Biochemical characterisation of urease from urease-positive thermophilic *Campylobacter* (UPTC)

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Accepted: 19 August 2011

ABSTRACT

This study aims to characterise biochemically urease from an atypical *Campylobacter lari*, namely urease-positive thermophilic *Campylobacter* (UPTC). Urease was purified from cells of a Japanese UPTC isolate (CF89-12) using phenyl-Sepharose chromatography. Two protein components (estimates molecular masses 24 kDa and 61 kDa) were obtained that appeared to be structural proteins of urease (subunits A and B), and these were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE). The native molecular weight for the final purified UPTC urease was estimated to be approximately 186,000 Da which is close to the calculated molecular weight (182,738 Da) based on all six open reading frames of UPTC CF89-12 urease genes (*ureA*, *B*, *E*, *F*, *G* and *H*), as described previously. Moreover, an active band was observed on phenol red staining after a non-denaturing native PAGE of the crude extract from the UPTC cells. In addition, the purified urease of UPTC CF89-12 showed enzyme activity over a broad pH range (pH 6–10), with maximal activity at pH 8.0. The urease was also stable against heat treatment, with almost no loss of enzyme activity seen following 60-min incubation at temperatures of 20–60°C. Urease subunits A and B were identified immunologically by Western blot analysis with rabbit anti-urease α (A) and β (B) raised against *Helicobacter pylori*.

KEY WORDS: Biochemical characterisation.
Campylobacter.
UPTC.
Urease.

Introduction

Campylobacter lari was first recognised as a nalidixic acid-resistant thermophilic *Campylobacter* (NARTC).[1] 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.[2] Thereafter, the characterisation of UPTC as a variant or biovar of *C. lari* was described.[3,4] After the original description of UPTC appeared, isolates of UPTC have been reported in France,[3,5] Northern Ireland,[6–9] The Netherlands,[10] England,[11] Japan[12,13] and, most recently, in Sweden.[14,15]

Many species of bacteria produce urease (urea amidohydrolase; EC3.5.1.5), a nickel-containing metalloenzyme that hydrolyses urea to ammonia and carbamate.[16] This organism, UPTC, is an atypical taxon among the genus *Campylobacter* that produces urease,[17] as does *C. sputorum* biovar paraureolyticus.[18]

The authors have already demonstrated the cloning and sequencing of a urease gene operon consisting of two putative promoters, six closely spaced and putative open reading frames (ORF), two structural genes (*ureA* and *ureB*) and four accessory genes (*ureE*, *ureF*, *ureG* and *ureH*), and a possible ρ-independent transcriptional termination region from a genome DNA library constructed from Japanese UPTC CF89-12 cells.[19] This urease gene operon was approximately 5.1 kbp in length and showed high nucleotide sequence similarity to those from some *Helicobacter* organisms.[19] Moreover, a neighbour-joining tree constructed based on the nucleotide sequence data of urease genes suggests a commonly shared ancestry between UPTC and *Helicobacter* urease genes.

Although the urease activity of UPTC has been described in a report on the immunological specificity of *Helicobacter pylori* urease by Shingaki and colleagues,[20] the biochemical study of urease from UPTC has only been reported in recent genetic descriptions.[19,21,22] Therefore, the present study aims to characterise urease biochemically from a Japanese *C. lari* UPTC isolate (CF89-12).

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Materials and methods

The cells of a Japanese UPTC isolate (CF89-12) used in the present study were cultured in Mueller-Hinton broth at 37°C for 48 h in an aerobic jar on Blood Agar Base No. 2 (Oxoid, Hampshire, UK) containing 7% (v/v) defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) and *Campylobacter*-selective medium (Virion, Zurich, Switzerland). An atmosphere of 5% (v/v) O_2 and 10% (v/v) CO_2 was produced by BBL Campypak microaerophilic system envelopes (Becton Dickinson, NJ, USA).

The cells were pelleted by centrifugation (7000 x*g*) at 4°C for 10 min and crude extracts were prepared from the

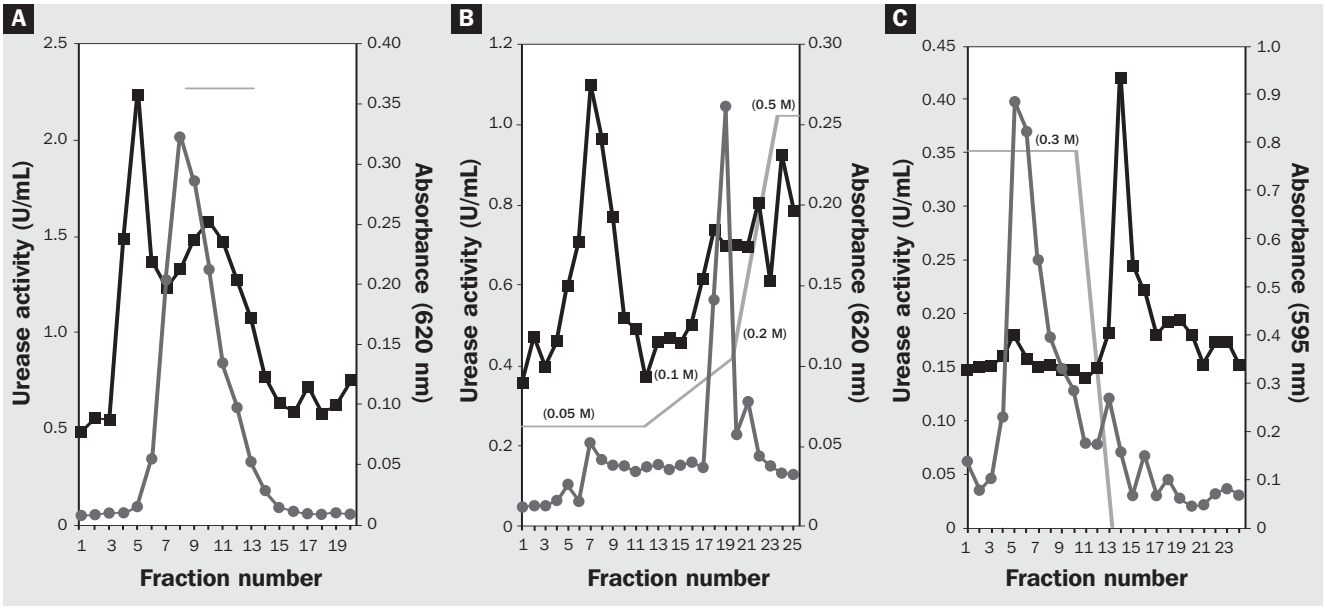

Fig. 1. Biochemical purification of UPTC CF89-12 urease using **A)** Bio-Gel A-5M, **B)** DEAE sepharose and **C)** phenyl-sepharose column chromatography procedures. Loading materials were separated using chromatography on each column with **A)** PEM buffer, **B)** 0.05 M-0.1M-0.2 M-0.5 M NaCl in PEM and **C)** 0.3 M-0.0 M KCl in PEM (C), respectively. Loading materials were monitored for protein concentration (●) and assayed for urease activity (■).

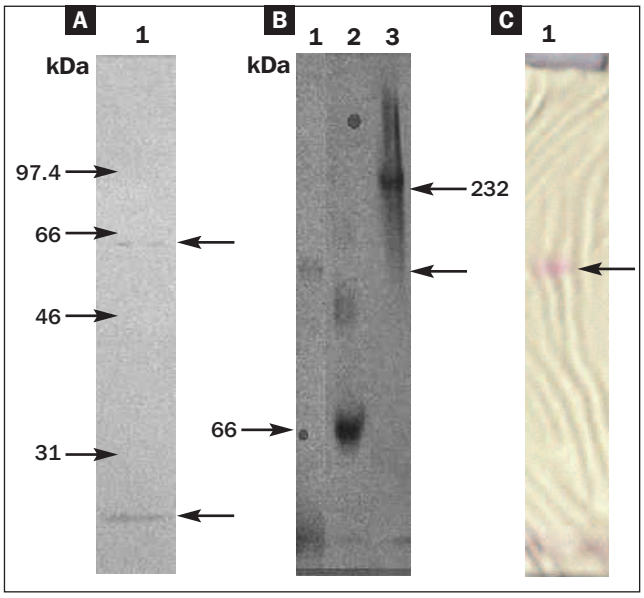

Fig. 2 SDS-PAGE **(A)** and non-denaturating native PAGE **(B)** of the final UPTC urease preparation, and its phenol red active stained band following the non-denaturing native PAGE of the crude extracts prepared from the UPTC cells **(C)**. Arrows (←) represent the purpose band obtained in the lane 1, respectively. (A), Some of the molecular weight markers from the Page Ruler™ Prestained Protein Ladder (Fermentas LIFE SCIENCES, Tokyo, Japan). (B) Lane 1, UPTC urease preparation after purification by phenyl-Sepharose chromatography; lane 2, Bovine serum albumin (66 kDa); lane 3, Catalase (232 kDa).

surface of UPTC cells by suspending 1.4 g (wet weight) cells in 10 mL 1% n-octyl glucopyranoside (n-OGP; Sigma, St. Louis, MO, USA) with gentle shaking at room temperature for 30 min. The crude extracts were also prepared from the outside and inside of the cells by sonication. Both extracts were centrifuged (7000 x*g*) at 4˚C for 10 min. The resultant supernatant was dialysed against PEM buffer (20 mmol/L sodium phosphate [pH 7.0], 1 mmol/L EDTA and 1 mmol/L 2-mercaptoethanol). After centrifugation (25,000 x*g*) at 4˚C for 30 min, urease was fractionated by chromatography on a Bio-Gel A-5M agarose column (2.5 x 9.5 cm; Bio-Rad. CA, USA), a DEAE Sepharose CL-6B column (1.5 x 20 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) and a phenyl-Sepharose high-performance column (1.5 x 3.5 cm; Amersham Pharmacia Biotech). Protein concentrations were determined using protein assay reagent (Pierce Chemical, Rockford, Ill, USA).

Enzyme activity in each sample was determined using the indophenol formation method at 530 nm with ammonium chloride as a standard.[23] Urease activity was determined as one unit of enzyme for the hydrolysation of 1 µmol urea/min.

Sodium dodecyl sulphate (SDS; 1% [w/v])–10% (w/v) PAG was employed in the electrophoresis to detect the urease protein component(s), which were stained with Coomassie brilliant blue.

The native molecular weight of the purified UPTC urease preparation was estimated using non-denaturing 7.5% (w/v) native PAGE, and 5% non-denaturing native PAGE and 0.04% (w/v) phenol red active staining in 50 mmol/L citrate

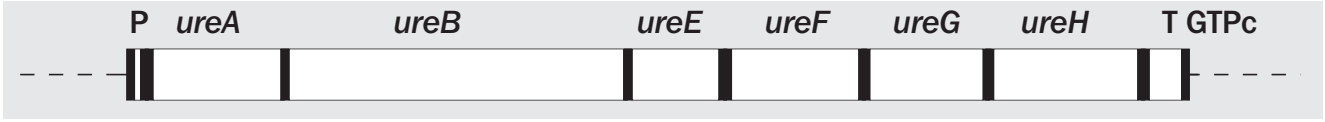

Fig. 3. An illustration of the full-length putative urease gene operon in the *C. lari* UPTC CF89-12 isolate (P: promoter, T: terminator, GTPc: GTP cyclohydrolase I).

Table 1. Urease purification from the Japanese *C. lari* UPTC isolate UPTC CF89-12.

Purification step	Specific activity*	Purification (fold)	Total activity*	Enzyme recovery (%)
Crude extract	4.1	1	1148	100
Bio-Gel A-5M agarose	7.3	1.8	940	82
DEAE-Sepharose	7.8	1.9	379	33
Phenyl-Sepharose	789.0	192.6	153	13

*µmol urea hydrolysed/min/mg.

buffer (pH 5.0) containing 0.1 mol/L urea were employed to detect urease.

Effects of pH activity, urease stability against heat treatment, and the effects of several inhibitors on UPTC CF89-12 urease activity were determined. Optimal temperature of urease from the UPTC CF89-12 cells was also determined.

For Western blot analysis, soluble extracts were subjected to PAGE on a mini-slab gel comprising 1% (w/v) SDS–10% (w/v) PAG with Tris-glycine buffer at 24 mA for 2 h. Proteins were transferred to PVDF membranes (Immobilon; Millipore, MA, USA) at 90 V for 2 h with cooling. The membranes were then blocked with 5% (w/v) non-fat dry milk in buffer, as described by Sambrook and Russell,[24] at room temperature for 30 min. Membranes were examined for reactivity with rabbit polyclonal anti-*H. pylori* urease (A) and (B) subunit antibodies (Santa Cruz Biotechnology. CA, USA) diluted in 0.5% (v/v) blocking buffer at 4˚C overnight. Immunoreactants were then detected using nitro blue tetrazolium (15-bromo-4-chlorl-3-indolyl phosphate-ρ toluidine salt solution).

Results and discussion

In the present study, after initial dialysis of the supernatant fluid of the extracted materials from the surface, outside and inside of *C. lari* UPTC CF89-12 cells, the crude extracts were used as a loading material on a Bio-Gel A-5M agarose column. Two protein peaks appeared using chromatography and the urease activity was demonstrated to be enriched in the preceding fractions (fraction numbers 6–12) of the second protein peak (Fig. 1A). When loading on the DEAE Sepharose column after dialysis of the pooled fractions (6–12), urease was eluted by 0.1–0.2 mol/L NaCl gradient buffer at approximately 0.14 mol/L NaCl and from the second protein peak (17–19) using chromatography of the column (Fig. 1B). Urease was finally purified by chromatography on the phenyl-Sepharose column (Fig. 1C). Overall urease yields from the UPTC cells are summarised in Table 1.

As demonstrated in Figure 2A, SDS-PAGE of the final urease preparation showed two protein components with approximate molecular masses of 24 kDa and 61 kDa. These were similar to the value for *H. pylori*.[25] The present UPTC urease preparation, after purification by phenyl–Sepharose chromatography, had a specific activity of approximately 789 µmol urea hydrolysed/min/mg (Table 1).

Although the specific activity determined for any enzyme

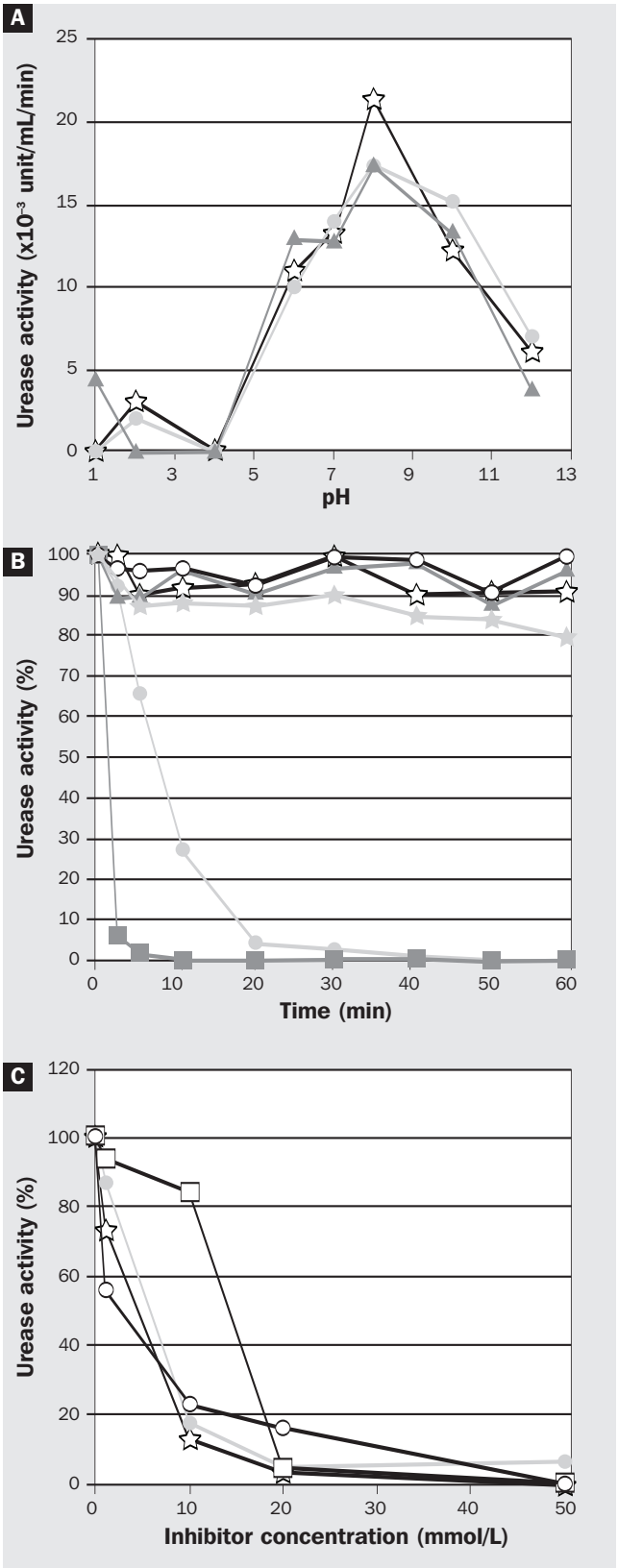

Fig. 4. Biochemical characteristics of the purified UPTC urease. **A)** pH activity, **B)** stability with heat treatment, and **C)** effects of inhibitors on urease activity ([A] ●: urease activity after incubation for 2 min; ▲: 10 min; ☆: 20 min; [B] ●: urease activity after incubation for 2 min at 30˚C; ▲: 40˚C; ☆: 50˚C; ★: 60˚C; ●: 70˚C; ■: 80˚C; [C] ❑: urease activity in the preasence of thiourea; ●: hydroxyurea; ☆: *N*-ethylmaleimide; ❍: acetohydroxamic acid).

depends on the state of purification, those for several purified microbial ureases have been described, and range from 1000 to 5500 µmol/min/mg.[16] Thus, the specific activity of UPTC urease was identified to be much lower than the average values of microbial ureases. In addition, the urease purification of UPTC was estimated to be 192.6-fold at the final phenyl-Sepharose step (Table 1). This purification value was mid-range among the several purification techniques for microbial ureases.[16]

As shown in Figure 2B, based on mobility on the non-denaturing native PAGE, the native molecular weight for the final purified UPTC urease by phenyl-Sepharose high-performance chromatography was estimated to be approximate 186,000 Daltons, a value lower than the average of microbial urease.[16] An active band was also identified by phenol red active staining on a non-denaturing native PAGE of the crude extract from the UPTC cells (Fig. 2C).

In relation to the urease genes of the UPTC CF89-12 isolate, the authors have already described the cloning and sequencing of a urease gene cluster, consisting of two putative promoters, six closely spaced and putative ORFs (two structural [*ureA* and *ureB*] and four accessory [*ureE*, *ureF*, *ureG* and *ureH*; Fig. 3] genes) and a putative ρ-independent transcriptional termination region, with a genome DNA library constructed from a Japanese UPTC CF89-12 isolate.[19] They were predicted to encode peptides of 223 amino acid residues for *ureA*, 565 for *ureB*, 155 for *ureE*, 222 for *ureF*, 199 for *ureG*, and 250 for *ureH*, with calculated molecular weights (CMWs) of 25,341; 61,420; 18,586; 25,937; 21,992 and 29,462, respectively. Apparent molecular sizes for UPTC urease A and B subunits obtained in a previous study[19] were shown to be consistent with these CMW values. Thus, the lower specific activity of UPTC urease described above may be due in part to the lack of the *urel* gene, as described already by Scott *et al.*, who reported low levels of urease activity in the *H. pylori urel* mutant.[26]

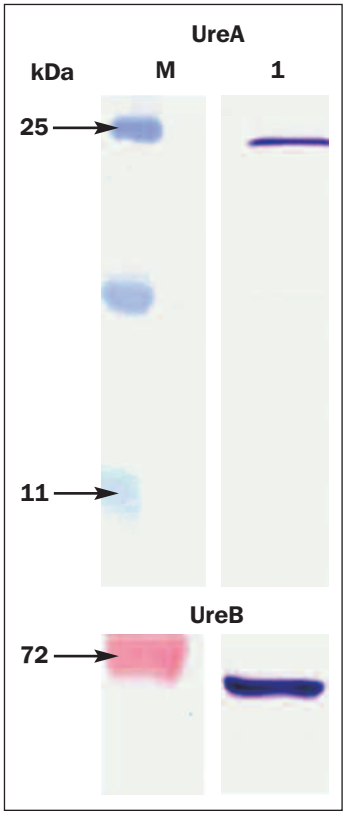

Fig. 6. Western blot analysis of soluble extract from the UPTC CF89-12 cells with rabbit polyclonal anti-*H. pylori* urease α (A) and β (B) subunit antibodies. Lane M: Page Ruler Prestained Protein Ladder (Fermentas), lane 1: purified UPTC CF89-12 urease.

Although UPTC urease activity was described in a report of the immunological specificity of *H. pylori* urease by Shingaki and colleagues,[20] the present study is the first to focus on urease from UPTC organisms. Regarding purified bacterial urease, one to three major protein bands have been described following SDS-PAGE analysis.[27,28]

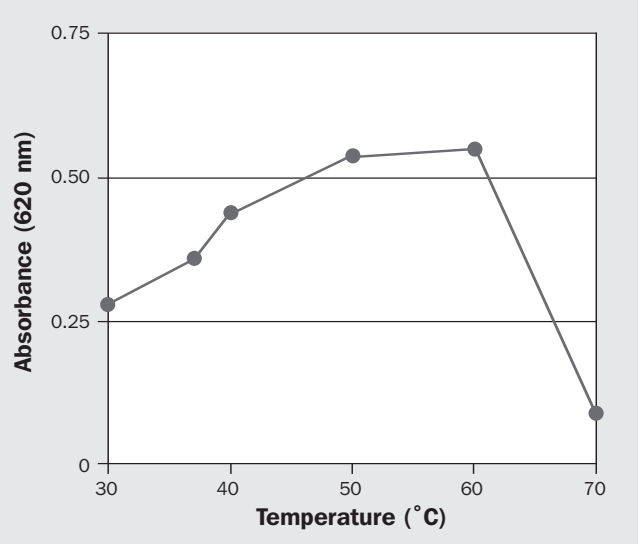

Fig. 5. Determination of the optimum temperature of urease activity from UPTC CF89-12.

In the present study, other biochemical characteristics of the purified UPTC CF89-12 (i.e., pH activity, stability against heat, effects of inhibitors on urease activity) have been clarified (Fig. 4). The UPTC urease showed enzyme activity over a broad pH range (pH 6–10, with maximal activity at pH 8.0) (Fig. 4A). Almost no loss of enzyme activity occurred after 60 min at temperatures between 20˚C and 60˚C. However, activity was progressively suppressed at temperatures above 60˚C (Fig. 4B). This was followed by a comparison of four UPTC urease inhibitors (Fig. 4C). Hydroxyurea, thiourea, *N*-ethylmaleimide and acetohydroxamic acid[29] inhibited UPTC urease by 50% at 7.0 mmol/L, 15.0 mmol/L, 4.9 mmol/L and 3.0 mmol/L, respectively.

As UPTC organisms are thermophilic, the study determined the optimum temperature of urease from the UPTC organisms, and was shown to be approximately 60˚C (Fig.5). This is higher than 42˚C at which thermophilic UPTC organisms are able to grow and survive optimally.

As shown in Figure 6, anti-*H. pylori* urease α (A) and β (B) subunit antibodies identified immunoreactive bands at approximately 24 kDA and 61 kDa, respectively, in UPTC cells (Fig. 6, Lane 1). The present study demonstrates that the approximate molecular weights of the urease subunits A and B estimated from the results of SDS-PAGE and Western blotting are similar to the calculated molecular weights of the putative ORFs for *ureA* (25,341 Da) and *ureB* (61,420 Da) of UPTC CF89-12, as described previously.[19] □

This study was partially supported by Promotion and Mutual Aid Corporation for Private Schools of Japan, Grant-in-Aid for Matching Fund Subsidy for Private Universities. JEM and MM were supported by a Butterfield Award from the Great Britain Sasakawa Foundation to examine the role of Campylobacter in food poisoning in the UK and Japan. MM was the recipient of a Grant-in-Aid for Scientific Research (C) (No. 20580346) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. AT and TN should be regarded as joint first authors.

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