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# Survival of *Escherichia coli* harboring nucleic acid-hydrolyzing 3D8 scFv during RNA virus infection

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ABSTRACT

Previously, *Escherichia coli* harboring the codon-optimized *3D8scFv* gene (*E. coli* 3D8scFv) was developed as a feed additive for use in preventing norovirus infection. Here, we evaluated whether the *3D8scFv* gene affects the colonization of *E coli* when *E. coli* 3D8scFv passes through the mouse gastrointestinal tract. To determine the colonization ability of *E. coli* 3D8scFv, *E. coli* cells with or without the *3D8scFv* gene were fed to mice. Total DNA was extracted from the animals' stools, stomach, small intestine and colon. All samples were amplified using *3D8scFv* gene-specific primer sets. *E. coli* 3D8scFv begins to be excreted 1 h after feeding and that all *E. coli* 3D8scFv cells were excreted between 12 and 24 h after the last feeding of the cells. The previously measured gastrointestinal transit time of the mice was between 8 h and 22 h. The results of this study therefore show that *E. coli* 3D8scFv cannot colonize the gastrointestinal tracts of mice. In addition, if the purified 3D8 scFv protein is used as a feed additive, any associated *E. coli* 3D8scFv bacteria will not colonize the gastrointestinal tracts of the livestock. Thus, this feed additive meets the safety assessment criteria for the commercial use of bacteria.

## 1. Introduction

Humans have been making efforts to combat novel diseases caused by rapidly evolving viruses. Recently, avian influenza A (H7N9) viruses from East Asia have spread to the Americas and Europe, and human infection with avian influenza A virus has been reported in China (Artois et al., 2017). In addition, Ebola virus and beta coronavirus, which cause Ebola hemorrhagic fever and Middle East respiratory syndrome (MERS), respectively, are known to be fatal to humans. These viruses have spread to Africa, the Middle East, and Asia and have raised the fear of new viruses (Mackay and Arden, 2015; Martinez et al., 2015). Notably, some viruses isolated from animals have been reported to induce fatal diseases in humans due to the mutation and evolution of the animal viruses (Parrish et al., 2008). Avian influenza A viruses from birds, Ebola viruses from bats and rodents, and beta coronaviruses from bats and camels have been transmitted to humans (Parrish et al., 2008). All of these viruses are single-stranded RNA viruses. Therefore, the prevention of virus infections in livestock is essential for eliminating the potential risk to human life caused by cross-species viral transmission between humans and livestock.

Nucleic acid antibodies are significantly increased in patients with autoimmune diseases (Marion et al., 1997). The antigens recognized by most of these anti-nucleic acid antibodies have been shown to be single- or double-stranded DNA or RNA molecules that contain no specific common target sequences; dinucleotides such as dTdT and dGdC have also been reported as antigens of these antibodies (Kim et al., 2006).

The nucleic acid antibody 3D8 was isolated from the spleen cells of mice with autoimmune syndrome (Kwon et al., 2002). The VH and VL of the mAb 3D8 IgG are connected by a flexible linker, $(G_4S)_3$, to create 3D8 scFv (Kim et al., 2006). This recombinant single-chain variable fragment can penetrate cells via caveolae-mediated endocytosis. Once endocytosed, 3D8 scFv remains in the cytoplasm without further trafficking into endosomes, lysosomes, the endoplasmic reticulum, the Golgi, or the nucleus. In the nucleus, 3D8 scFV induces the hydrolysis of both DNA and RNA (Jang et al., 2009). 3D8 scFv has also been reported to exert an antiviral effect against classical swine fever virus (CSFV), which is an RNA virus that primarily infects pigs (Jun et al., 2010). A strain of *Escherichia coli* harboring nucleic acid-hydrolyzing codon-optimized 3D8 scFv (*E. coli* 3D8scFv) was developed for use as a feed additive to prevent norovirus infection, and *Lactobacillus paracasei*

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harboring nucleic acid-hydrolyzing 3D8 scFv was developed for use as a preventive probiotic. The use of these strains was shown to prevent the induction of apoptosis during murine norovirus (MNV) infection and to decrease mRNA expression for the viral capsid protein VP1 (Hoang et al., 2015).

Genetically modified microorganisms must be evaluated for safety before use as commercial food or as food additives for humans or livestock, because genetically modified microorganisms may produce toxic materials and allergens and it can also destruct the microbial ecosystem. The test of the colonization of genetically modified microorganisms is also the main issue in human safety, because the colonization can affect microbiome of human or livestock microbiome negatively. For assessing the risk of genetically modified organisms, the concept of substantial equivalence has been adopted by the Organization for Economic Co-operation and Development (OECD) and is used globally. According to this concept, *E. coli* 3D8scFv should not have any effect, except an anti-viral effect, on the animals to which it is administered. For example, oral ingestion of *E. coli* 3D8scFv must not induce changes in weight or mortality in comparison to oral ingestion of control *E. coli*.

Here, we evaluated whether *E. coli* 3D8scFv colonizes the gastrointestinal tracts of mice. To comply with the substantial equivalence concept that is required for safety assessment when purified 3D8 scFv protein is used as a feed additive, we also tested whether ingested *E. coli* 3D8scFv are excreted rapidly.

## 2. Material and methods

### 2.1. Bacterial strains

*E. coli* BL21 (DE3) (*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*) harboring a codon-optimized *3D8scFv* gene in pET42 was used to express 3D8 scFv in *E. coli*. This *E. coli* strain was used both as an animal feed and in the protein purification reported in this study. The pET42 vector was cleaved by the DNA restriction enzymes *NdeI* and *XhoI* to remove the GST tag. *E. coli* BL21 (DE3) was used in animal feed as a control.

### 2.2. Cell growth curves and colony-forming unit (CFU) assays

*E. coli* BL21 (DE3) harboring either pET42-*3D8scFv* or empty pET-42 lacking the GST coding region was streaked on M9 plates containing 50 μg/ml of kanamycin and incubated at 37 °C. Three colonies from each plate were separately cultured in M9 liquid medium containing 0.4% glycerol at 37 °C for 12 h. After this time, 3 ml of the cell culture was used to inoculate 100 ml of fresh M9 liquid medium, and the starting $OD_{600}$ of the culture was measured (0 min). Aliquots of the cell cultures were collected at various time intervals (1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d), and the $OD_{600}$ of the culture and the number of CFUs present were measured. To calculate the number of CFUs present in cultures of *E. coli* BL21 (DE3) containing either pET42-*3D8scFv* or pET-42, the collected cells were washed three times with saline and spread on M9 plates containing 50 μg/ml of kanamycin. The plates were incubated for 18 h at 37 °C, and the number of colonies per ml of cell suspension plated was counted.

### 2.3. Protein turnover assay

pET42-*3D8scFv* was transformed into *E. coli* BL21 (DE3) cells, and the cells were incubated at 37 °C. When the $OD_{600}$ of the culture reached 0.6, 0.5 mM isopropyl-β-D-1-thiogalactoside (IPTG) was added to induce the expression of the 3D8 scFv protein, and aliquots of the cell cultures were collected 0, 3, 6, 12, 24 and 48 h after induction. The collected cells were lysed by sonication in lysis buffer (50 mM $Na_2H_2PO_4$, 500 mM NaCl, 10 mM β-mercaptoethanol, pH 8.0) and centrifuged at 12,000 rpm for 10 min at 4 °C. The proteins present in the cell pellets were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene difluoride) membranes. The blots were probed with a polyclonal anti-His tag antibody (#2365, Cell Signaling Technology, Danvers, MA, USA) and with a monoclonal anti-OmpF antibody (orb13626, Biorbyt, San Francisco, CA, USA).

### 2.4. Animals and diets

A total of twenty-five male specific pathogen-free (SPF) ICR mice aged 8 weeks were used. The animals were purchased from DBL Co., Ltd. (Umsung, Korea). The mice were maintained in an SPF facility under a 12 h light-dark cycle (lights on 6:00; lights off 18:00) at 22 ± 0.5 °C and 40–60% relative humidity and were allowed free access to chow (Teklad Global 18% Protein Rodent Diet (cat. #2018S), Harlan Laboratories Inc., WI, USA) and water. All experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Korea Research Institute of Bioscience and Biotechnology (approval No: KRIBB-AEC-16145).

### 2.5. Experimental design

The mice were acclimatized and fasted for 24 h. Supplemented drinking water and feed were changed daily. Mortality and individual clinical signs were also assessed daily. *E. coli* with or without the *3D8scFv* gene ($2x10^9$ cells/time) or saline was fed to individual groups of mice three times at 24-h intervals. The mice were weighed, and stools were collected at various time intervals (0 h, 1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 6 d, 15 d, 18 d, and 21 d) after the last feeding of either cells or saline. After 21 days, the animals were sacrificed, necropsies were performed, and total DNA was extracted from the stomachs, small intestines, and colons of the animals.

### 2.6. Bacterial survival study

Total DNA was extracted from the stools obtained at each time interval (1 h, 3 h, 6 h, 9 h, 12 h, 1 d, 3 d, 7 d, 15 d, 18 d, and 21 d) after the last feeding of either cells or saline. For bacterial cultures on plates, the stool from each time interval was dissolved in saline and centrifuged, and the supernatants were applied to the culture plates. Total DNA was extracted from the stomachs, small intestines, and colons of all mice sacrificed 21 days after the last feeding. The DNA samples were amplified using *3D8scFv* gene-specific primer sets and electrophoresed on 1.5% agarose gels.

### 2.7. Statistics

The $OD_{600}$ values and CFU/ml values obtained for *E. coli* 3D8scFv were normalized to the values obtained for the control (**$p < 0.001$, *$p < 0.05$ for the comparison of the control with *E. coli* 3D8scFv). The *p* values for the comparison to the control were determined using a two-tailed *t*-test. The error bars indicate the SEM; n = 3 experiments. The mean body weights of the animals in the three experimental groups (mice fed *E. coli* 3D8scFv, mice fed control *E. coli* pET42 without a GST tag, and mice that received no treatment) were normalized to that of the control group (**$p < 0.001$, *$p < 0.05$ comparing the control to *E. coli* 3D8scFv or to no treatment). The *p* values were determined via one-way ANOVA followed by Dunnett's multiple comparisons test vs. the control for each time point. The error bars indicate the SEM; n = 10 experiments for the *E. coli* 3D8scFv and control groups and n = 5 experiments for the "no treatment" group.