize those in *E. coli* cells, in order to clarify the roles of the accessory genes products in the UPTC urease activation.

Materials and methods

Bacterial isolate and its culture condition

The Japanese isolate, UPTC CF89-12 (Matsuda et al. 1996), was used in the present study. The cells were cultured as described already (Nakanishi et al. 2010ab).

PCR amplification of the UPTC urease gene cluster

For the amplification of approximately 5.1-kbp urease gene operon from UPTC CF89-12 consisting of the promoter region and six urease genes, np 389 through 5,396 bp (AB201709), we designed a PCR primer pair for UPTC ureP-f [5′-AAGACTATGAAACTGAATTA-3′, nucleotide position (np) 389-408 bp] and ureH-r (5′-TTATAATCCTTAGTTTGTT-3′; np 5,396-5,378 (Fig. 1). PCR amplification and its product purification were carried out, as described by Sambrook and Russell (2001) (Sambrook and Russell 2001). TA cloning of the amplified urease gene operon using the pGEM-T vector and *E. coli* JM109 cells was also carried out, as described by Sambrook and Russell (2001) (Sambrook and Russell 2011).

In the present study, we also attempted to construct several large deletion recombinant variants of UPTC urease subunits genes by using their specific PCR primer pairs and inverse (I)-PCR procedures with TA cloned full-length UPTC urease gene operon. The primer pairs shown in Figure 1 for the I-PCR were designed based on the nucleotide sequence data of approximately 5.1-kbp full-length urease gene operon from the UPTC CF89-12

isolate (DDBJ/EMBL/GenBank accession number AB201709).

The PCR mixture contained 1 x iProof HF buffer, 200 μM each dNTP, 0.5μM each primer, a total of 1 unit iProof DNA polymerase (Bio-Rad Laboratories, Tokyo, Japan) and 100 ng template DNA. The PCR reaction was performed in 50 μL reaction volumes at 98°C for 30 sec, with 35 cycle at 98°C for 5 sec, 50°C for 10 sec, and 72°C for 10 sec to 3 min, followed by a final extension at 72°C for 5 min.

Amplified PCR products were separated by 0.7% (w/v) agarose gel electrophoresis in 0.5x TBE at 100 V and detected by staining with ethidium bromide. The PCR products amplified by the newly constructed primer pairs for the urease gene operon were purified by using a QIAEXII gel extraction kit (Qiagen, Tokyo, Japan). The purified amplicons were then subjected to cycle sequencing with BigDye Terminator (Applied Biosystems, Tokyo, Japan), with the PCR primers or the I-PCR primers and other sequence primers constructed by primer walking procedures, if necessary. The reaction products were separated and detected on an ABI 310 genetic analyzer (Applied Biosystems).

Urease activity measurement of recombinant UPTC urease gene operon

Urease activities of recombinant molecule of the full-length UPTC urease gene operon and several deletion recombinant variants of urease subunits genes (each *ureA*-, *ureB*-, *ureE*-, *ureF*-, *ureG*- and *ureH*-large deficient) constructed *in vitro* were determined, with log-phase cultured *E. coli* JM109 cells, transformed with pGEM-T vector carrying the TA cloned

full-length UPTC urease gene cluster and deletion recombinant variants with isopropyl-β-D-thiogalactoside (IPTG) (0.1 mM) and without IPTG. Recombinant *E. coli* JM109 cslls were cultured in L Broth medium containing 750 μM of $NiCl_2$ at 37°C (Hu and Mobley 1993).

The *E. coli* cells containing the recombinant UPTC urease gene operon were pelleted by centrifugation (7,000x g) at 4°C for 20 min. The cells were washed with PBS and resuspended in the same buffer. After the cells were disrupted by sonication, cell lysates extracts were collected by centrifugation at 27,000x g for 30 min at 4°C. Protein concentration determination of the extracts was conducted by using the DC Protein Assay Kit (Bio-Rad Laboratories). Quantitive detection of urease activity was achieved by using the indophenol method (μmole/min/mg protein) (Huizenga and Gips 1982) following the urease reaction of the freshly extract (4 μg) by adding to the 50 mM urea in PBS at 37°C for 4 h.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and western blotting

Soluble extracts were analyzed by the polyacrylamide gel electrophoresis (PAGE) on a mini-slab gel, comprising a 1% (w/v) SDS -12% (w/v) PAG with Tris-glycine buffer at 24 mA for 2 h. Proteins were transferred to a PVDF membrane, Immobilon™ (Millipore Corp. MA, USA), at 90 V for 2 h with cooling. The PVDF membrane was then blocked with 5% (w/v) non-fat dry milk in basis buffer, as described by Sambrook and Russell (2001) (Sambrook and Russell 2001), at room temperature for 30 min.

The membrane was then examined for reactivity with rabbit polyclonal anti-*Helicobacter pylori* urease α (A) and β (B) subunits antibodies (Santa Cruz Biotech Inc. CA, USA) diluted in 0.5% (v/v) with blocking buffer at 4°C overnight. Immunoreactants were then detected by using the ECL Western Blotting Detection System with horseradish peroxidase-labeled secondary antibody (GE Healthcare Life Sci, Tokyo, Japan).

Results

Construction of *in vitro* recombinant molecule of full-length urease gene operon from the UPTC CF89-12 and its expression

In the present study, the authors first constructed *in vitro* recombinant molecule of full-length urease gene operon from the UPTC CF89-12 isolate represented schematically in Figure 1 and expressed the recombinant urease in the *E. coli* JM109 cells. As shown in Figure 2B, the recombinant UPTC urease genes including full-length two structural (*ureA* and *ureB*) and four accessory (*ureE, ureF, ureG* and *ureH*) genes were expressed catalytically active urease in *E. coli*, *E. coli* cell lysate with recombinant urease gene in Figure 2B.

Thus, urease enzyme activity of the recombinant full-length molecule of UPTC CF89-12 isolate urease gene operon was detected with the transformed and log-phase cultured *E. coli* JM109 cells by using the indophenol method. However, no activity was detected with the cultured *E. coli* JM109 not carrying the recombinant urease gene operon analyzed as a negative control, *E. coli* cell lysate without recombinant urease gene in Figure 2B.

Construction of large deletion variants of UPTC CF89-12 urease subunits genes and their expression

In addition, we constructed large deletion variants of UPTC CF89-12 urease subunits genes, as schematically shown in Figure 2A. The details of the large deletion were shown in Table 1. Consequently, among seven large deletion variants of UPTC

CF89-12 urease subunits genes, *ureA*-(ΔA), *ureB*-(ΔB), *ureE*-(ΔE), *ureF*-(ΔF), *ureG*-(ΔG), and *ureH*-(ΔH) and ureE-H-(ΔE-H) deficient recombinant variants represented schematically in Figures 2A and summarized in Table 1, only the *ureE*-deficient recombinant variant (remaining amino acid residues of the positions 1 to 43 and 142 to 155, namely from amino acid position of 44 to 141 deleted UreE, 63% deficient ; 98 amino acids residues deletion out of the UreE 155 amino acids of the full-length; Table 1), showed a change in the colour of the assay to red judged as a positive reaction for urease with IPTG. Moreover, surprisingly, the urease activity in the *ureE* 63% deficient recombinant variant fraction was accelerated more than 10-fold in the recombinant full-length UPTC urease gene operon in *E. coli* cells. Interestingly, this urease activity value is almost equivalent to that in the UPTC CF89-12 cell lysate (Figure 2B). Regarding the 7th large deletion variant, ΔE-H, shown in Figure 2 and Table 1, the variant would generate a partial UreE (aar 1-43) and partial UreH (aar 162-250) fusion protein, which lacks the partial UreE (aar 44-155), complete UreF (aar 1-223), complete UreG (aar 1-199) and partial UreH (aar 1-161).

Urease genes recombinant variants containing 100% deletion of the UPTC *ureE* subunit gene

In Figure 3, deduced amino acid sequence alignment analysis of the putative ORFs of the full-length and large deletion recombinant variant of the UPTC *ureE* (63% deletion) was carried out. Since, in the present study, urease enzyme activity of the *ureE* deletion recombinant variant (63% deficient of the full

length UreE subunit) showed an accelerated urease activity to approximately 15-fold (Figure 2B), it is very interesting if within the urease genes recombinant variant containing 100% deletion of the UPTC UreE subunit gene, the urease activity is detected or not. Then, the authors constructed a 100% *ureE* complete deletion variant (155 amino acid residues deficient) of the UPTC CF89-12 full-length urease genes operon and examined the urease enzyme activity. The urease activity in the 100% *ureE* deletion recombinant variant constructed also showed a positive acceleration (approximately 18-fold), similarly in that of the 63% *ureE* deletion variant described above (Table 2). In addition, we constructed both 100% *ureE* and *ureG* deletion recombinant variant. When the recombinant variant was subjected to examine its urease activity, no activity was detected (Table 2).

Effects of $NiCl_2$ on the urease activity

Effect of $NiCl_2$ (750 μM) in the *E. coli* culture medium on the urease activity of the full-length recombinant UPTC urease gene operon and the recombinant UPTC urease gene variants of the large *ureE* deletion (63% segment deficient) and 100% *ureE* deletion were examined. Urease activity was positively affected by $NiCl_2$ as shown in Table 3. Consequently, the recombinant molecule of full-length urease gene operon and a recombinant variant containing large deletion of the *ureE* subunit from the UPTC CF89-12 showed a positive urease activities when cultured in the medium only containing the $NiCl_2$.

Western blot analysis

As shown in Figure 4, no A and B subunits in the large deletion variants of UPTC CF89-12 urease genes *ureA* (ΔA) and *ureB* (ΔB) were identified immunoreactively by the anti-*H. pylori* urease α(A) and β(B) subunits antibodies, respectively. In addition, anti-*H. pylori* urease α(A) and β(B) subunits antibodies identified immunoreactive bands at approximately 59 kDa and 26 kDa, respectively in the UPTC cells (CF89-12) and *E. coli* JM109 cells transformed with pGEM-T vector ligating the recombinant full-length UPTC urease gene operon of about 5.1 kbp (WT), and the five *ureE* (ΔE) - , *ureF* (ΔF) - , *ureG* (ΔG) - , *ureH* (ΔH) - and *ureE-H* (ΔE-H) - large deficient recombinant variants. However, no band was identified for *E. coli* JM109 transformed with pGEM-T vector only (T-v in Figure 4).

Discussion

This is the first demonstration of the construction, expression and characterization of the recombinant molecule of full-length UPTC urease gene operon from the UPTC CF89-12 isolate with transformed and log-phage cultured *E. coli* JM109 cells.

In the present study, the recombinant full-length UPTC urease genes cluster, two structural *ureA* and *ureB*, four accessory *ureE*, *ureF*, *ureG* and *ureH* genes constructed, and promoters structures, was identified to express a urease enzyme activity in the transformed and log-phase *E. coli* cells. As described previously (Kakinuma et al. 2007), some accessory genes, such as *ureI* other than the four *ureE*, *ureF*, *ureG* and *ureH*(*D*) genes, were undetectable in the UPTC CF89-12 urease genes cluster operon. In addition, only the *ureE*-deficient recombinant large deletion variant showed a urease-positive reaction.

Regarding the *ureE* from the UPTC, the previous sequence analysis indicated that the putative *ureE* ORF was identified to be 465 nucleotide sequence and it was predicted to encode 155 amino acid residues with the calculated molecular weight (CMW) of 18,586 (Kakinuma et al. 2007). Regarding the deduced amino acid sequence identities of the six ORFs of UPTC CF89-12 urease genes to those of *H. hepaticus*, *H. pylori*, *H. heilmannii* and *H. mustelae* (for the latter two, *ureA* and *ureB* only), the three accessory genes of *ureE*, *ureF* and *ureH* were characteristically demonstrated to give relatively lower identities (57.8 - 70.9%) among the three organisms, UPTC, *H. hepaticus* and *H. pylori*, when compared with the other three genes (Kakinuma et al. 2007). When, in addition, a neighbour-joining tree was constructed based on the complete nucleotide sequence

information of UPTC CF89-12 and both the urease structural and accessory gene sequences accessible in DDBJ/EMBL/GenBank, UPTC formed a cluster together with *H. pylori* and *H. hepaticus*, separating from the other urease-producing bacteria (Kakinuma et al. 2007).

In *Klebsiella aerogenes*, extensive biochemical studies showed that three accessory subunits, UreD, UreF and UreG are required *in vivo* for the assembly of the nickel metallocenter in the urease (Moncrief and Hausinger 1996). UreE was shown to be a metallochaperone that delivers nickel to urease (Colpas et al. 1999). The *K. aerogenes* UreE contains a histidine-rich carboxyl terminus sequence in which 10 of the last 15 residues are histidine (Lee et al. 1993), and is able to bind five to six Ni^{2+} ions per dimmer (Colpas et al. 1999; Lee et al. 1999). Although the histidine-rich motif has been thought to be essential to UreE function, not all UreE peptides possess a histidine-rich region (Moncrief and Hausinger 1997).

In the present study, the UPTC urease accessory *ureE* gene and its deduced amino acid sequence of the putative ORF sequenced and analyzed lacked the histidine-rich carboxyl terminus (Figure 3).

Thus, some bacterial UreEs did not possess a histidine-rich region. Regarding the *ureE* gene of *H. pylori*, the *ureE* did not contain a histidine-rich motif; however, its presence (genetically modified histidine-rich versions of UreE) resulted in a significant increase of urease activity (Benoit and Maier 2003; Maier et al. 2007; Shi et al. 2010). In addition, recently, Bellucci et al. (2009) described the metal-binding properties of the *H. pylori* UreE and its interaction with the related

accessory subunit UreG, a GTPase involved in the assembly of the urease active site (Bellucci et al. 2009). An alternative possible physiological role for UreE of *K. aerogenes* was suggested by the observation that the GTP concentration needed for optimal activation of urease *in vitro* is greatly reduced in the presence of UreE as compared with that required in its absence (Soriano et al. 2000).

In the present study, two kinds of *ureE* deletion recombinant variants, both approximately 63% deficient and 100% deficient variants showed accelerated urease activity to approximately 10-fold activity. In addition, a *ureE* and *ureG* deletion recombinant variant showed no urease activity (Table 2). Thus, the present study revealed that the three (*ureF, G* and *H*) of the four accessory genes within the urease gene operon of the UPTC CF89-12 may possibly be necessary for the expression of the urease. Whereas the UreE may possibly regulate the activity with the physiological and modulate level in the cells, long or complete *ureE* deletion recombinant variants of the gene may accelerate the urease activity without possible regulation.

In addition, regarding the report by Bellucci et al. (Bellucci et al. 2009), the results were discussed in relation to available evidence of a UreE-UreG functional interaction *in vivo* (Bellucci et al. 2009). In our present study on the *Campylobacter* UPTC urease, two *ureE* deletion recombinant variants accelerated the urease activity to approximately 10-fold as compared with a recombinant full-length urease gene operon, but both the *ureE* and *ureG* deletion recombinant variant generated no urease enzyme activity. Thus, in the UPTC CF89-12 cells, no UreE-UreG functional interaction may exist.

This is the first report of the construction, expression and characterization of a full-length recombinant urease gene operon and several large deletion recombinant variants of urease subunits genes from UPTC organism. In the present study, the target DNA was transformed into the *E. coli* cell by employing the pGEM-T vector, and the urease gene operon (approximately 5.1-kbp) containing the promoter region and its UPTC urease activity was detected in the *E. coli* cells. It remains uncertain, at present, as to which promoter in the urease gene operon or in the pGEM-T vector was responsible. In addition to the expression of the urease activity in the *E. coli* cells harbouring the recombinant UPTC operon, urease subunits A and B encoded on *ureA* and *ureB*, respectively, were also confirmed immunologically by western blot analysis with anti-urease α(A) and β(B) raised against *H. pylori*. This result strongly suggests that these subunits are immunologically reactive within UPTC and *H. pylori* to each other.

Acknowledgements

This research was partially supported by a research project grant awarded by Azabu University (Research Services Division) and by Grant-in-Aid for Scientific Research (C) (20580346) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to MM). MM and JEM were supported by a Butterfield Award from the Great Britain Sasakawa Foundation to jointly examine the role of *Campylobacter* in food-poisoning in the UK and Japan.

References

Bellucci M, Zambelli B, Musiani F, Turano P, Ciurli S (2009) *Helicobacter pylori* UreE, a urease accessory protein: specific Ni^{2+}- and Zn^{2+}-binding properties and interaction with its cognate UreG. Biochem J 422: 91-100

Benoit S, Maier R J (2003) Dependencce of *Helicobacter pylori* urease activity on the nickel-sequestering ability of the UreE accessory protein. J Bacteriol 185: 4787-4795

Bezian M C, Ribou G, Barberis-Giletti C, Mégraud F (1990) Isolation of a urase positive thermophilic variant of *Campylobacter lari* from a patient with urinary tract infection. Eur J Clin Microbiol Infect Dis 9: 895-897

Bolton F J, Holt A V, Hutchinson D N (1985) Urease-positive thermophilic campylobacters. Lancet I: 1217-1218

Bullman S, Corcoran D, O'Leary J, Lucey B et al. (2011) *Campylobacter ureolyticus*: an emerging gastrointestinal pathogen? FEMS Immunol Med Microbiol 61: 228-230

Colpas G J, Brayman T G, Ming L J, Hausinger R P (1999) Identification of metal-binding residues in *Klebsiella aerogenes* urease nickel metallochaperone UreE. Biochemistry 38: 4078-4088

Endtz H P, Vliegenthart J S, Vandamme P, Waverink H W et al (1997) *Campylobacter lari* isolated from mussels and oysters in the Netherlands. Int J Food Microbiol 34: 79-88

Fitzgerald C, Jones K, Anderton S, Andrew S (1998) Campylobacters in wild birds: identification and molecular characterization. In: Lastovica A J, Newell D G, Lastovica E E. (ed) *Campylobacter, Helicobacter and related organisms*. Rustica Press, Cape Town

Hu L-T, Mobley HLT (1993) Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in *Escherichia coli*. Infect Immun 61: 2563-2569

Huizenga J R, Gips C H (1982) Determination of ammonia in saliva using indophenol, an ammonium electrode and enzymatic method: a comparative investigation. J Clin Chem Clin Biochem 20: 571-574

Kakinuma Y, Iida H, Sekizuka T, Usui K et al (2007) Cloning, sequencing and characterization of a urease gene operon from urease-positive thermophilic *Campylobacter* (UPTC). J Appl Microbiol 103: 252-260

Kakinuma Y, Hayashi K, Tazumi A, Hirayama J et al (2011) Molecular analysis and characterization of a urease gene operon from *Campylobacter sputorum* biovar paraureolyticus. Folia Microbiol 52: 159-165

Kaneko A, Matsuda M, Miyajima M, Moore J E, Murphy P G (1999) Urease-positive thermophilic *Campylobacter* isolates from seagulls (*Larus* spp.) Lett Appl Microbiol 29: 7-9

Lee M H, Pankratz H S, Wang S, Scott R A et al (1993) Purification and characterization of *Klebsiella aerogenes* UreE protein-a nickel-binding protein that functions in urease metallocenter assembly. Protein Sci 2: 1042-1052

Maier R J, Benoit S L, Seshadri S (2007) Nickel-binding and accessory proteins facilitating Ni-enzyme maturation in *Helicobacter pylori*. Biometals 20: 655-664

Matsuda M, Kaneko A, Fukuyama M, Itoh T et al (1996) First finding of urease-positive thermophilic strains of *Campylobacter* in river water in the Far East, namely, in Japan, and their phenotypic and genotypic characterization. J Appl Bacteriol

81: 608-612

Matsuda M, Shibuya T, Itoh Y, Takiguchi M et al (2002) First isolation of urease-positive thermophilic *Campylobacter* (UPTC) from crows (*Corvus levaillantii*) in Japan. Int J Hyg Environ Health 205: 321-324

Matsuda M, Kaneko A, Stanley T, Millar B C et al (2003) Characterization of urease-positive thermophilic *Campylobacter* subspecies by multilocus enzyme electrophoresis typing. Appl Environ Microbiol 69: 3308-331

Matsuda M, Moore J E (2004) Urease-positive thermophilic *Campylobacter* species. Appl Environ Microbiol 70: 4415-4418

Mégraud F, Chevrier D, Desplaces N, Sedallian A, Guesdon J L (1988) Urease-positive thermophilic *Campylobacter* (*Campylobacter laridis* variant) isolated from an appendix and from human feces. J Clin Mirobiol 26: 1050-1051

Mobley H L T, Hausinger R P (1989) Microbial ureases: significance, regulation, and molecular characterization. Microbiol Rev 53: 85-108

Moncrief M B C, Hausinger R P (1996) Nickel incorporation into urease. In: Hausinger R P, Eichorn G L, Marzilli L G (ed) Mechanisms of metallocenter assembly. VCH Publishers, New York. pp 151-171

Moncrief M B C, Hausinger R P (1997) Characterization of UreG, identification of a UreD-UreF-UreG complex, and evidence suggesting that a nucleotide-binding site in UreG is required for *in vivo* metallocenter assembly of *Klebsiella aerogenes* urease. J Bacteriol 179: 4081-4086

Moore J E, Gilpin D, Crothers E, Canney A et al (2002) Occurrence of *Campylobacter* spp. and *Cryptosporidium* spp. in seagulls

(*Larus* spp.) Vect Borne Zoo Dis 2: 111-114

Nakanishi S, Tazumi A, Aihara N, Sekizuka T, Amano K, Moore JE, Millar BC, Matsuda M (2010a) Structural analysis and expression of the full-length cytochrome *P450* gene operon in *Campylobacter lari*. Br J Biomed Sci 67:133-139

Nakanishi S, Tazumi A, Moore JE, Millar BC, Matsuda M (2010b) Molecular and comparative analyses of the full-length cytolethal distending toxin (*cdt*) gene operon and its adjacent genetic loci from urease-positive thermophilic *Campylobacter* (UPTC) organisms. Br J Biomed Sci 67:208-215

On S L W, Atabay H I, Corry J E L, Harrington C S, Vandamme P (1998) Emended description of *Campylobacter sputorum* and revision of its infrasubspecific (biovar) divisions, including *C. sputorum* biovar paraureolyticus, a urease-producing variant from cattle and humans. Int J Syst Bacteriol 48: 195-206

Owen R J, Costas, Sloss L, Bolton F J (1988) Numerical analysis of electrophoretic protein patterns of *Campylobacter laridis* and allied thermophilic campylobacters from the natural environment. J Appl Bacteriol 65: 69-78

Sambrook J, Russell D W (2001) Molecular cloning: a laboratory manual. 3rd edn. Cold Spring Harbor Laboratory Press, New York

Shi R, Munger C, Asinas A, Benoit S L et al (2010) Crystal structures of apo and metal-bound forms of the UreE protein from *Helicobacter pylori*: role of multiple metal binding sites. Biochemistry 49: 7080-7088

Skirrow M B, Benjamin J (1980) '1001' campylobacters: cultural characteristics of intestinal campylobacters from man and animals. J Hyg (Camb) 85: 427-442

Soriano A, Colpas G J, Hausinger R P (2000) UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. Biochemistry 39: 12435-12440

Vandamme P, Debruyne L, De Brandt E, Falsen E (2010) Reclassification of *Bacteroides ureolyticus* as *Campylobacter ureolyticus* comb. Nov. Int J Syst Evol Microbiol 60; 2016-2022

Waldenström J, On S L W, Ottvall R, Hasselquist D, Olsen B (2007) Species diversity of campylobacteria in a wild bird community in Sweden. J Appl Microbiol 105: 424-432

Wilson I G, Moore J E (1996) Presence of *Salmonella* spp. and *Campylobacter* spp. in shellfish. Epidemiol Infect. 116: 147-153

Table 1. Details of the large deletion recombinant variants of UPTC CF89-12 urease subunit genes

Large deletion recombinant variant	Deletion			Note (mutation and so on)
	Nucleotide position (AB201709)	aar position	% deletion (remaining aar/putative ORF aar)	
ureA-deletion variant (ΔA)	603 ... 1025	41 ... 181	63%(82/223)	
ureB-deletion variant (ΔB)	1348 ... 2754	67 ... 535	83%(96/565)	
ureE-deletion variant (ΔE)	2977 ... 3270	44 ... 141	63%(57/155)	T3273C
ureF-deletion variant (ΔF)	3369 ... 3905	18 ... 196	81%(43/223)	T3368A
ureG-deletion variant (ΔG)	4084 ... 4551	30 ... 185	78%(43/199)	A4554G T4083C
ureH-deletion variant (ΔH)	4670 ... 5080	25 ... 161	55%(113/250)	
ureE,F,G,H-deletion variant (ΔE-H)	2977 ... 5080	UreE 44 ... UreH 161	16%(132/827)	*ureE* 1-43 and *ureH* 162-250 remaining

aar, amino acid residues

Table 2. Urease activities in the 100% *ureE* and 100% *ureE-G* deletion recombinant variants of the UPTC CF89-12 full-length urease gene operon

Deletion recombinat variant	μmol/min/mg protein	% activity of full-length wild type recombinant
E. coli cell lysate without urease gene	0.004	5.7
E. coli cell lysate with full-length urease gene operon	0.070	100
E. coli cell lysate with urease gene (63%ΔE)	1.176	1,680
E. coli cell lysate with urease gene (100%ΔG, E)	0.003	4.3
E. coli cell lysate with urease gene (100%ΔG)	0.002	2.9
E. coli cell lysate with urease gene (100%ΔE)	1.258	1,797

Table 3. Effects of $NiCl_2$ on the urease activity in the large *ureE* deletion recombinant variant and full-length urease gene operon of the UPTC CF89-12

Deletion recombinant variant	µmol/min/mg protein	% of wild type
E. coli cell (grown in LB without $NiCl_2$) lysate without urease gene	0.001	4.8
E. coli cell (grown in LB without $NiCl_2$) lysate with urease gene	0.000	0
E. coli cell (grown in LB without $NiCl_2$) lysate with urease gene (ΔE)	0.001	4.8
E. coli cell [grown in LB containing $NiCl_2$ (750 µM)] lysate without urease gene	0.002	9.5
E. coli cell [grown in LB containing $NiCl_2$ (750 µM)] lysate with urease gene	0.021	100
E. coli cell [grown in LB containing $NiCl_2$ (750 µM)] lysate with urease gene (ΔE)	0.469	2,233

Legends to Figures

Fig. 1 Schematic representations of the genetic organization of the urease gene operon from UPTC CF89-12 isolate and the primer sites for the amplification of the operon (A), and primers (B).

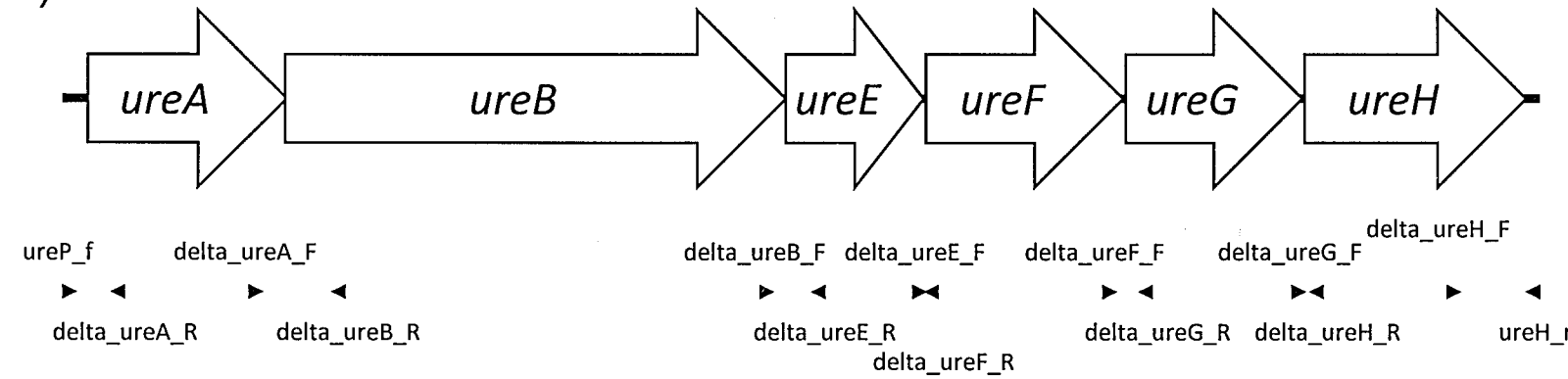
Fig. 2 Schematic representations of the genetic organization of the recombinant full-length urease gene operon molecule from the UPTC CF89-12 isolate and several urease subunits genes as large deletion recombinant variants (A). Urease activity determination was also carried out by using the indophenol method (Huizenga and Gips 1982) (B). ΔA, ΔB, ΔE, ΔE-H, ΔG andΔH show the deficient regions schematically within the urease subunits genes large deletion recombinant variants (A).

Fig. 3 Deduced amino acid sequence aligment analysis of the putative ORFs of the full-length UPTC *ureE* (ClUreE) and UPTC *ureE* large deletion (63%) variants (ClUreEld), as well as the putative ORFs of the full-length urease gene *ureE* from *H. pylori* ATCC43504 (HpUreE), *K. aerogenes* (KaUreE) and *B. pasteurii* (BpUreE).

Fig. 4 Western blot analysis of the UPTC urease in the UPTC CF89-12 cells and the recombinant UPTC urease in *E. coli* cells using polyclonal anti-*H. pylori* urease α(A) and β(B) subunits antibodies. T-v, cell lysate of *E.coli* JM109 cells transformed with pGEM-T vector only; WT, cell lysate of *E. coli* JM109 cells transformed with pGEM-T vector ligating the recombinant full-length UPTC CF89-12 urease gene operon; ΔA~ΔE-H, cell lysates of *E. coli* JM109 cells transformed with pGEM-T vector ligating large deletion

variants of UPTC CF89-12 urease subunits genes; CF89-12, UPTC CF89-12 cell lysate; 299, UN *C. lari* 299 cells lysate.

A)

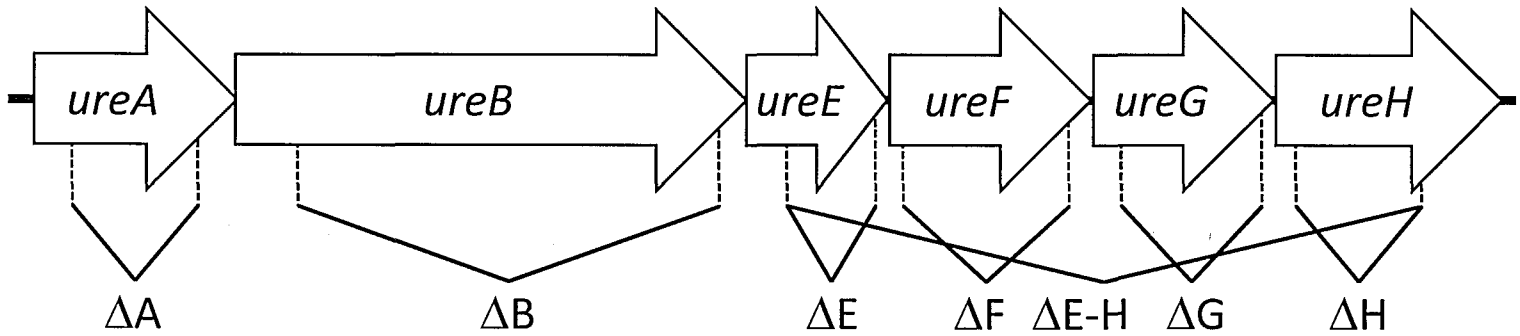


B)

Primer	Nucleotide sequence (5'→3')	Nucleotide position
ureP-f	AAGACTATGAAACTGAATTA	389...408
ureH-r	TATAATCCTTAGTTTGTT	5378...5396
ure_lac_F_SacII	AACAACCGCGGACAGGAAACAGCTATGACC	
ure_lac_R_ApaI	TTGGAGGGCCCGCTTGAGTATTCTATAGTG	
delta_ureA_F	GAATGTACAGTAAGTTTAATAGAATTTGG	1020...1048
delta_ureA_R	TTCTGTACATATATAAGCTAAAGCTTC	582...608
delta_ureB_F	AGAAGTGGATCCACAAACTTATGAAGTA	2746...2773
delta_ureB_R	ACTGGATCCGGAAAGTCTCCCTCACTC	1327...1353
delta_ureE_F	GATATGGCTAGCTTTATCCAAGTTGATCC	3262...3290
delta_ureE_R	TTTTGCTAGCGACATCTAGTCCTTTTAAAGTCG	2951...2983
delta_ureF_F	AAAACCATGGTGTGAAAATCACAATATC	3899...3926
delta_ureF_R	CTCCCCATGGAAAAGAAGAATCACTAATTTG	3345...3375
delta_ureG_F	GAAGGTTTACTCGAGATCATTACTTGGAT	4540...4568
delta_ureG_R	GCCAACTCGAGCTCATCTTTTAAAGCTTGAC	4061...4091
delta_ureH_F	GAGTTTAACTCAAATTTCTAGAAATGATACT	5062...5092
delta_ureH_R	AGGTGTGAATCTAGAATTTTTTATGATAGT	4652...4681

Fig. 1

A)



B)

Deletion recombinant variant	μmol/min/mg protein	% activity of full-length wild type recombinant
E. coli cell lysate without urease gene	0.002	4.1
E. coli cell lysate with urease gene	0.049	100
E. coli cell lysate with urease gene (ΔA)	0.002	4.1
E. coli cell lysate with urease gene (ΔB)	<0.002	0
E. coli cell lysate with urease gene (ΔE)	0.723	1,476
E. coli cell lysate with urease gene (ΔF)	<0.002	0
E. coli cell lysate with urease gene (ΔG)	0.003	6.1
E. coli cell lysate with urease gene (ΔH)	0.003	6.1
E. coli cell lysate with urease gene (ΔE-H)	<0.002	0
UPTC CF89-12 cell lysate	2.403	4,904
UN*C. lari* 299 cell lysate	<0.002	0

Fig. 2

```
ClUreE      1:MIL--LQNKIKHYD-LNKECDFLELSWFDTFKKILRTTTLKGLDVAIKMPDNK--GLNHN 55
ClUreEld    1:MIL--LQNKIKHYD-LNKECDFLELSWFDTFKKILRTTTLKGLDVA------------- 43
HpUreE      1:MIIERLVGNLRDLNPLDFSVDHVDLEWFETRKKIARFKTRQGKDIAIRLKDAPKLGLSQG 60
KaUreE      1:-------MLYLTQRLEIPAAATASVTLPIDVRVKSRVKVTLNDGRDAGLLLPRGLLLRGG 53
BpUreE      1:MLITKIVGHIDDYESSDKKVDWLEVEWEDLNKRILRKETENGTDIAIKLENSG--TLRYG 58
                                                 *

ClUreE     56:DCLY-DE-DFLILVKIKPEKVLKIHIENEYNLALISYQVGNMHLNLFYKDHKL-LTLE-Q 111
ClUreEld   43:---------------------------------------------------------- 43
HpUreE     61:DILFKEE-KEIIAVNILDSEVIHIQAKSVAEVAKICYEIGNRHAALYYGESQFEFKTPFE 119
KaUreE     54:DVLSNEEGTEFVQVIAADEEVSVVRCDDPFMLAKACYHLGNRHVPLQIMPGEL--RYHHD 111
BpUreE     59:DVLYESD-DTLIAIRTKLEKVYVIKPQTMQEMGKMAFEIGNRHTMCIIEDDEI--LVRYD 115

ClUreE    112:NSIIRFLEKFNIKYEKCEEILEPKYMLDMPSFIQVDPNFKLIKE-------- 155
ClUreEld   44:-----------------------------SFIQVDPNFKLIKE-------- 57
HpUreE    120:KPTLALLEKLGVQNRVLSSKLDSKERLTV-SMPHSEPNFKVSLASDFKVVVK 170
KaUreE    112:HVLDDMLRQFGLTVTFGQLPFEPEAGAYASESHGHHHAHHDHHAHSH----- 158
BpUreE    116:KTLEKLIDEVGVSYEQSERRFKEPFKYRGHQH------------------- 147
```

Fig. 3

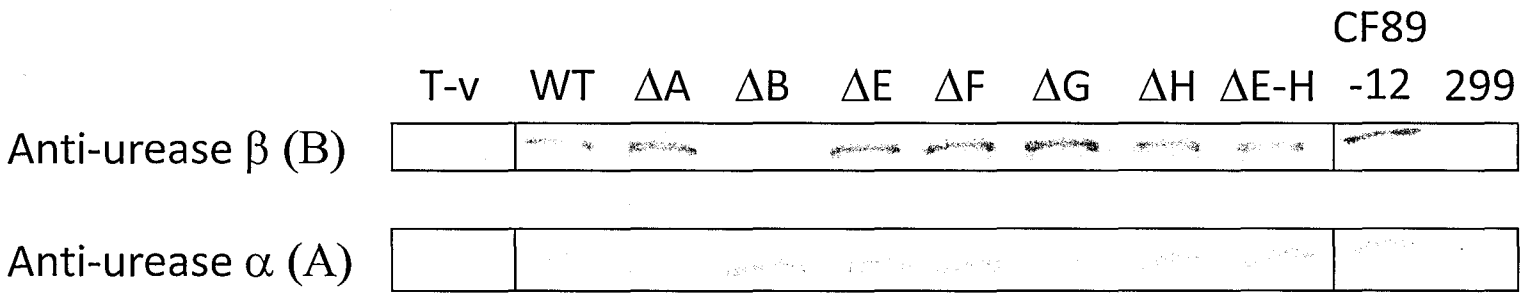

CF89
T-v
WT
ΔA
ΔB
ΔE
ΔF
ΔG
ΔH
ΔE-H
-12
299
Anti-urease β (B)
Anti-urease α (A)

Fig. 4

Acknowledgments

The author wishes to thank Professor Dr. M. Matsuda, Graduate school of Environmental Health Sciences, Azabu University for the main examine to this thesis and Professor Dr. K. Iwahashi and Associate Professor Dr. K. Furuhata for the sub examine. The author also thanks Professors Dr. J. E. Moore, Northern Ireland Public Health Laboratory, Belfast City Hospital, UK.