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Cloning, Expression, Purification, Regulation, and Subcellular Localization of a Mini-protein from *Campylobacter jejuni*

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Abstract The Cj1169c-encoded putative protein of *Campylobacter* was expressed and purified from *E. coli* after sequence optimization. The purified protein allowed the production of a specific rabbit antiserum that was used to study the protein expression in vitro and its subcellular localization in the bacterial cell and putative interactions with other proteins. This protein is produced in *Campylobacter* and it clearly localizes into the periplasmic space. The level of protein production depends on factors, including pH, temperature, osmolarity, and growth phase suggesting a role in the *Campylobacter* environmental adaptation. The cysteine residues present in the sequence are probably involved in disulfide bridges, which may promote covalent interactions with other proteins of the *Campylobacter* envelope.

Introduction

Campylobacter is responsible for campylobacteriosis, a human gastroenteritis. With about 200,000 human cases reported every year, this disease is the most frequently reported food-borne illness in the European Union. However, this is underestimated and the actual number of cases is believed to be around 9 million each year. The cost of campylobacteriosis to public health systems and to lost productivity in the EU is estimated by European Food Safety Authority to be around EUR 2.4 billion a year (http://www.efsa.europa.eu). In the developing world, the burden of *Campylobacter* infections in children is largely more important [18]. In addition, post-infectious sequelae including Guillain Barré Syndrome and other neurological disorders may arise, with a limited morbidity but high mortality [8].

Campylobacter jejuni and *C. coli* are the two main species responsible for human gastrointestinal infection by *Campylobacter* [2]. While it is shown that the origin of contamination differs from the two species, *C. coli* being mainly transmitted by pork and *C. jejuni* by chicken consumption, there is no clear evidence for differences between these species that may account for their origin of transmission. Numerous genome sequences of both species are now available and comparisons identified several open reading frames that may represent species-specific proteins [12, 13]. Among them, some may be associated with the preferred reservoir of infection; however, this needs to be demonstrated. In a detailed study, comparing 96 genome sequences of *C. jejuni* and *C. coli* Lefébure et al. identified core genome comprising specific genes for each species and 17 genes that were conserved in all strains of *C. jejuni* (43 strains tested) but less frequent in *C. coli* (42 strains tested), suggesting they are indispensable regarding the physiology of *C. jejuni* compared to that of *C. coli* [15, 18]. Thus, the study of these genes and of the corresponding proteins may help in better understanding the reasons for this prevalence.

The Cj1169c open reading frame belongs to the 17 genes identified by Lefébure et al. [8, 15]. According to a previous comparison analyses of several strains of *C. jejuni*, *C. coli*, and *C. lari*, Cj1169c was associated to the

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Cj1170c ORF in all strains of *C. jejuni* tested [2, 9]. Cj1170c encodes for Omp50, an outer membrane protein that was shown to have pore forming activity in black lipid bilayers [4] and more recently to exhibit tyrosine kinase activity [7, 13]. Transcriptomic studies showed that Cj1169c and Omp50 production was stimulated in rabbit infection model [11, 20] and in iron-depleted environments [5, 6, 14] suggesting an involvement of these genes during host colonization. Moreover, temperature sensitive expression study of *C. jejuni* showed that these two open reading frames were rapidly overproduced after temperature increase from 37 to 42 °C [19] which should be in agreement with their putative role in chicken colonization that have a body temperature of 42 °C. In addition, both genes were shown to be involved in macrolide adaptation [23].

While the Omp50 protein, encoded by the Cj1170c ORF, was purified from *Campylobacter* [4, 7] and its various function studied, no data are already available regarding Cj1169c.

In the present study, we performed cloning, expression, and purification of the Cj1169c-encoded protein in *E. coli* and thanks to the production of a specific rabbit antiserum we studied its production regarding culture conditions and subcellular localization in *Campylobacter*.

Materials and Methods

SDS-PAGE and Western Blotting

SDS-PAGE was performed in 14 % (w/v) polyacrylamide gel. Samples were mixed with a loading buffer (Tris160 mM, EDTA 4 mM, SDS 3.6 %, bromophenol blue 0.01 %, sucrose 800 mM, 2-mercaptoethanol (BME) 1 %, and dithiothreitol (DTT) 60 mM) and incubated for 10 min at 96 °C before being loaded onto SDS-PAGE. The gel was run in SDS-Tris–glycine buffer (Tris–HCl 186 mM, glycine 24 mM, and SDS 0.1 %) at a constant 180 V (Bio-Rad Protean II system, Marnes-la-Coquette, France). To visualize the protein profiles, gels were stained with Coomassie Brilliant blue R-250.

For Western blotting, proteins were electro-transferred onto a nitrocellulose membrane (Proteigene, Saint-Marcel, France) in transfer buffer (Tris 20 mM, glycine 150 mM, isopropanol 20 %, and SDS 0.05 %). Immuno-detections were performed with a 1:1000 dilution of specific rabbit antiserum. After washing, membranes were then probed either with a 1:30,000 dilution of anti-rabbit IgG-phosphatase alkaline conjugate antibody (Sigma, Saint-Quentin Fallavier-France) according to the manufacturer's instructions and revealed by colorimetric detection, or with a 1/5000 anti-rabbit IgG-peroxidase linked antibody (Invitrogen, Saint Aubin, France). In this latter case, the targeted band was detected by Clarity ^{TM}ECL Western blotting detection reagents (Bio-Rad). A Coomassie blue staining of an identical gel was performed for each immunoblot to ensure that an equal amount of total proteins was loaded in each lane.

Tris–EDTA Extraction of Soluble Proteins

After a pre-culture of *C. jejuni* strains during 24 h at 42 °C on COS-BW, bacteria were grown on GC for 24 h as described in online Resource 1. Then, the bacteria were harvested in TE (Tris–HCl 10 mM, EDTA 1 mM pH 7.5). The suspension was adjusted to OD600 = 3 and centrifuged at 10,000×*g* for 45 min at 4 °C. Soluble proteins were precipitated by ethanol 66 %. Samples were stored at −20 °C until use.

Protein Concentration Determination

The concentrations of protein samples were determined by the bicinchoninic acid (BCA) assay (Thermoscientific, Villebon-sur-Yvette, France). Samples were diluted in 20 mM potassium phosphate buffer (pH 7.2) supplemented with 1 mM $MgCl_2$ (PPB). A standard curve was developed using a series of Bovine Serum Albumin (BSA) standards in the 25 to 2,000 µg/ml range. PPB was used as a negative control. The absorbance of each sample was measured at 562 nm and plotted on the standard curve versus the concentration of BSA. To determine the relative protein amount in Tris–EDTA fractions, an equal amount of each sample was submitted to Western blotting analysis using the adequate antisera. The immunoblots were developed with Clarity ^{TM}ECL Western reagents. Protein levels were quantified by densitometric analysis of proteins bands performed as indicated in the Biorad ChemiDocTM XRS + imager documents system and expressed relative to the same protein in the whole bacterial sample.

Nitrocefin Hydrolysis Assay

To detect the activity of ß-lactamase, a fresh culture of F38011 strain was adjusted to 1.5×10^9 bacteria/ml and either extracted with TE as indicated above or lysed by cetyl trimethylammonium bromide (CTAB). The obtained fractions were diluted in 20 mM potassium phosphate buffer (pH 7.2) supplemented with 1 mM $MgCl_2$ (PPB) and nitrocefin was added extemporaneously at a final concentration of 50 µg/ml [16]. Hydrolysis was followed by monitoring absorbance at 490 nm with an Infinite M200 spectrophotometry microplate reader (Tecan) over 60 min. Experiment was performed in triplicate. The protein concentration in each fraction was measured as described above and allowed the determination of the specific activity of the enzyme.

Results

Prevalence of Cj1169c in *Campylobacter* Species

To study the Cj1169c production in *Campylobacter* species by Western blotting, a rabbit antiserum specific to Cj1169c was prepared after cloning of the coding gene into an expression vector. Protein production and purification, together with antiserum preparation, are described in Online Resource 2 and 3. The prevalence of Cj1169c was evaluated in 12 strains of C. *jejuni*, 3 strains of *C. lari*, and 4 of *C. coli*. Culture conditions and strains used in this study are described in Online Resource 1 and 4, respectively. Protein production was analyzed by Western blotting and compared to the previous study about Omp50 prevalence [9]. Using the Cj1169c specific antibodies, a signal at 8 kDa corresponding to Cj1169c was detected in all *C. jejuni* and *C. lari* strains but not in the *C. coli* strains. The protein signal was not detected in *C. jejuni* LD1 and LD3 in which the gene encoding for Cj1169c is lacking. Conversely, the two proteins were detected in the *C. jejuni* LD2 and in the *C. coli* LD4 recombinant strains. Data are summarized in Table 1.

Table 1 Cj1169c and Omp50 prevalence in *Campylobacter* strains

Strains	Omp50	Cj1169c
C. jejuni		
F38011	+	+
LD1	–	–
LD2	+	+
81176	+	+
LD3	–	–
85H	+	+
79AH	+	+
NCTC1168	+	+
85AF	+	+
87AF	+	+
85X	+	+
85S	+	+
C. lari		
85AB	+	+
96C15	+	+
96C22	+	+
C. coli		
85 N	–	–
85 W	–	–
79 K	–	–
87AC	–	–
87AGC	–	–
LD4	+	+

Western blotting analysis of *Campylobacter* strains *C. jejuni*, *C. lari*, and *C. coli* performed using Cj1169c specific antibodies (this study) and with Omp50 specific antibodies (adapted from Dedieu et al. [9].)

Regulation of Cj1169c Expression

Production of Cj1169c Protein Depends on Growth Phase

In order to evaluate the protein production in the function of growth conditions, *C. jejuni* F38011 strain was grown in MH broth during 12, 24, 36, 48, 60, and 72 h at 42 °C. As shown in Fig. 1, a signal with high intensity was obtained after 12 h and 24 h of growth followed by a decrease of production. According to the growth curve, this increase corresponds to the exponential growth phase. During the stationary phase, the protein was no longer detectable, suggesting a strong degradation and/or an inhibition of synthesis. Similar results were obtained with bacteria grown on agar plates, such as MH agar and COS (data not shown).

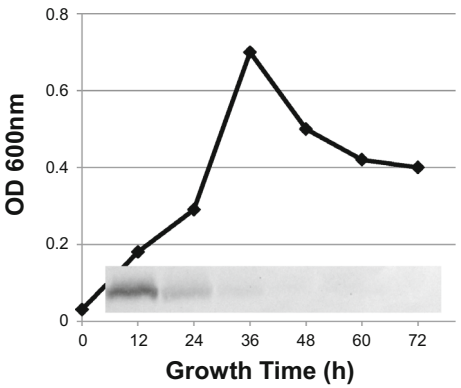

Fig. 1 Cj1169c evolution of production in function of time in MH broth. Graph represents the growth curve of *C. jejuni* strain F38011 grown during 12, 24, 36, 48, 60, and 72 h in MH broth medium. Western blotting was performed with the rabbit antiserum directed against Cj1169c

Effect of Temperature on Protein Production

To characterize the temperature influence on Cj1169c production, *C. jejuni* F38011 strain was grown at 32, 37, and 42 °C during 24 h. We observed that the increasing the growth temperature from 32 to 42 °C caused a gradual increase in the Cj1169c production. The optimal temperature for the protein production was 42 °C (Fig. 2, panel a).

In order to determine whether Cj1169c production was also dependent on temperature in different strains, 11 strains of C. *jejuni*, 3 strains of *C. lari*, and 4 strains of *C. coli* used as negative controls were subjected to the same experiment (Fig. 2, panel a, and data not shown). Results confirmed that Cj1169c production reached a maximum at

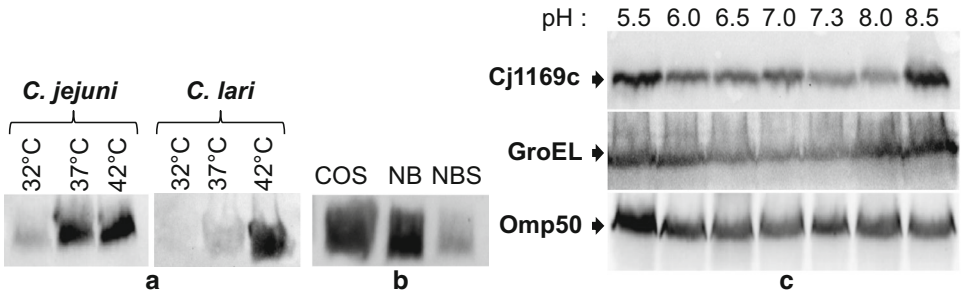

Fig. 2 Cj1169c production in different physicochemical conditions. Western blottings were performed on whole bacterial cells using Cj1169c antibodies, *panels* **a** and **b**; and Cj1169c, GroEL and Omp50 antibodies as indicated, *panel* **c**. *Campylobacter* strains *C. jejuni* and *C. lari* as indicated were grown at 32, 37, and 42 °C, *panel* **a**; *C. jejuni* strain F38011 was grown at 42 °C on GC, NB, (low osmolarity); and NBS, (high osmolarity), *panel* **b**; *C. jejuni* strain F38011 was grown at 42 °C on COS-BW at pH 5.5; 6.0; 6.5; 7.0; 7.3; 8.0 and 8.5, *panel* **c**

higher temperature independently of the strain studied. In addition, thermoregulation was also observed in *C. lari*, showing that Cj1169c regulation is conserved among species.

Effect of Medium pH and Osmolality

In order to assess the pH influence on protein production, *C. jejuni* F38011 strain was grown at 42 °C in MH medium buffered at pH 5.5, 6, 6.5, 7, 7.3, 8, and 8.5 during 24 h.

The results from Coomassie blue staining showed that the protein patterns were highly affected by the pH of the medium (data not shown). In addition, Fig. 2 panel c shows that Cj1169c production was considerably affected by the pH of growth medium. The Cj1169c production at pH 5.5 and pH 8.5 was higher while a weak signal was observed at all other pH tested. These results indicated that the Cj1169c production was pH dependent. Similarly, we observed that Cj1169c production is higher in low osmolarity than in high osmolarity (see Fig. 2, panel b).

Biochemical Analysis of Cj1169c, Effect of Thiol Reducing Agents

Sequence analysis of Cj1169c showed that the protein contained 2 cysteines at the 1st and 41st position of the putative mature sequence. Furthermore, the putative localization of this protein in the periplasm, an oxidant compartment, makes it susceptible to establish intra- or inter-chain disulfide bonds with itself or with other periplasmic partners.

To determine the existence of Cj1169c disulfide bond, we treated the bacterial samples with or without the reducing agents β-mercaptoethanol (BME) 0.01 % and dithiothreitol (DTT) 60 mM. As shown in Fig. 3 panel a, the protein signal was significantly affected by the absence of these agents. When treated with DTT and BME, a signal corresponding to Cj1169c was observed at about 8 kDa. In

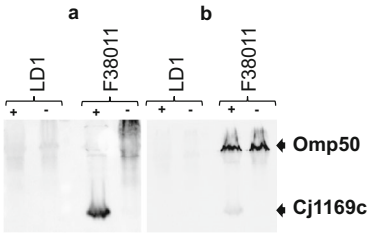

Fig. 3 Effect of thiols reducing agents on Cj1169c and Omp50 conformation in F38011 strain. Western blotting analysis of whole bacterial lysates were solubilized in the presence and absence of DTT and BME in wt and LD1 strains. Immuno-detection was performed with the rabbit antiserum directed against Cj1169c (*panels* **a** and **b**) and after extensive washing and overnight blocking, it was re-developed with Omp50 specific antibodies (*panel* **b**)

their absence, the signal at 8 kDa was not observed and a smear at high molecular weight was detected (Fig. 3, panel a). The smear was not observed in the lane corresponding to the LD1 sample (Fig. 3, panel a). Conversely the Omp50 apparent migration was not modified by these agents in accordance with the sequence of the protein (GeneBank accession number CAE46532.1) which does not contain cysteine residue in its mature form (Fig. 3 panel b). In order to eliminate the possibility that the protein can engage disulfide bridges during cell breakage and/or protein solubilization the same experiment was repeated in the presence of the alkylation agent iodoacetamide 5 (IAM) in order to block-free cysteine residues. The protein profile was identical to the one presented in Fig. 3 panel a, after IAM incubation (data not shown). Taken together, these data supported the hypothesis that Cj1169c can interact with protein(s) by disulfide bonds inside the bacterial periplasm and that the resulting complex have a high molecular weight.

Subcellular Localization of Cj1169c

An hydrophilicity plot of Cj1169c (not shown) (http://web.expasy.org/protscale/) revealed that the protein is mainly

hydrophilic with a small hydrophobic domain. Its N-terminal region contains a short sequence with polar basic amino acids residues followed by a longer stretch of hydrophobic amino acids residues and followed by polar amino acids residues. Such characteristics design a typical signal sequence. In addition, according to Parkhill et al. [17], Cj1169c is predicted to be localized in the periplasmic compartment of *Campylobacter*.

The method previously described [3] was first used to determine the subcellular localization of the protein. This method first includes a treatment of bacteria with EDTA, in order to improve membrane disruption by sonication. Then, sonication and ultra-centrifugation allow isolation of soluble versus membrane (pellet) fractions.

Surprisingly, the signal corresponding to Cj1169c was only detected in the Tris–EDTA extract. This might suggest that the protein was weakly associated to the *Campylobacter* envelope. While observing the sample after Coomassie blue staining, it clearly appears that this fraction contained a large amount of proteins of different sizes. Thus, it could result from the extraction of many soluble proteins, and at this stage we could not exclude that it was the result of envelope disruption. In order to better analyze the content of this fraction, we first performed Western blotting with the outer membrane Omp50 and the cytoplasmic GroEL-specific antibodies. As shown in Fig. 4, neither the membranous nor the cytoplasmic proteins were detected in the fraction suggesting that this fraction mainly contained periplasmic proteins of *Campylobacter*.

In order to ascertain this hypothesis, we used the β-lactamase as a periplamic marker. *Campylobacter* strains naturally produced a β-lactamase that allows resistance to penicillin and cephalosporin [21]. The ß-lactamase activity can be observed by monitoring the hydrolysis of nitrocefin, a chromogenic substrate.

Nitrocefin was added to the TE soluble fraction and the corresponding pellet, and to the whole bacterial pellet, which was previously treated with CTAB to disrupt *Campylobacter* membranes as a control (Fig. 4, panel a). We found that about 60 % of β-lactamase activity was recovered in the TE fraction. Only 1–2 % were present the TE pellet. Hence, the majority of the β-lactamase activity was found in the TE supernatant demonstrating that this fraction contains the soluble periplasmic proteins of *Campylobacter*. To confirm the protein localization, the reference method of isolation of the periplasmic fraction of Gram-negative bacteria using chloroform was implemented [1]. It was followed by SDS-PAGE analysis and Western blotting using Cj1169c-specific antibodies. A signal at 8 kDa was observed in the periplasmic fraction. Contaminations of fractions were also checked (Fig. 4, panel b). Taken together, these data demonstrated that Cj1169c is a periplasmic protein of *Campylobacter*.

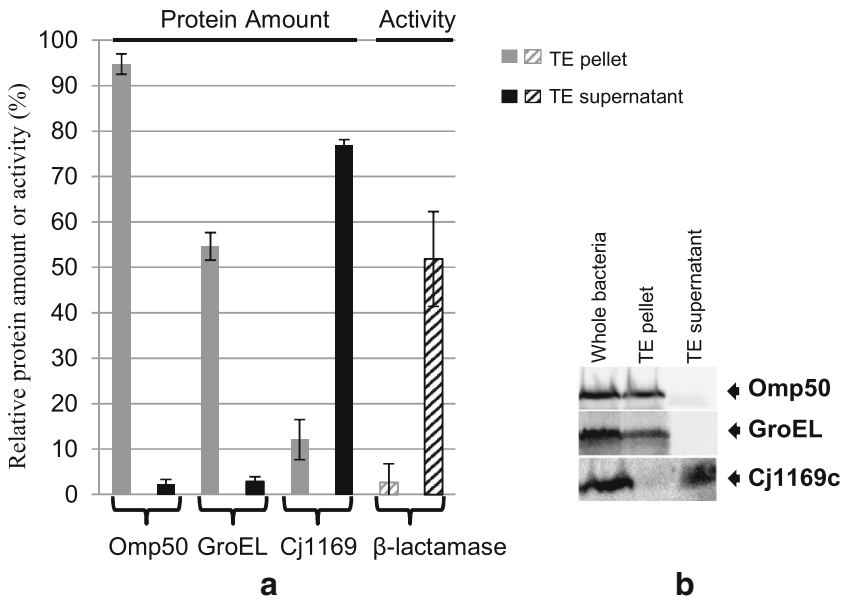

Fig. 4 Omp50, GroEL, Cj1169c, and β-lactamase localization in Tris–EDTA fraction. Relative protein amount of Omp50, GroEL, and Cj1169c were determined based on the total protein amount present in the whole bacteria. Relative β-lactamase activity was calculated based on the activity of the β-lactamase found in the whole bacteria F38011 treated with CTAB. The results presented correspond to the mean of 3 independent experiments, *panel* **a**; Western blotting of a representative experiment is presented in *panel* **b**, Omp50, GroEL, and Cj1169c indicate the position of the bands detected by the respective antiserum

Discussion

The Cj1169c open reading frame first described by the pioneer work of Parkhill and co-workers [17] was annotated as a putative periplasmic protein in the genome of *Campylobacter* NCTC11168. In a comparative study of *C. jejuni*, *C. lari*, and *C. coli* strains, Cj1169c was found associated to the Cj1170c ORF encoding the outer membrane protein Omp50 [9]. Transcriptomic studies showed that both ORF were regulated by environmental conditions, including temperature [19] and iron limitation [5, 6, 14], and were expressed in vivo [11, 20]. Taken together, these data suggested that these two ORF might have important functions in *Campylobacter* adaptation. In addition, Omp50 was first characterized as a porin [4] and was also found to exhibit phosphotyrosine kinase activity [7]. However, no function was attributed to the Cj1169c while both ORF were suspected to be in operonic structure even if a second transcription initiation sequence was found between the two ORFs in the detailed analysis of *Campylobacter* RNA sequences [10].

In the present study, we produced a recombinant Cj1169c protein in *Escherichia coli* and used the purified protein to generate a specific antiserum. Thanks to this tool we analyzed the level of the protein expression in *Campylobacter*.

Cj1169c encodes a 75 amino acid residues polypeptide with a predicted signal sequence of 17 residues of amino-acids. The mature protein was found in all the tested strains of *C. jejuni* and *C. lari*, demonstrating its innate expression in *Campylobacter*. Cj1169c was absent in the *C. coli* species tested in our study that apparently could be in disagreement with the data of Lefébure et al. [15]. However, in their report, authors analyzed a larger number of strains and showed that Cj1169c ORF belongs to core genes of *C. jejuni* that are dispensable in *C. coli* which is in accordance with the present data.

We demonstrated here that Cj1169c expression depends on many factors that refer to environmental conditions, such as temperature, pH, and osmolarity. Noticeably, we observed a highest production at 42 °C, which is the body temperature of chicken. The higher prevalence of *C. jejuni* in chicken, and the characteristics of Cj1169c, may suggest that this protein plays an important role during host colonization.

The size of the polypeptide identified, classes Cj1169c among mini-proteins. Mini-proteins represent about 10 percent of total ORFs in bacteria and only 30 % possesses functional or structural description [22]. In addition, their functions identified are mainly as cytoplasmic regulators [22]. Thus, it appeared essential to precisely localize the protein into the bacteria. We have used different methods and demonstrated the periplasmic localization of Cj1169c. Beyond the accordance of our data with the prediction [17], this open several hypotheses on the putative role of this protein. Taking into account that its gene is associated with Cj1170c and that Omp50 is an outer membrane protein that exhibits pore forming activity, one may hypothesize that Cj1169c interacts with the periplasmic side of Omp50. These putative interactions may modify the pore function and/or the kinase function of Omp50. Another possibility would be that Cj1169c is secreted through the Omp50 pore; however, we did not detect any Cj1169c in the culture supernatant, even when the intracellular amount of the protein was strongly decreased at the end of the exponential growth phase (Fig. 1). The presence of two cysteine residues in the sequence of the mature form of the protein suggested that they could be involved in disulfide bridges. The SDS-PAGE analyses carried out in various solubilization conditions showed that the protein was not detectable by the antiserum in non-reducing conditions. However, while a smear in the high molecular weight part of the gel was detected (Fig. 3), we did not identified a single-protein species involved in this interaction.

Further studies are needed to determine the role and the interactions of the Cj1169c-encoded protein.

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