N-Acetyl-D-Glucosamine Induces the Expression of Multidrug Exporter Genes, *mdtEF*, via Catabolite Activation in *Escherichia coli*†

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Received 1 March 2006/Accepted 25 May 2006

The expression of MdtEF, a multidrug exporter in *Escherichia coli*, is positively controlled through multiple signaling pathways, but little is known about signals that induce MdtEF expression. In this study, we investigated compounds that induce the expression of the *mdtEF* genes and found that out of 20 drug exporter genes in *E. coli*, the expression of *mdtEF* is greatly induced by *N*-acetyl-D-glucosamine (GlcNAc). The induction of *mdtEF* by GlcNAc is not mediated by the *evgSA*, *ydeO*, *gadX*, and *rpoS* signaling pathways that have been known to regulate *mdtEF* expression. On the other hand, deletion of the *nagE* gene, encoding the phosphotransferase (PTS) system for GlcNAc, prevented induction by GlcNAc. The induction of *mdtEF* by GlcNAc was also greatly inhibited by the addition of cyclic AMP (cAMP) and completely abolished upon deletion of the cAMP receptor protein gene (*crp*). Other PTS sugars, glucose and D-glucosamine, also induced *mdtEF* gene expression. These results suggest that *mdtEF* expression is stimulated through catabolite control.

The emergence of bacterial multidrug resistance has become an increasing problem in the treatment of infectious diseases. One of the important mechanisms underlying antibiotic resistance involves the extrusion of compounds by an efflux pump. The most intriguing mechanisms of drug extrusion are those that include a wide variety of structurally unrelated compounds as substrates for multidrug exporters. Multidrug exporters are found in a variety of bacterial species. Among them, AcrAB in *Escherichia coli* and MexAB in *Pseudomonas aeruginosa* play major roles in bacterial intrinsic tolerance against various drugs and toxic compounds (17, 26).

Previous comprehensive expression cloning studies revealed that the *E. coli* chromosome encodes 20 intrinsic drug exporter systems that actually contribute to drug resistance when they are overexpressed (19). Among them, MdtEF (formerly named YhiUV) confers hyperresistance against various antibiotics, antitumor reagents, pigments, and detergents, although it is hardly expressed under normal laboratory conditions (18, 19, 28). In previous studies, it was found that expression of the *mdtEF* genes is up-regulated by the bacterial two-component signal transduction system EvgSA (20) and the AraC/XylS acid resistance regulator YdeO (14), while their expression is repressed by a histone-like protein, H-NS (21). The multidrug resistance caused by overexpression of EvgA and YdeO is due to the induction of *mdtEF* gene expression (6, 7, 14, 20). On

the other hand, the multidrug resistance caused by H-NS is partly dependent on *mdtEF* (21). We also found that *mdtEF* expression is highly dependent on the growth phase; that is, the expression is induced at the stationary phase (10).

Although *mdtEF* expression is controlled in multiple ways through these signaling pathways, little is known about the signals that induce *mdtEF* expression. Recently, we reported that indole greatly induces expression of the *mdtEF* genes. Indole signaling for *mdtEF* expression is independent of the EvgSA pathway but mediated by GadX, which is also an AraC/XylS regulator that cooperates with YdeO (5). These results suggest that there are at least two independent pathways for the induction of *mdtEF* expression, that is, the indole-driven GadX pathway and the two-component system, EvgSA.

In this study, we screened for chemical compounds that induce expression of the *mdtEF* genes and found that among the drug exporter genes, only the expression of *mdtEF* genes is greatly induced by *N*-acetyl-D-glucosamine (GlcNAc). This is due to “catabolite induction,” which is the third pathway for the induction of *mdtEF* expression and which is dependent on neither the EvgSA nor the GadX pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table S1 in the supplemental material. Chromosomal DNA of *Escherichia coli* MG1655 (8) was used as the PCR template. KAM3 (16), a derivative of TG1 (29) that lacks *acrB*, and gene deletion mutants of KAM3 were used to search for compounds that induce *mdtEF* transcription and to verify the regulatory mechanism. The construction of gene deletion mutants was performed using the plasmid pKO3 (12). The whole sequence of the targeted gene was replaced with a 33-bp linker sequence that retained the start and stop codons in the gene deletion mutants. Gene deletion was verified by PCR using the primers listed in Table S2 in the supplemental material. The plasmids for Crp expression were constructed as follows. The *crp* gene was cloned into the pQE30 vector (QIAGEN) by using the primers listed in Table S2 in the supplemental material. The resulting plasmid was named pQE30crp (Table

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† Supplemental material for this article may be found at <http://jb.asm.org/>.

TABLE 1. Induction of promoter activity and drug exporter gene transcripts attributed to GlcNAc^a

Gene	Induction (fold change ^b)	
	Reporter gene assay	Quantitative PCR
<i>acrA</i>	0.4	0.6
<i>acrD</i>	0.4	0.6
<i>acrE</i>	0.5	0.9
<i>bcr</i>	0.7	0.7
<i>cusB</i>	0.2	0.5
<i>emrA</i>	0.6	0.7
<i>emrD</i>	0.8	0.8
<i>emrE</i>	0.8	1.5
<i>emrK</i>	0.7	0.2
<i>fsr</i>	0.5	0.2
<i>macA</i>	0.4	0.4
<i>mdfA</i>	0.6	0.3
<i>mdtA</i>	0.7	1.4
<i>mdtE</i>	9.7	48.0
<i>yceE</i>	0.7	1.9
<i>yceL</i>	0.6	0.3
<i>YidY</i>	0.5	1.1
<i>ydgF</i>	1.9	0.1
<i>ydhE</i>	1.3	0.4
<i>YjiO</i>	0.5	1.8

^a Averaged results of three independent experiments are shown.

^b Values indicate changes in transcript levels and promoter activities of cells cultured with 10 mM GlcNAc compared to those of the host strain cultured without GlcNAc.

S1). *E. coli* cells were grown in Luria-Bertani (LB) medium and supplemented with appropriate antibiotics, when necessary, under aerobic conditions at 37°C. Construction of a *ptsG* mutant derived from KAM3 was performed by P1 transduction with strain IT1168 (W3110 *ptsG*::Tn5) (9), kindly provided by Teppei Morita and Hiroji Aiba.

Reporter gene assay. The reporter fusion plasmids (3) listed in Table S1 in the supplemental material were used to determine the effects of several compounds on the transcription of *mdtEF* and the effects of GlcNAc on the other genes of the 19 multidrug exporter systems of *E. coli*. Each bacterial strain was grown at 37°C in LB medium containing 10 µg/ml chloramphenicol until the optical density (OD) at 600 nm reached 0.3, and then compounds were added to the LB medium, and cultures were incubated for 2 hours. β-Galactosidase activity in cell

lysates was monitored by the method of Miller, using *o*-nitrophenyl-β-galactopyranoside (ONPG) as a substrate (15).

Transcriptional analysis of drug exporter genes of *E. coli* responding to GlcNAc. KAM3 cells were grown in LB medium until the optical density at 600 nm reached 0.3, and then the cells were incubated for 2 hours with or without 10 mM GlcNAc. Purification of total RNA from a KAM3 lysate was performed using an SV total-RNA isolation system (Promega). Total RNA (1.0 µg) was used as a template and converted to cDNA. The conditions for obtaining cDNA were those recommended by PE Applied Biosystems. The specific primer pairs used are listed in Table S3 in the supplemental material. Real-time reverse transcription (RT)-PCR was performed with each specific primer pair, using SYBR green PCR master mix (PE Applied Biosystems). Normalization of the cDNA loaded for each PCR was performed using the *rnsA* 16S rRNA gene. The real-time PCR was performed with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems).

Rhodamine 6G resistance assay. The rhodamine 6G resistance assay was carried out by the method described previously (5). Each bacterial strain was grown at 37°C in LB medium with or without 10 mM GlcNAc. After the cells were incubated with rhodamine 6G for 30 min, survival was calculated as the number of CFU ml⁻¹ remaining after rhodamine 6G treatment divided by that before drug treatment (expressed as a percentage). Each experiment was repeated at least three times.

Purification of Crp. The His₆-Crp fusion protein was overexpressed from the plasmid pQE30crp in the *E. coli* M15 strain. Cells were grown at 37°C in LB broth until the absorbance at 600 nm reached 0.6. Then, IPTG (isopropyl-β-D-thiogalactopyranoside) was added (final concentration, 1 mM), and the culture was allowed to grow for an additional 2 hours at 37°C. The His₆-Crp fusion protein was purified from *E. coli* crude soluble lysates using Ni affinity resin (Amersham Pharmacia Biotech).

DNase I footprinting analysis. DNase I footprinting analysis was performed by a nonradiochemical capillary electrophoresis method, as previously described (5, 33). The 6-carboxyfluorescein (6-FAM) DNA fragments were synthesized with the PCR primers listed in Table S2 in the supplemental material. The forward primer (*gadE*-F-6-FAM) was labeled with 6-FAM prior to PCR. A 215-bp 6-FAM-labeled fragment (from positions -1 to -215 relative to the *gadE* start codon), including a *gadE* promoter and the region upstream of it (0.45 pmol), was mixed with purified His₆-Crp (0 to 35 pmol) in a 50-µl reaction mixture consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA-K, 50 mM KCl, 1 mM dithiothreitol, and 100 µM cyclic AMP (cAMP). After incubation for 10 min at 37°C, 0.6 units of DNase I (Promega) was added. After incubation for 60 s at room temperature, samples were purified for GeneScan sequencing analysis (PE Applied Biosystems). The prepared samples were electrophoresed using an ABI PRISM 310 sequencer/genetic analyzer equipped with an ABI PRISM 310 GeneScan, and fragment sizes were determined with GeneScan analysis software.

FIG. 1. GlcNAc-induced rhodamine 6G resistance of *E. coli* KAM3 and KAM3Δ*mdtEF* cells. The survival rates of the cells exposed to 100 mg/ml rhodamine 6G were measured after induction of *mdtEF* in the absence or presence of GlcNAc as described in Materials and Methods. Solid and open bars indicate noninduced and GlcNAc-induced cells, respectively.

FIG. 2. Effects of deletion of the *evgSA*, *ydeO*, *gadX*, *rpoS*, *nagE*, *nagC*, and *crp* genes on GlcNAc-induced expression of the *mdtEF* genes. *E. coli* KAM3 and derivative strains were grown in LB medium with (open bars) or without (solid bars) 10 mM GlcNAc. (A) β -Galactosidase activity of the *mdtEF* promoter-fused reporter enzyme. (B) Amounts of *mdtE* mRNA. The ordinate indicates the *mdtE* mRNA amounts relative to the amount of *rsA* 16S rRNA. The data correspond to mean values for three independent experiments. Error bars correspond to standard deviations.

RESULTS AND DISCUSSION

Screening of compounds that induce *mdtEF* gene expression. In the first screening, we searched for compounds that induce *mdtEF* gene expression by means of a promoter-fused reporter gene assay. The *mdtEF* genes are located downstream of the *gadE* gene, which encodes an acid regulator. Since RT-PCR analysis revealed that *mdtEF* were cotranscribed with *gadE* (see Fig. S1 in the supplemental material), the *gadE* promoter-fused *lacZ* carried on the plasmid pNNGadE was

used as a reporter for *mdtEF* expression in the first screening. The exporter gene expression was measured as the reporter enzyme activity in cells cultured in the presence of MdtEF substrates (antibiotics, pigments, antiseptics, and antitumor reagents) and the other compounds that are not substrates of MdtEF (bile acids, oxidation-reduction reagents, quinones, and the components of peptidoglycan). GlcNAc, which was chosen from among these compounds as a component of peptidoglycan, caused the highest induction (10-fold increase),

FIG. 3. Induction of *mdtEF* expression by glucose (Glc), GlcNAc, and glucosamine (GlcN) and inhibition by cAMP. The *E. coli* KAM3 strain was grown in LB medium with 10 mM concentrations of the indicated sugars in the presence (open bars) or absence (solid bars) of 10 mM cAMP. (A) β -Galactosidase activity of the *mdtEF* promoter-fused reporter enzyme. (B) Amounts of *mdtE* mRNA. The relative amount of mRNA indicates the expression ratio of *mdtE* to *rnsA* of the 16S rRNA. The data correspond to mean values of three independent experiments. Error bars correspond to the standard deviation.

while phosphomycin and novobiocin, which are substrates for MdtEF, caused moderate induction (two- and threefold increases, respectively) (see Fig. S2 in the supplemental material). Although the expression of *mdtEF* was induced on cessation of cell growth (our unpublished observation), the induction was not due to growth cessation, because these compounds at the indicated concentrations hardly affected cell growth ($OD_{600} = 2.4$). Other compounds either did not induce the *mdtEF* promoter activity at all or reduced it only slightly.

GlcNAc induces *mdtEF* gene expression without a significant effect on the expression of other drug exporter genes. *E. coli* cells carry genes for 20 drug exporter systems, including *mdtEF* (19). Using quantitative RT-PCR analysis and a reporter gene assay, we then investigated whether GlcNAc in-

duces drug exporter genes other than *mdtEF* (Table 1). The transcriptional levels of the *mdtE* and *mdtF* genes in *E. coli* cells cultured with 2.2 mg/ml (10 mM) GlcNAc were about 48- and 33-fold higher, respectively, than those cultured without GlcNAc. The expression of none of the other exporter genes was affected at all by GlcNAc.

MdtEF induction by GlcNAc actually confers drug resistance. When the expression of *mdtEF* is induced by GlcNAc, GlcNAc-induced drug resistance is expected. Since rhodamine 6G is one of the substrates for MdtEF, we wanted to determine whether GlcNAc-treated *E. coli* cells actually confer rhodamine 6G resistance (19). *E. coli* KAM3, which is hypersusceptible to various drugs including rhodamine 6G, showed a very low survival rate (0.22%) when cells were treated with 100

FIG. 4. DNase I footprinting analysis of CRP binding to the *gadE* promoter region. (A) A DNA fragment (0.45 pmol), including the *gadE* promoter region, was labeled with 6-FAM at the 5' end, incubated with His₆-CRP, and then subjected to a DNase I footprinting assay using an ABI PRISM 310 Gene Scan. The fluorescence intensities (ordinate) of 6-FAM-labeled DNA fragments are plotted against the sequence lengths of the fragments (abscissa). Protein-binding sites are shown in squares. (B) The DNA sequence around the CRP binding site of *gadE*. Protein-binding sites are enclosed by a square. The sequence homologous to the CRP binding motif is indicated.

mg/liter rhodamine 6G (Fig. 1). In contrast, after being cultured in 10 mM GlcNAc, *E. coli* KAM3 cells showed a very high survival rate (31.7%). On the other hand, the *mdtEF* gene deletion mutant KAM3 Δ *mdtEF*, even when cultured with GlcNAc, showed a drastically lower survival rate (8.39%) than that of KAM3 cells cultured with rhodamine 6G (Fig. 1). The residual increase in rhodamine 6G resistance from 0.35% to 8.39% by GlcNAc in the Δ *mdtEF* mutant is probably due to the induction of unknown resistance factors other than drug efflux.

GlcNAc-induced *mdtEF* gene expression is mediated by neither the EvgSA-YdeO nor the RpoS-GadX signaling pathway. It is known that expression of the *mdtEF* genes is controlled through two independent signaling pathways. One pathway is mediated by EvgSA (a two-component regulatory system)-

YdeO (an AraC/XylS regulator for *E. coli* acid resistance) (13, 20). The other pathway is mediated by the RpoS (a stress-inducible sigma factor)-GadX (an RpoS-dependent acid inducible regulator) cascade (5, 14, 22). The latter pathway is also stimulated by indole. In order to determine which pathway mediates the induction by GlcNAc, the effect of the deletion of these genes on *mdtEF* induction by GlcNAc was investigated. Deletion of these genes itself did not affect *mdtEF* expression in the absence of GlcNAc, because these signaling systems are silent under normal laboratory conditions. As shown in Fig. 2, the deletion of neither *evgSA*, *ydeO*, *gadX*, nor *rpoS* prevented the induction of *mdtEF* by GlcNAc. Although the absolute promoter activity and mRNA levels differed in some of these mutants, the degree of induction by GlcNAc was not significantly changed by these mutations. This observation indicates

that the GlcNAc signal is mediated by neither the EvgSA-YdeO nor the RpoS-GadX pathway.

***mdtEF* induction by GlcNAc requires the PTS system for GlcNAc.** GlcNAc is taken up into cells through the GlcNAc-specific phosphotransferase system (PTS), including NagE (23, 25, 30). In a *nagE* gene deletion mutant, *mdtEF* gene expression induced by GlcNAc was largely prevented (Fig. 2). On the other hand, the deletion of the *nagC* gene, which encodes a GlcNAc-6-P-binding transcriptional regulator, did not affect the induction by GlcNAc (Fig. 2). Although the possibility that the intracellular GlcNAc-6-P regulates *mdtEF* gene expression through unknown GlcNAc-6-P-responding regulators is not excluded, these findings strongly suggest that the PTS system for GlcNAc confers the induction of *mdtEF*. The reason why *mdtEF* genes were still slightly induced even in the *nagE* mutant might be that GlcNAc can be transported by another PTS system, ManXYZ, but with low efficiency (30).

***mdtEF* gene expression is also induced by D-glucose and D-glucosamine, and the induction is abolished by cAMP.** GlcNAc is known to cause catabolite control via the cAMP receptor protein (CRP) (24). Thus, the effect of *crp* gene deletion on *mdtEF* expression was examined. In the *crp* deletion mutant, the expression level of *mdtEF* was significantly higher than that in the parent KAM3 strain without GlcNAc (Fig. 2). The expression level without GlcNAc in the Δ *crp* mutant is almost the same as that of the wild type induced with GlcNAc. In addition, the *mdtEF* expression level was no longer affected by GlcNAc at all in the Δ *crp* mutant (Fig. 2), indicating that the induction of *mdtEF* gene expression by GlcNAc is mediated by a CRP-dependent catabolite control system. In this case, CRP seems to act as a repressor of the *mdtEF* genes.

If the induction by GlcNAc is a CRP-dependent catabolite control, the effect should be prevented by cAMP. When cAMP was added to *E. coli* cells, the induction of *mdtEF* by GlcNAc was almost cancelled (Fig. 3). In addition, *mdtEF* gene expression was also induced by other PTS carbohydrates, i.e., D-glucose (Glc) and D-glucosamine (GlcN) (Fig. 3). Glc-treated *E. coli* cells showed high-level induction of the *mdtEF* genes, while GlcN moderately induced *mdtEF* gene expression. The addition of cAMP also cancelled the *mdtEF* induction by Glc and GlcN.

CRP-cAMP directly binds to the *mdtEF* promoter region. The binding of the CRP-cAMP complex to the upstream region of the *gadE-mdtEF* operon was examined by means of DNase I footprinting analysis. A 215-bp DNA fragment that included the putative promoter region (13) was used as a 6-FAM-labeled probe, and CRP binding sites were determined (Fig. 4). CRP actually bound to the sequence between positions -54 and -14 from the transcriptional start (Fig. 4). This 41-bp region contains the putative promoter of *gadE* (13). It has been reported that the CRP recognition motif is TGTGA(N₆ or N₈)TCACA (2, 32). There is no completely consistent motif in this region, but a homologous sequence, aaTGA(N₆)TgACA, exists between positions -27 and -12 from the transcriptional start. Our results suggest that the CRP-cAMP complex represses expression of the *gadE-mdtEF* operon by directly binding to its promoter region and that the catabolite induction is due to the derepression caused by a decrease in the intracellular concentration of cAMP.

FIG. 5. Effect of the *nagE* deletion and the *ptsG* mutation on the induction of *mdtEF* by sugars. (A) Effect of the *nagE* deletion on the *gadE-mdtEF* promoter activity. β -Galactosidase activities of the *mdtEF* promoter-fused reporter enzyme in the wild-type strain (WT) and in the Δ *nagE* mutant were observed in the presence of sugars as indicated. (B) The effects of the *ptsG* mutation on the expression level of *mdtE* were investigated by quantitative RT-PCR. The wild-type strain (WT) and the *ptsG* mutant (*ptsG::Tn5*) were grown with or without sugars as indicated, and the expression ratio of *mdtEF* to *rnsA* of the 16S rRNA was determined by quantitative RT-PCR analysis.

Effects of *nagE* deletion and *ptsG* mutation on the gene expression levels of *mdtE* induced by Glc, GlcNAc, and GlcN. To investigate the roles of sugar transporters in the induction of *mdtEF* by Glc, GlcNAc, and GlcN, we investigated the effects of *nagE* deletion and *ptsG* mutation on expression levels of *mdtE*. The wild-type strain, the *nagE* mutant, and the *ptsG* mutant were grown with or without sugars, and the promoter activities of *mdtE* (Fig. 5A) and the mRNA amounts of *mdtE* (Fig. 5B) were determined. In the *nagE* mutant, induction of *mdtEF* expression by GlcNAc was not observed, while the levels induced by glucose and glucosamine were almost similar to those in the wild type. In the *ptsG* mutant, the induction of *mdtE* by GlcNAc was also similar to that in the wild type, while the induction by glucose was almost nonexistent. As for glucosamine, it is mainly transported by the phosphotransferase system for mannose (PTS^{Mann}) (1), but it is also transported by PTS^{Glc} (27). The large decrease in the level of induction by glucosamine in the *ptsG* mutant suggests that the efficiency of the catabolite control by glucosamine via PTS^{Glc} may be higher

FIG. 6. Model for the regulation of *mdtEF* expression by GlcNAc activation. The circled plus symbol indicates activation. A cAMP-CRP complex acts as a repressor for the *gadE-mdtEF* operon.

than that via PTS^{Mann} . These results indicate that the corresponding PTS systems are required for the induction of *mdtEF* by sugars.

Schematic model for *mdtEF* expression regulation by GlcNAc.

In this study, we revealed that *mdtEF* expression is induced by catabolite control by PTS sugars, including GlcNAc. Figure 6 shows a schematic model of the induction of *mdtEF* expression by GlcNAc. Since NagE does not directly stimulate adenylate cyclase (31), it must stimulate adenylate cyclase via phosphorylation of enzyme IIA for glucose (IIA^{Glc}). When GlcNAc is transported by NagE, enzyme IIB for GlcNAc (IIB^{Nag}) is dephosphorylated. Because IIA^{Nag} (C-terminal region of NagE) is homologous with IIA^{Glc} (27), IIB^{Nag} can then dephosphorylate IIA^{Glc} (11), which in turn stops the activation of adenylate cyclase, followed by a decrease in the intracellular concentration of cAMP. The decrease in cAMP concentration causes dissociation of the cAMP-CRP complex followed by the release of CRP from the *mdtEF* operator region. Because, in this case, the cAMP-CRP complex acts as a negative regulator of this operon, the release causes derepression of *mdtEF* expression. An alternative pathway

mediated by a GlcNAc-6-P-binding regulator other than NagC might be possible; however, the possibility is small because the induction by GlcNAc is strictly dependent on CRP.

Although most of the catabolite controls consist of catabolite repressions, the catabolite control of *mdtEF* comprises catabolite activation. In other words, the cAMP-CRP complex functions as a positive regulator for many genes controlled by catabolites, whereas, in the case of *mdtEF*, it is actually a negative regulator. Similar cases were reported by Gosset et al. (4), who found in their transcriptome study that some genes, including *aceE*, *gusB*, and *ptsG*, are activated through catabolite control.

Although the *mdtEF* genes are transcribed with the *gadE* gene, which encodes a glutamate decarboxylase-dependent acid resistance regulator, overexpression or deletion of the *mdtEF* genes did not affect *E. coli* acid resistance at all (data not shown). Because catabolite control generally has a relationship with energy metabolism (4), we suspect that MdtEF may be an exporter for waste metabolites, which have not been identified.

In this study, we revealed that the expression of the *E. coli* multidrug exporter MdtEF is induced through catabolite control. Our current results not only are useful for understanding the regulation mechanisms of bacterial intrinsic multidrug exporter genes but also are highly suggestive as to the physiological roles of the intrinsic drug exporter MdtEF.

ACKNOWLEDGMENTS

We thank Teppei Morita and Hiroji Aiba for the strain IT1168, George M. Church for the plasmid pKO3, Ronald W. Davis for the plasmid pNN387, and Tomofusa Tsuchiya for the *E. coli* KAM3 strain.

H. Hirakawa was supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Zoonosis Control Project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

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