**In vitro genotoxicity analyses of colibactin-producing E. coli isolated from a Japanese colorectal cancer patient**

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**ABSTRACT** — Colibactin is a polyketide-peptide genotoxin produced by enteric bacteria such as *E. coli*, and is considered to contribute to the development of colorectal cancer. We previously isolated *E. coli* strains from Japanese colorectal cancer patients, and in the present study we investigated the genotoxic potency of the colibactin-producing (*clb*+) *E. coli* strains that carry the polyketide synthases “*pks*” gene cluster (*pks*+) and an isogenic *clb*- mutant in which the colibactin-producing ability is impaired. Measurement of phosphorylated histone H2AX indicated that DNA double strand breaks were induced in mammalian CHO AA8 cells infected with the *clb*- E. coli strains. Induction of DNA damage response (SOS response) by crude extract of the *clb*+ strains was 1.7 times higher than that of the *clb*- E. coli in an umu assay with a *Salmonella typhimurium* TA1535/pSK1002 tester strain. Micronucleus test with CHO AA8 cells revealed that infection with the *clb*+ strains induced genotoxicity, i.e., the frequencies of micronucleated cells infected with *clb*+ strain were 4-6 times higher than with the *clb*- strain. Since the intestinal flora are affected by dietary habits that are strongly associated with ethnicity, these data may contribute to both risk evaluation and prevention of colorectal cancer in the Japanese population.

**Key words:** Colibactin, Genotoxicity, DNA damage, Mammalian cells

**INTRODUCTION**

Colibactin is a genotoxic small molecule implicated in colorectal cancer pathogenesis, and produced by *Enterobacteriaceae*, including certain *Escherichia coli* strains harbored in the human gut. These microorganisms possess a 54-kilobase genomic island encoding polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), and PKS-NRPS hybrid megasynthetases (Nougayrède *et al.*, 2006). Colibactin induces DNA double-strand breaks (DSBs) and interstrand cross-links in human cell lines and in animals to generate gene mutations (Nougayrède *et al.*, 2006). The colibactin-producing (*clb*) *E. coli* stimulate growth of colon tumors under conditions of host inflammation (Cuevas-Ramos *et al.*, 2010; Vizcaino and Crawford, 2015), and are found with increased frequency in inflammatory bowel disease, familial adenomatous polyposis, and colorectal cancer patients. However, the chemical structure of colibactin and the molecular mechanism of its mutagenesis/carcinogenesis have not been fully revealed yet.

According to the Patient Survey 2014 of Japan, the...
total number of patients with malignant neoplasm of the colon and rectum has increased remarkably, and the mortality rate from colorectal cancer has also risen (Watanabe et al., 2018). Presumably, aging and dietary changes in Japan have primarily led to these increases in colorectal cancer. However, colibactin might also be involved in the development of colorectal cancer among Japanese patients. Therefore, in our previous study we investigated *E. coli* strains isolated from 34 Japanese colorectal cancer patients, and revealed that *clb* strains produced *N*-myristoyl-d-asparagine (*N*-myr-Asn) as a by-product of colibactin biosynthesis (Hirayama et al., 2019).

Dietary and eating habits are strongly associated with culture and ethnicity, and these factors influence the intestinal environment. The present study aimed to assess the genotoxic potency of the *E. coli* strains isolated from a Japanese colorectal cancer patient. We also evaluated the induction of DSBs in cultured rodent cells infected with *E. coli* (*clb*) by measuring the phosphorylation of H2AX. Using the *umu* test, DNA damage in a *Salmonella* tester strain treated with crude extracts of the *E. coli* were evaluated. We also examined the genotoxicity/clastogenicity of the *E. coli* in rodent cells using the *in vitro* micronucleus (MN) test. The resulting data will contribute to risk evaluation and the prevention of colorectal cancer in the Japanese population.

**MATERIALS AND METHODS**

**E. coli strains and in vitro infection assay**

Isolation and gene disruption of *E. coli* strains were described in our previous report (Hirayama et al., 2019). Briefly, the #50 and #253 strains were isolated from the T1 section of surgically-resected human colorectal cancer tissue from a 79-year-old male patient. The adenocarcinoma had a diameter of 9.5 cm and was present in the ascending colon. Deletion of the *clbP* gene (encoding periplasmic peptidase) in *E. coli* #50 was carried out by homologous recombination conferred to the strain by transforming it with the Red/ET expression plasmid. The *clbP* disruption cassette was comprised of the selection marker *Kan* flanked on both sides by a 50-base pair fragment. It was introduced to *E. coli* #50 harboring the Red/ET expression plasmid to replace *clbP* with the kanamycin resistance marker *Kan*. Disruption of *clbP* was confirmed by amplifying the disrupted segment of the genomic DNA by PCR using the three primer sets. The resultant strain was designated as #50clbP. Production of *N*-myr-Asn, thought to reflect colibactin synthesis, in #50clbP decreased significantly while #50 and #253 showed a same level of *N*-myr-Asn production (Hirayama et al., 2019; Yoshikawa et al., unpublished data).

The strains were cultured overnight at 37°C in LB media, inoculated in Infection Medium (IM) (DMEM High Glc (Thermo Fisher Scientific K.K., Tokyo, Japan) + 25 mM HEPES, 5% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), and cultured until the indicated OD<sub>595</sub> at 37°C. Infection to Chinese hamster ovary (CHO) cells was carried out as described previously (Cuevas-Ramos et al., 2010). Cells were incubated in the IM containing *E. coli* at the indicated multiplicity of infection (MOI) (number of bacteria per cell at the onset of infection) after appropriate dilution of the IM culture with fresh IM for the indicated MOI. Volume of the IM culture did not exceed 10% in total IM volume during the infection. To prepare IM for MOI=1000, *E. coli* was collected from the IM culture by centrifugation at 5,000 × g for 5 min and resuspended in an appropriate volume of fresh IM. After a 4-hr infection at 37°C and 5% CO<sub>2</sub>, cells were washed four to six times and incubated until analysis in cell culture medium supplemented with 200 µg/mL gentamicin (Nacalai Tesque, Kyoto, Japan).

**Fluorescent immunostaining of γH2AX**

To detect DSBs, immunostaining of γH2AX was carried out using phospho-H2AX antibody (Ser139, 20E3, Cell Signaling Technologies Japan, K.K., Tokyo, Japan) with a BD Accuri C6 Plus flow cytometer (Becton, Dickinson and Company Japan, Tokyo, Japan) according to the manufacturer’s instructions. Briefly, CHO AA8 cells obtained from the RIKEN Cell Bank (Wako, Saitama, Japan) were cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were then seeded in plastic cell culture dishes (φ60 mm) at 4 × 10<sup>5</sup> cells/dish one day before the treatment procedure. The infection was carried out in 3 mL of IM containing the *E. coli* at exponential phase (OD<sub>595</sub>=0.5) as mentioned above. The CHO cells treated for 4 hr were harvested and fixed with 4% formaldehyde for 15 min at room temperature. Permeabilization of the cells was performed with incubation in ice-cold methanol for 30 min. The cells were stained with the antibody in PBS containing 0.5% bovine serum albumin for 1 hr at room temperature, and then resuspended in PBS and analyzed on the flow cytometer.

**umu test**

Extracts of *E. coli* were prepared with BugBuster protein extraction reagent (Novagen, Merck Millipore Co., Japan, Tokyo, Japan) according to the manufacturer’s instructions. *E. coli* cells were harvested from 10 mL of overnight culture in LB media by centrifugation at
5,000 × g for 5 min. The bacterial pellet was resuspended in 1 mL of the BugBuster reagent and incubated for 20 min on a rotating mixer at room temperature. Insoluble cell debris was removed by centrifugation at 16,000 × g for 20 min at 4°C. The umu assay was conducted as previously reported (Oda et al., 1985; Oda, 2016). Briefly, the umu tester strain was grown overnight at 37°C in LB broth containing ampicillin (50 µg/mL), and then the cultures were diluted 50-fold with TGA medium and incubated at 37°C for 3 hr until the cell density reached OD600 = 0.3. Aliquots (1 mL) of the TGA culture and 20 µL of the BugBuster extracts from the E. coli strains were incubated with agitation for 3 hr at 37°C. As a solvent and positive controls, 20 µL of BugBuster solution and 10 µL of 4-nitroquinoline 1-oxide (4-NQO) (Nacalai Tesque) were used, respectively. Induction of the umuC gene in S. typhimurium TA1535/pSK1002 was determined by measuring cellular β-galactosidase activity.

Micronucleus test

The MN test was carried out as previously described (Kawanishi et al., 2013). Briefly, CHO cells (4 × 10⁵ cells/dish) were seeded in φ60 mm plastic cell culture dishes one day before the infection procedure. The infection was carried out with 3 mL of IM containing the E. coli as described above. After being treated with bacteria, the CHO AA8 cells were cultured for a further 20 hr, and then trypsinized, counted, and centrifuged. Growth inhibition was calculated using the following formula:

\[
\text{Growth rate} = \frac{\text{the number of treated cells}}{\text{the number of non-treated cells}}
\]

The cells were then resuspended in 0.075 M KCl and incubated for 5 min, before being fixed four times in methanol:glacial acetic acid (3:1) and washed with methanol containing 1% acetic acid. Finally, the cells were resuspended in methanol containing 1% acetic acid. The cell solution was dropped onto slides, and the nuclei were stained by mounting the cells with 40 µg/mL acridine orange (Nacalai Tesque) solution and immediately observed by fluorescence microscopy using blue excitation. The number of cells with MN was recorded based on the observation of 1,000 interphase cells.

RESULTS AND DISCUSSION

Induction of DNA double strand breaks (DSBs) in mammalian cells

First, we assessed the S139-phosphorylation of histone H2AX (γH2AX), a sensitive marker of DSBs (Rogakou et al., 1998; Motoyama et al., 2018). Chinese hamster ovary CHO AA8 cells were infected with #50 (wild-type, clb+), the isogenic clbP mutant #50clbP- (clb-), and #253 (wild-type, clb+) E. coli in exponential growth phase (OD595=0.5). The peptidase ClbP cleaves precolibactin, and liberates colibactin and N-myristoylated Asn (Bian et al., 2013; Brotherton and Balskus, 2013; Vizcaino et al., 2014; Pérez-Berezo et al., 2017). Therefore, in the present study, this mutant was used as a negative control strain since the mutant lacks the ability to produce colibactin (i.e., clb-). After 4 hr of infection, the CHO cells were collected and the phosphorylation was quantified using FCM. Increased intensities of γH2AX were found in the cells exposed to clb+ E. coli compared with the isogenic clbP mutant (Fig. 1). The fluorescent inten-

![Fig. 1. Flow cytometric analysis of γH2AX induction in CHO AA8 cells infected with E. coli. Distribution of γH2AX signals in the cells after 4 hr-infection with indicated E. coli strains at exponential phase (OD595=0.5). In the graph, MOI 0 represents the solvent control (treatment with IM only) and wt represents wild-type.](image-url)
sities increased in the cells infected with #50 and #253 both at MOI=500 and 1,000. Infections of #50 and #253 at MOI=500-1,000 increased the mean intensities 2.2-3.3 and 1.7-2.1-fold as compared with MOI=0, respectively. Strain #50 showed slightly stronger potency of DSB induction than #253. No increased intensity was observed in the cells treated with #50clbP. Cuevas-Ramos et al. (2010) reported the γH2AX induction in CHO cells at 16 hr or longer after infection with the E. coli strain DH10B hosting a BAC bearing the pks island. However, we did not observe clb+ dependent induction at 16 hr after infection, since #50clbP also induced γH2AX after 16 hr (data not shown). The precolibactin, that #50clbP also produces, might have weak DNA damaging potency (Vizcaino and Crawford, 2015). Although we did not identify the inducer(s) of DSBs by #50clbP, we concluded that no clb+ dependent DSB induction occurred at 16 hr after infection.

Induction of DNA damage response in bacteria by the E. coli extracts

Next, we used the umu test to examine the extracts of clb+ E. coli for induction of DNA damage response in Salmonella typhimurium TA1535/pSK1002. In order to prepare the extracts, overnight culture of the E. coli strains was mixed with BugBuster reagent that disrupts the cell walls and liberates the cytosol. Insoluble cell debris was removed by centrifugation, and the DNA-damaging potency of the supernatant was measured with the umu tester strain of which LacZ reporter activity represents the degree of DNA damage response (SOS response) induction. Increased SOS response was found in the cells exposed to the extracts of clb+ E. coli compared with those of the isogenic clbP mutant (Table 1). The relative degrees of SOS-induction in the extracts of #50 and #253 were 1.7 times and 1.6 times higher than that of #50clbP, respectively. A positive control agent, 4-NQO (0.3 μg/mL), induced a 3.2-fold response. Therefore, the results indicate that the extracts of clb+ E. coli have moderate or weak potency for DNA damage-induction. Bossuet-Greif et al. (2016) reported that the clbS gene in pks encodes a resistance protein blocking the genotoxicity of colibactin, and that the SOS response is activated in a colibactin-producing clbS mutant strain, probably due to colibactin-autotoxicity. Presumably, in the present study the presence...
of ClbS protein in the extracts could attenuate their DNA-damaging potency.

**Induction of micronucleus formation in mammalian cells**

Since DNA damage including DSBs is known to induce MN (Hayashi, 2016), the MN-inducing activity of the E. coli strains was examined using the Chinese hamster ovary cell line CHO AA8. We also determined the induction levels for different growth phases of E. coli. As shown in Fig. 2, infection with E. coli increased the number of micronucleated cells in a dose-dependent manner, but the degree of induction varied among the strains and growth phases of E. coli. The background frequency of micronucleated cells was 1.6-1.7%, and the frequencies rose to 20% and 12% by infections at MOI=1,000 with #50 and #253 cultured until OD_595=0.5 (exponential phase), respectively. However, the frequency was 3.2% by infection at MOI=1,000 with the negative control strain #50clbP (OD_595=0.5). Infection of E. coli at the early stationary phase (cultured till OD_595=1.75) and late stationary phase (cultured for 16 hr) inhibited the growth of the CHO cells more than 50% at MOI=1,000 (data not shown), and therefore we conducted MN tests with the highest dose as MOI=100. The frequencies were 7.8% and 4.3% by the infections at MOI=100 with #50 and #253 at the early stationary phase, respectively. In contrast, the frequency was 1.7% by infection at MOI=100 with #50clbP of early stationary phase. The MN-inducing activity of #50 was higher than that of #253. Although DNA damaging potency of #50 was slightly stronger in the γH2AX and SOS induction than #253 (Fig. 1 and Table 1), the reason for the difference in the MN induction is unclear.

Inductions of MN in the bacteria at the late stationary phase were not significant (i.e., the frequencies were 1.70 ± 0.44%, 2.73 ± 0.25% and 3.37 ± 1.33% in CHO cells infected with #50clbP, #50 and #253 at MOI=100, respectively). Homburg et al. (2007) reported that colibactin gene cluster expression peaks in the late logarithmic or early stationary phase of the E. coli growth curve, which is concordant with the fluctuations of MN-induction potency through the growth phases of the bacteria in the present study. Incidentally, E. coli #50 shows nearly 30-times greater activity of ClbP, namely a production of N-myristoyl-D-Asn that indicates the degree of Clb^+ than Nissle 1917, which is a representative strain of clb^+ (Hirayama et al., 2019). We also evaluated Nissle 1917; at exponential phase (OD_595=0.5), it was more potent than the #50 in MN induction, i.e., the frequencies were 1.92 ± 0.12% and 14.1 ± 0.56% in CHO cells infected at MOI=0 and 100, respectively. The mechanisms of secretion and delivery of colibactin are still unclear (Thakur et al., 2019). Therefore, the extent of genotoxicity in the infected cells could not be completely explained by the degree of clb^+ in E. coli.

In conclusion, we found that clb^+ E. coli strains isolated from a Japanese colorectal cancer patient displayed genotoxicity in in vitro assay systems. Our data indicate that the E. coli strains induce DNA strand breaks directly or indirectly, leading to MN formation in mammalian cells. We also found that cytosol of the E. coli induces DNA damage in a Salmonella strain. Intestinal flora is known to depend on dietary habits that are strongly associated with culture and ethnicity. Therefore, the present study may contribute to both risk evaluation and the prevention of colorectal cancer in the Japanese population.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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