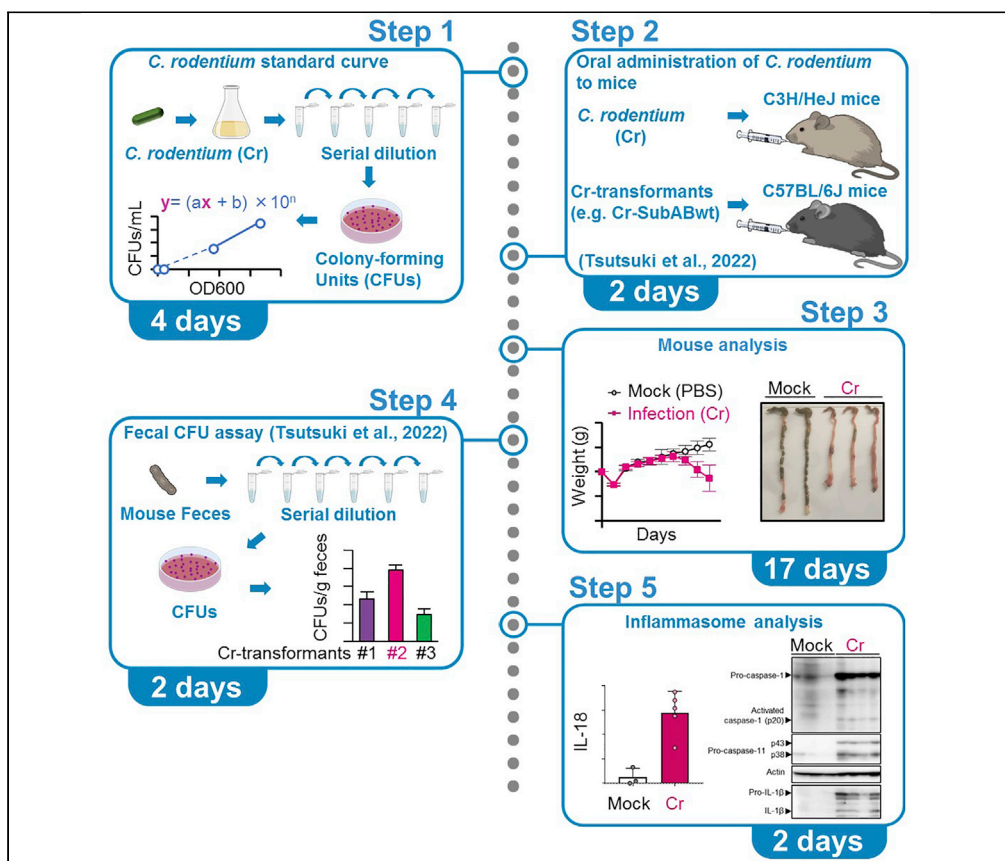


Protocol

Non-canonical inflammasome activation analysis in a mouse model of *Citrobacter rodentium* infection



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Highlights
Infection of mice by oral administration of *C. rodentium*

Analysis of daily weight changes and collection of intestinal tissues from mice

Determination of the number of viable bacteria in feces by CFU counting

Evaluation of intestinal inflammasome activation

Infection of mice with *Citrobacter rodentium* is a useful model for studying the pathogenicity of enteropathogenic and enterohemorrhagic *Escherichia coli*, pathogens that have a close association with humans. Here we provide a protocol detailing the approaches for non-canonical inflammasome analysis in a mouse model of *C. rodentium* infection, including preparation of bacteria, oral administration of bacteria to mice, counting colony-forming units to quantify bacterial colonization, and analysis of expression and activation of inflammasome-related factors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Non-canonical inflammasome activation analysis in a mouse model of *Citrobacter rodentium* infectionHiroyasu Tsutsuki,^{1,3,4,*} Tianli Zhang,¹ Kinnosuke Yahiro,² Touya Toyomoto,¹ and Tomohiro Sawa^{1,*}¹Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan²Department of Microbiology and Infection Control Sciences, Kyoto Pharmaceutical University, 5 Misasagi-Nakauchi-cho, Yamashina-ku, Kyoto 607-8414, Japan³Technical contact⁴Lead contact*Correspondence: tsutsuki@kumamoto-u.ac.jp (H.T.), sawat@kumamoto-u.ac.jp (T.S.)
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SUMMARY

Infection of mice with *Citrobacter rodentium* is a useful model for studying the pathogenicity of enteropathogenic and enterohemorrhagic *Escherichia coli*, pathogens that have a close association with humans. Here, we provide a protocol detailing the approaches for non-canonical inflammasome analysis in a mouse model of *C. rodentium* infection, including preparation of bacteria, oral administration of bacteria to mice, counting colony-forming units to quantify bacterial colonization, and analysis of expression and activation of inflammasome-related factors.

For complete details on the use and execution of this protocol, please refer to Tsutsuki et al. (2022).

BEFORE YOU BEGIN

Institutional permissions

Acquire appropriate licenses

This protocol describes experimental methods that use laboratory animals. Appropriate licenses for animal experiments from institutional and regional ethical review boards should be obtained before conducting experiments.

Ethics statement

All animal experiment procedures were approved by the Kumamoto University Ethics Review Committee for Animal Experimentation and were performed with an effort to minimize the number of animals used and their suffering.

Preparation of the standard growth curve of *Citrobacter rodentium*

⌚ Timing: 4 days

1. Streak *Citrobacter rodentium* (*C. rodentium*, Cr) ATCC 51459 bacteria on a MacConkey agar plate (Nissui Pharmaceutical Co., Ltd), and incubate the bacteria at 37°C overnight (12–18 h). Pick a single colony, inoculate it in 2 mL Luria-Bertani (LB) broth (Becton, Dickinson and Company), and culture it overnight at 37°C with shaking at 150 rpm. Dilute the culture 1:100 with 50 mL of fresh medium and sub-culture it for 0, 2, 4, and 6 h.
2. Monitor the growth of *C. rodentium* by measuring the optical density at 600 nm (OD₆₀₀) via a spectrophotometer at the different culture time points of 0, 2, 4, and 6 h.



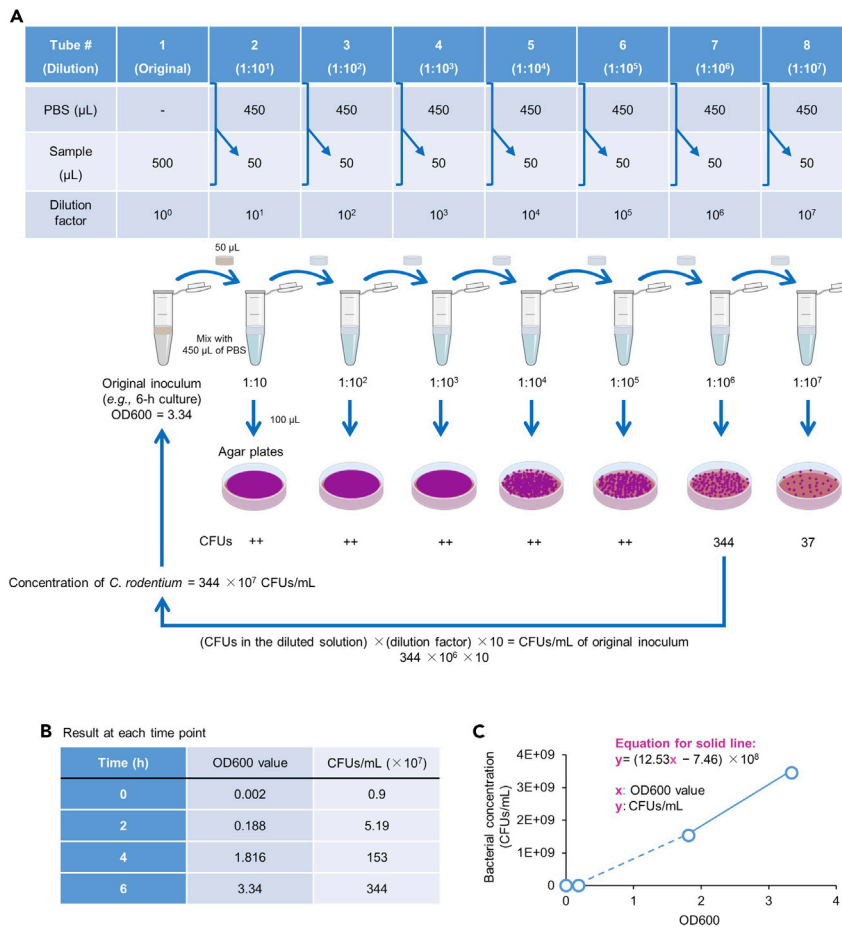


Figure 1. Preparation of the standard growth curve of *C. rodentium* for CFU measurements

(A) Serial dilution procedure for CFU assay.

(B) Correlation between turbidity (OD600) and bacterial concentration (CFUs/mL) at each culture time determined by using the CFU assay in (A).

(C) Preparation of the standard growth curve and calculation of the standard equation.

- Determine the bacterial concentration of *C. rodentium* by counting the number of colony-forming units (CFUs) on the LB agar plate. At each time point, repeat the following steps:
 - Prepare eight sterile 1.5-mL tubes and label them with dilution factors (10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , and 10^7) including the original inoculum (as 10^0) (Figure 1A, #1–8 tubes).
 - Add 450 μ L of sterile PBS into tubes #2–#8.
 - Transfer 0.5–1 mL of the original inoculum to tube #1. After mixing well by vortexing and pipetting, transfer 50 μ L of the original inoculum to tube #2 and mix by vortexing.
 - In the same manner, dilute at a 1:10 ratio from tube #3 (10^2) to #8 (10^7), one by one, after mixing by vortexing and then pipetting.
 - Spread 100 μ L of each dilution onto LB agar plates by using sterile bacterial cell spreaders.
 - Culture the plates overnight in the incubator at 37°C.
 - Count the CFUs of the last two countable plates (Figure 1A). The ratio of the two adjacent CFUs should be approximately (approx.) 10.

Note: Diluted samples should preferably be spread on agar plates immediately after preparation.

- Draw the standard growth curve of *C. rodentium*.

- a. Determine the bacterial concentration (CFUs/mL) of the original inoculum (tube #1), according to the dilution ratio (Figure 1A).
- b. Repeat the same calculation at each culture time point: 0, 2, 4, 6 h (Figure 1B).
- c. Plot the values of the CFUs/mL (y-axis) and OD600 (x-axis) and draw the standard growth curve (Figure 1C).

Note: The example in Figure 1C shows the linear curves drawn between 2 to 4 h (dotted line) and 4–6 h (solid line) of culture, respectively. Generate an equation “ $y = (ax + b) \times 10^n$ ” by using the linear curve between each time point. We showed the example of equation only for the solid line (See Figure 1C).

5. Use the equation to determine the *C. rodentium* bacterial CFUs per administration unit (CFUs/mouse) with the inoculum that was prepared for infection (See [step-by-step method details](#)).

△ CRITICAL: Tubes #2–6 should be diluted 1:10 because otherwise the bacterial concentration would be too high to accurately count colonies on the LB agar plate. Count the number of properly formed colonies on the plates, and calculate the concentration of bacteria.

Preparation of the *C. rodentium* inoculum for mouse infection

⌚ **Timing:** 2 days

6. Culture *C. rodentium* bacteria overnight as in step 1. Dilute the culture 1:100 with 50 mL of fresh medium and sub-culture it for 4–6 h.

Note: Bacteria cultured overnight can also be used in infection experiments if diluted to fit the equation for calculating CFU/mL against OD600.

7. Calculate the CFUs/mL of bacteria.
 - a. Measure the OD600 value of the *C. rodentium* culture inoculum and calculate the CFUs/mL according to the standard growth curve as determined above.
 - b. Harvest enough *C. rodentium* bacteria at $6,000 \times g$ for 10 min at 4°C.
 - c. Discard the supernatant and resuspend the pellet with sterile PBS at the proper concentration.

Note: If the infective dose is 2.5×10^8 CFUs/100 μ L of PBS per mouse, resuspend the bacteria at 2.5×10^9 CFUs/mL of PBS. If the infective dose is 5×10^9 CFUs/100 μ L of PBS per mouse, resuspend the bacteria at 5×10^{10} CFUs/mL of PBS. If 10 mice are required to be infected, prepare enough *C. rodentium* for at least for 15 mice to prevent potential loss. For example, if the OD600 of *C. rodentium* culture medium is 3.34, the concentration will be 3.44×10^9 CFUs/mL (Figure 1). If 10 mice are required to be infected at the dose of 2.5×10^8 CFUs/100 μ L per mouse, take 1.0901 mL of the *C. rodentium* culture to spin down at $10,000 \times g$ for 2 min, then wash the sample with PBS twice and resuspend the bacteria with 1.5 mL of PBS to dilute the *C. rodentium* bacteria 1.376-fold. The 1.5 mL of *C. rodentium* inoculum will be at the concentration of 2.5×10^9 CFUs/mL and enough for 10 mice (100 μ L/mouse). See [problem 1](#) in [troubleshooting](#).

△ CRITICAL: C3H/HeJ mice are relatively susceptible to *C. rodentium* infection, whereas C57BL/6J mice are relatively resistant to *C. rodentium* infection (Vallance et al., 2003). Because the susceptibility to *C. rodentium* varies depending on the mouse strain, consider the mouse strain, gender, age, and dose of bacteria to be administered (CFUs per mouse) in terms of the research purpose.

For example, when we investigated the pathogenicity of the virulence factor (VF) subtilase cytotoxin (SubAB), we used the less susceptible C57BL/6J mice (female, 8 weeks old) and administered SubAB-expressing *C. rodentium* (Cr-SubABwt) at a high dose of 5×10^9 CFUs (Tsutsuki et al., 2022). In another report, a dose of 2×10^9 CFUs per mouse was used when investigating a host immune response with specific knockout mice bred in a C57BL/6 background (Guo et al., 2015).

When developing a therapeutic drug and evaluating its therapeutic effect, or when using a *C. rodentium* strain with reduced pathogenicity, use C3H/HeJ mice and administer a relatively low dose of bacteria (for example, approx. 10^8 CFUs per mouse). Previous studies have suggested that gender was not associated with the susceptibility against *C. rodentium* in C3H/HeJ mice (Tea-tero et al., 2011).

Preparation of *C. rodentium* carrying a plasmid expressing VF and antibiotic-resistant gene (optional)

⌚ Timing: 7 days

8. Preparation of an expression vector containing the VF of interest.

Note: This section introduces preparation of *C. rodentium* (Cr) transformants for infection of mice as reported in a previous publication in which we used C57BL/6J mice and Cr-SubABwt (wild-type SubAB), Cr-SubABmt (catalytically inactive mutant SubAB), and Cr-empty (vector control Cr transformants) (Tsutsuki et al., 2022). Here, we provide a protocol that uses SubAB as a representative VF.

Note: Clone the gene of interest according to the general procedure of the *E. coli* cloning system. In several instances, plasmid DNA vectors for protein expression in *E. coli* may be used for *C. rodentium*.

Note: Antibiotic resistance genes such as ampicillin resistance gene (amp^R) are useful as selection markers on antibiotic-containing agar plates in fecal CFU assay for mice that received Cr transformants carrying the drug-resistant plasmids.

- a. Amplify the *subAB* gene by using PCR and subclone into a pCR2.1-TOPO vector (Thermo Fisher Scientific). Digest the *subAB*-inserted TOPO plasmid and expression plasmid containing amp^R (e.g., pET23b, Novagen) with *Bam*H I and *Xho* I restriction enzymes (Morinaga et al., 2007).
- b. Ligate the digested gene (e.g., *subAB*) to the digested expression plasmid with the Rapid DNA Ligation Kit (Merck) according to the manufacturer's instruction (<https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/366/473/11635379001bul.pdf>).
- c. Transform the ligated plasmid into the laboratory strain of *E. coli* such as DH5 α (Takara Bio Inc.) and select a clone by using ampicillin resistance. Culture the selected clone and purify the plasmid by using a plasmid purification kit (e.g., QIAprep Spin Miniprep Kit, QIAGEN).

Note: The expression vector containing the VF of interest can be stored at -30°C for more than 1 month.

9. Preparation of *C. rodentium* electrocompetent cells.

- a. Inoculate a single colony of *C. rodentium* in 2 mL LB broth, and culture it overnight (12–18 h) at 37°C with shaking at 150 rpm. Dilute the culture 1:100 with 50–100 mL of fresh medium and sub-culture it until the OD₆₀₀ is 0.5–1.0 (approximately 2.5–3 h).

- b. Keep the sub-culture on ice for 10 min. Harvest bacteria by centrifugation at 6,000 × g for 5 min at 4°C and carefully pour off and discard the supernatant.
- c. Gently resuspend the bacteria in 40 mL of sterile cold 10% glycerol. Centrifuge at 6,000 × g for 5 min at 4°C; carefully pour off and discard the supernatant (Wash 1).
- d. Repeat the washing step three more times for a total of four times (Wash 2–4).
- e. Resuspend the bacteria in 1 mL of sterile cold 10% glycerol. Dispense 100 μL of the final re-suspension into sterile 1.5-mL tubes.

Note: The electrocompetent cells can be stored at –80°C for more than 1 month.

10. Transformation of the plasmid into competent *C. rodentium* cells and plating onto an LB agar plate containing ampicillin.
 - a. Add the plasmid vector containing VF or empty vector (5 μg each) to 100 μL of the electrocompetent cells, and transfer the cells into an ice-cold 1-mm gap electroporation cuvette (Nepa Gene). Perform electroporation at 1,800 V, 25 μF, and 600 Ω by using a Bio-Rad MicroPulser (Bio-Rad).
 - b. Resuspend the cells in 1 mL of LB medium, followed by plating 100 μL of the resuspension onto LB agar containing 100 μg/mL ampicillin.
 - c. After overnight incubation of the plate at 37°C, isolate ampicillin-resistant colonies. Confirm expression of the target gene by using Western blotting with antibody or antiserum.
11. Prepare the standard growth curve and equation for the Cr transformants as in the protocol shown above (Figure 1).
12. Culture the Cr transformants and treat the culture with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37°C before infecting mice. After washing the sample by centrifugation at 6,000 × g for 10 min at 4°C (15-mL tube) or 10,000 × g for 2 min at 4°C (1.5-mL tube) and resuspension with PBS, prepare the inoculum by calculating the CFU of the Cr transformants according to “preparation of the *C. rodentium* inoculum for mouse infection” as shown above.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-actin (working dilution 1:1000)	Sigma-Aldrich	Cat#A2066; RRID: AB_476693
Mouse monoclonal anti-caspase-1 p20 (clone Casper-1) (working dilution 1:1000)	AdipoGen	Cat#AG-20B-0042-C100; RRID: AB_2755041
Goat polyclonal anti-IL-1β (working dilution 1:1000)	R&D Systems	Cat#AF-401-NA; RRID: AB_416684
Mouse monoclonal anti-BiP/Grp78 (working dilution 1:3000)	BD Biosciences	Cat#610978; RRID: AB_398291
Rat monoclonal anti-caspase-11 (9D11) (working dilution 1:1000)	BioLegend	Cat#647201; RRID: AB_1937283
Anti-mouse IgG Veriblot for IP secondary antibody (HRP) ab131368 (working dilution 1:3000)	Abcam	Cat#Ab131368; RRID: AB_2895114
HRP-conjugated anti-goat secondary antibody (working dilution 1:3000)	R&D Systems	Cat#HAF017; RRID: AB_562588
Anti-mouse IgG, HRP-linked antibody (working dilution 1:3000)	Cell Signaling Technology	Cat#7076; RRID: AB_330924
Anti-rabbit IgG, HRP-linked antibody (working dilution 1:3000)	Cell Signaling Technology	Cat#7074; RRID: AB_2099233
HRP-conjugated anti-rat secondary antibody (working dilution 1:5000)	GE Healthcare	Cat#NA9350; RRID: AB_772192
Bacterial and virus strains		
<i>Citrobacter rodentium</i> DBS100	ATCC	Cat# 51459
<i>Escherichia coli</i> DH5α	Takara Bio Inc.	Cat#9057
Chemicals, peptides, and recombinant proteins		
Immobilon-P PVDF membrane	Merck Millipore	Cat#IPVH00010
Protease inhibitor cocktail set I, Animal-derived free (for general use) (x100)	FUJIFILM Wako Pure Chemical Corp.	Cat#161-26023

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>N,N,N',N'</i> -Tetramethylethylenediamine (TEMED) -	FUJIFILM Wako Pure Chemical Corp.	Cat#205-06313
Ammonium Persulfate (APS)	FUJIFILM Wako Pure Chemical Corp.	Cat#016-08021
30% Acrylamide and bis-acrylamide solution, 29:1	Bio-Rad	Cat#1610156
Immobilon Western Chemiluminescent HRP Substrate	Merck Millipore	Cat#WBKLS0500
MacConkey agar	Nissui Pharmaceutical Co., Ltd	Cat#05037
Difco LB Broth, Miller (Luria-Bertani)	Becton, Dickinson and Company	Cat#244620
Powdered Agar "Nissui"	Nissui Pharmaceutical Co., Ltd	Cat#05835
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat#14190144
Sodium Chloride (NaCl)	Nacalai Tesque	Cat#31320-05
Tween 20	Sigma-Aldrich	Cat#P1379
IGEPAL CA-630	MP Biomedicals, LLC	Cat#198596
Tris(hydroxymethyl)aminomethane	Nacalai Tesque	Cat#35434-21
Skim milk for immunoassay	Nacalai Tesque	Cat#31149-75
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750
Sodium Dodecyl Sulfate (SDS)	Nacalai Tesque	Cat#30400-85
Glycerol	Nacalai Tesque	Cat#17018-83
Bromophenol Blue (BPB)	FUJIFILM Wako Pure Chemical Corp.	Cat#029-02912
2-Mercaptoethanol (2-ME)	FUJIFILM Wako Pure Chemical Corp.	Cat#139-07525
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	FUJIFILM Wako Pure Chemical Corp.	Cat#094-05144
Ampicillin Sodium	FUJIFILM Wako Pure Chemical Corp.	Cat#012-23303
BamH I	Takara Bio Inc.	Cat#1010A
Xho I	Takara Bio Inc.	Cat#1094A
Critical commercial assays		
Mouse IL-18 ELISA Kit	MBL	Cat#7625
Protein Assay BCA Kit	FUJIFILM Wako Pure Chemical Corp.	Cat#297-73101
QIAprep Spin Miniprep Kit (50)	QIAGEN	Cat#27104
Rapid DNA Ligation Kit	Roche	Cat#11635379001
Can Get Signal	Toyobo	Cat#NKB-101
Experimental models: Organisms/strains		
C57BL/6J Mice (Female, 8-week-old)	Japan SLC Inc.	N/A
C3H/HeJ Mice (Male, 5-week-old)	Japan SLC Inc.	N/A
Recombinant DNA		
pET23b(+)	Novagen	Cat#69771
pET23b-SubABwt	(Morinaga et al., 2007)	N/A
pET23b-SubABmt	(Morinaga et al., 2007)	N/A
TOPO™ TA Cloning™ Kit for Subcloning, without competent cells	Thermo Fisher Scientific	Cat#451641
Software and algorithms		
GraphPad Prism 7.0	GraphPad Software	https://www.graphpad.com
Image Lab Software for PC Version 6.0.1	Bio-Rad	https://www.bio-rad.com/ja-jp/product/image-lab-software?ID=KRE6P5E8Z
Other		
Disposable feeding needle	Fuchigami	Cat#5202K
Asnol Petri Dish JP ϕ 90 × 15 mm	AS ONE Corp.	Cat#3-1491-01
Nepa Electroporation Cuvettes 1 mm gap	Nepa Gene	Cat#EC-001S
Polytron homogenizer	Kinematica	Cat#PT1200E
Bioruptor UCD-250	Tosho Electric	Cat#UCD-250
iMark Microplate Reader	Bio-Rad	Cat#1681130
ChemiDoc™ XRS+ system	Bio-Rad	Cat#1708265
MicroPulser Electroporator	Bio-Rad	Cat#1652100

MATERIALS AND EQUIPMENT

LB liquid medium (Difco™ LB Broth, Miller, Becton, Dickinson and Company)

Approx. formula per Liter	Final concentration	Amount
Pre-mixed powder consisting of:	2.5%	25 g
Yeast extract	0.5%	5.0 g
Tryptone	1.0%	10.0 g
NaCl	1.0%	10.0 g
ddH ₂ O	N/A	1,000 mL
Total	N/A	1,000 mL

Dissolve the powder in 1,000 mL of ddH₂O. Mix thoroughly and autoclave at 121°C for 20 min. Store for up to 6 months at room temperature (approx. 25°C). If necessary, add 1 mL of filtered sterilized 100 mg/mL ampicillin after cooling down (to less than 60°C). Store ampicillin containing LB for up to 1 week at 4°C.

LB agar plate (Difco™ LB Broth, Miller) (for 50 plates)

Approx. formula per Liter	Final concentration	Amount
Pre-mixed powder consisting of:	2.5%	25 g
Yeast extract	0.5%	5.0 g
Tryptone	1.0%	10.0 g
NaCl	1.0%	10.0 g
Agar	1.5%	15.0 g
ddH ₂ O	N/A	1,000 mL
Total	N/A	1,000 mL

Prepare as shown above. If necessary, add 1 mL of filtered sterilized 100 mg/mL ampicillin after cooling down (to approx. 60°C). Pour the solution directly from the bottle onto the plate. Store for up to 1 month at 4°C.

MacConkey agar plate (MacConkey agar, Nissui Pharmaceutical) (for 50 plates)

Approx. formula per liter	Final concentration	Amount
Pre-mixed powder consisting of:	5.0%	50 g
Gelatin peptone	1.7%	17.0 g
Casein peptone	0.15%	1.5 g
Meat peptone	0.15%	1.5 g
Sodium deoxycholate	0.1%	1.0 g
Lactose	1%	10.0 g
NaCl	0.5%	5.0 g
Crystal violet	0.0001%	0.001 g
Neutral red	0.003%	0.03 g
Agar	1.5%	15.0 g
ddH ₂ O	N/A	1,000 mL
Total	N/A	1,000 mL

Prepare as shown above. If necessary, add 1 mL of filtered sterilized 100 mg/mL ampicillin after cooling down (to approx. 60°C). Pour the solution directly from the bottle onto the plate. Store for up to 1 month at 4°C.

RIPA buffer

Reagents	Final concentration	Amount
1.0 M Tris-HCl, pH 7.5	10 mM	1.0 mL
NaCl	150 mM	0.8775 g
IGEPAL CA-630	1%	1.0 mL

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Reagents	Final concentration	Amount
Sodium deoxycholate	0.1%	0.1 g
Sodium dodecyl sulfate (SDS)	0.1%	0.1 g
ddH ₂ O	N/A	up to 100 mL
Total	N/A	100 mL

Store for up to 6 months at 4°C. Add Protease inhibitor cocktail (Fujifilm Wako Pure Chemical Corp.) and chill on ice before use.

10% glycerol (for electrocompetent cell preparation)

Reagent	Final concentration	Amount
Glycerol	10%	50 mL
ddH ₂ O	N/A	up to 500 mL
Total	N/A	500 mL

Mix thoroughly and autoclave at 121°C for 20 min. Store for up to 1 week at 4°C. Chill on ice before use.

D-PBS (Gibco)

Pre-mixed solution consisting of:	Final concentration	Amount
NaCl	137.93103 mM	4.0 g
KCl	2.6666667 mM	0.1 g
Sodium phosphate dibasic (Na ₂ HPO ₄ · 7H ₂ O)	8.059702 mM	1.08 g
Potassium phosphate monobasic (KH ₂ PO ₄)	1.4705882 mM	0.1 g
ddH ₂ O	N/A	up to 500 mL
Total	N/A	500 mL

This product is sterile, endotoxin-free, and commercially available from Thermo Fisher Scientific.

6 × SDS sample buffer containing 2-mercaptoethanol (2-ME)

Reagent	Final concentration	Amount
1.0 M Tris-HCl, pH 6.8	375 mM	15.0 mL
Glycerol	35.5%	14.2 mL
SDS	12%	4.8 g
Bromophenol blue	0.03%	12.0 mg
2-ME	15%	6.0 mL
Total	N/A	40 mL

Store for up to 6 months at room temperature. Mix the protein sample with the 6 × SDS sample buffer (5:1). If necessary, heat the mixed solution and then cool down. The solution is ready for SDS-PAGE. Dilute 1:5 with ddH₂O to prepare 1 × SDS sample buffer.

10 × Tris-buffered saline containing Tween 20 (TBST)

Reagent	Final concentration	Amount
Tris(hydroxymethyl)aminomethane	200 mM	96.8 g
NaCl	1.37 M	320 g
Tween 20	1%	40 mL
ddH ₂ O	N/A	up to 4 L
Total	N/A	4 L

Mix thoroughly and adjust the pH to 7.4 with HCl. Store for up to 6 months at room temperature. Dilute 1:10 with ddH₂O before use.

12% Acrylamide resolving gel solution

Reagent	Final concentration	Amount
ddH ₂ O	N/A	3.3 mL
30% Acrylamide and bis-acrylamide solution, 29:1	12%	4.0 mL
1.5 M Tris-HCl (pH 8.8)	375 mM	2.5 mL
10% SDS	0.1%	0.1 mL
10% APS	0.1%	0.1 mL
TEMED	0.04%	0.004 mL
Total	N/A	10 mL

15% Acrylamide resolving gel solution

Reagent	Final concentration	Amount
ddH ₂ O	N/A	2.3 mL
30% Acrylamide and bis-acrylamide solution, 29:1	15%	5.0 mL
1.5 M Tris-HCl (pH 8.8)	375 mM	2.5 mL
10% SDS	0.1%	0.1 mL
10% APS	0.1%	0.1 mL
TEMED	0.04%	0.004 mL
Total	N/A	10 mL

Thoroughly mix ddH₂O, 30% acrylamide solution, Tris-HCl, and 10% SDS in a 50-mL tube. Add APS and TEMED and swirl. Pour the mixture immediately into the assembled glass gel plate sandwich. Add water on top of the resolving gel solution to form a layer and incubate it until the gel polymerizes and solidifies. After solidification, discard the water and prepare the following acrylamide stacking gel solution.

Acrylamide stacking gel solution

Reagent	Final concentration	Amount
ddH ₂ O	N/A	6.8 mL
30% Acrylamide and bis-acrylamide solution, 29:1	5.1%	1.7 mL
1.0 M Tris-HCl (pH 6.8)	125 mM	2.5 mL
10% SDS	0.1%	0.1 mL
10% APS	0.1%	0.1 mL
TEMED	0.1%	0.01 mL
Total	N/A	10 mL

Thoroughly mix ddH₂O, 30% acrylamide solution, Tris-HCl, and 10% SDS in a 50-mL tube. Add APS and TEMED and swirl. Pour the mixture immediately onto the solidified resolving gel. Insert the comb to form sample lanes in the stacking gel layer, and incubate it until the gel polymerizes and solidifies. After solidification, remove the comb. Store the gel under humidified conditions at 4°C for up to 1 week.

Note: APS and TEMED are polymerizing agents and should be added last. The percentage of acrylamide can be adjusted (replaced with ddH₂O) accordingly depending on the molecular weight of the protein of interest.

1 M IPTG

Dissolve 238 mg of IPTG in 1 mL ddH₂O and filter-sterilize the solution with 0.2- or 0.45- μ m filters. Store for up to 1 week at -30°C .

100 mg/mL ampicillin

Dissolve 1 g of ampicillin sodium in 10 mL ddH₂O and filter-sterilize the solution with 0.2- or 0.45- μ m filters. Store for up to 3 months at -30°C .

5% skim milk in TBST

Dissolve 5 g of skim milk in 100 mL TBST. Do not store the solution.

10% SDS

Dissolve 10 g of SDS in 100 mL ddH₂O. Store at room temperature (20°C–25°C) for up to 1 year.

10% APS

Dissolve 1 g of APS in 10 mL ddH₂O. APS solution should preferably be used for gel preparation immediately after preparation, but solution can be kept frozen below –20°C. Avoid repeated freeze-thaw cycles.

Disposable feeding needle

A disposable plastic feeding needle 38 mm long (Fuchigami, 5202K) was used in this protocol. This needle is straight but flexible. Stainless steel feeding needles are also available.

Mice

Both 5-week-old male C3H/HeJ mice and 8-week-old female C57BL/6J mice were purchased from Japan SLC Inc. and housed in the Center for Animal Resources and Development, Kumamoto University. Mice were maintained in a 12-h light/12-h dark cycle with free access to water and a standard mouse diet (CE-2, CLEA Japan).

STEP-BY-STEP METHOD DETAILS

Oral infection of mice with *C. rodentium*

⌚ Timing: 17 days

This section describes procedures for oral infection of mice with *C. rodentium*.

1. One day before infection (at day –1).
 - a. Prepare the required number of mice.

Note: In this protocol, 5-week-old male C3H/HeJ mice (Figure 2A) were chosen for normal *C. rodentium* infection. The 8-week-old female C57BL/6J mice (Figure 2B) were used for infection with Cr transformants carrying the plasmid encoding VF and *amp*^R genes (In this article, we used Cr-SubABwt as Cr-VF transformants; Tsutsuki et al., 2022).

- b. Weigh the mice and divide them into the required number of groups.
 - c. Stop food intake for 8–12 h before infection.
2. At day 0.
 - a. Prepare the bacterial solution according to the section “preparation of the *C. rodentium* inoculum for mouse infection” in “before you begin”.

Note: In this article, we infected C3H/HeJ mice with 2.5×10^8 CFUs of *C. rodentium* and the C57BL/6J mice with 5×10^9 CFUs of Cr-VF transformants via oral gavage with a disposable 1-mL syringe (e.g., Terumo) and a flexible plastic feeding needle (e.g., Fuchigami, 5202K) (Figure 2C). Before the feeding needle is inserted, hold the mouse and confirm the insertion position of the feeding needle from the mouth to the stomach of the mouse from the outside (Figure 2D).

- b. Introduce the bacterial solution into the syringe, attach the feeding needle, and remove air bubbles as much as possible. Insert the feeding needle from the side of the mouth to avoid teeth and slowly push inward, so that the needle is parallel to the upper jaw, and advance into the throat (Figure 2E).

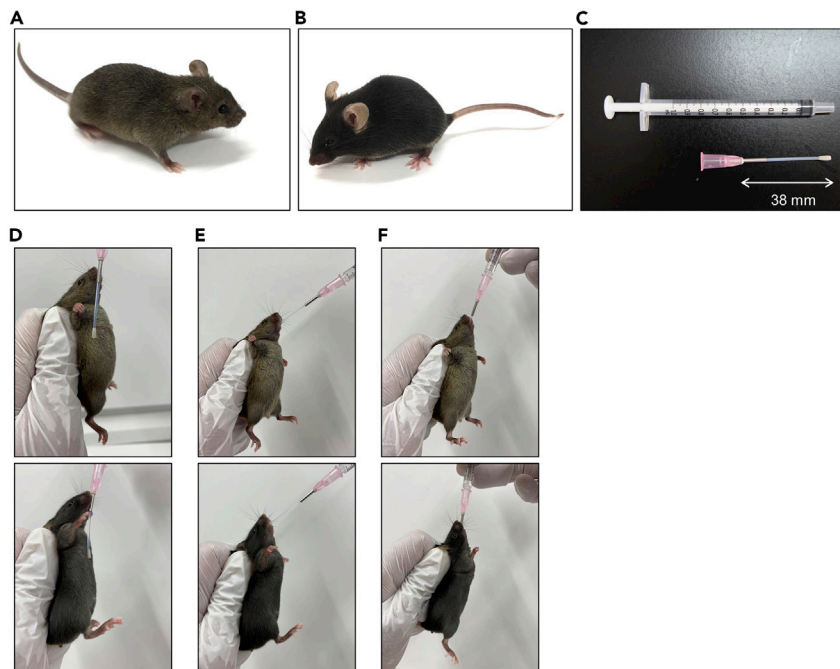


Figure 2. Oral administration of *C. rodentium* bacteria to C3H/HeJ and C57BL/6J mice

(A) C3H/HeJ mouse. Male, 5-week-old mice were used for infection with normal *C. rodentium* (Cr).

(B) C57BL/6J mouse. Female, 8-week-old mice were used for infection with VF-expressing Cr transformants.

(C) Disposable syringe and feeding needle used for this protocol.

(D–F) Oral administration of *C. rodentium* bacteria. Simulation of insertion position of the feeding needle (D).

Insertion into the mouth (E). Injection of the bacterial solution (F). (Upper panel: C3H/HeJ mouse, Lower panel: C57BL/6J mouse).

- c. Keep the mouse head vertically aligned with the body as much as possible by holding the needle and syringe vertical. Once the needle has entered the esophagus, gently push the needle down until the needle of the confirmed length is inserted (Figure 2F).
- d. Gently inject 100 μ L of bacterial solution without overflow from the mouth.
- e. After 1–2 h of infection, resume food intake. See [problem 2](#) in [troubleshooting](#).
3. At day 1 to day 16.
 - a. Record the body weight of the mice daily and analyze the weight change according to the initial weight at day –1 or 0 (Figure 3). See “CRITICAL” below.
 - b. To analyze inflammasome activation in the intestine and the CFUs of bacteria in feces, euthanize mice at 8–10 days post infection (dpi) (C3H/HeJ mice) and 4–16 dpi (C57BL/6J mice).
 - c. Collect intestinal tissue segments and feces into sterile microcentrifuge tubes. Prepare a homogenate of the mouse intestine and examine the activation and expression of inflammasome-related proteins, according to the protocol sections “[collection of intestines and feces from euthanized mice](#)” and “[analysis of non-canonical inflammasome activation in the mouse intestine](#).” Determine the CFUs of Cr-VF transformants (e.g., Cr-SubABwt) in the feces as in the protocol “[determination of the CFUs in feces of *C. rodentium* transformants carrying the plasmid encoding VF and *amp*^R genes \(Cr-VF\)](#).”

Note: Intestinal tissue can be stored at -80°C for approximately 1 month. Tissues may dry out and lose weight if stored for longer than a month. We recommend homogenizing the tissue and storing it as a lysate at -80°C . Solubilized samples can be used for IL-18 analysis by ELISA for at least 1 month and for caspase-1 and caspase-11 analysis by Western blotting

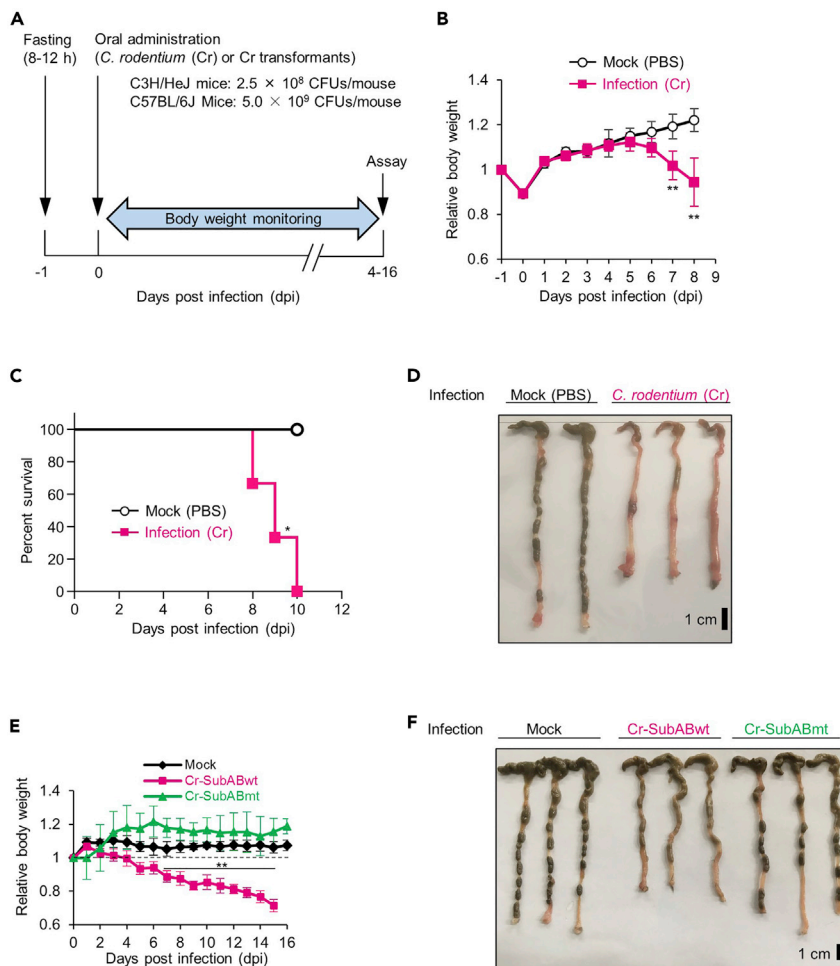


Figure 3. Analysis of daily weight change and intestinal tissue collection of mice

(A) Time schedule of experimental design including oral administration of *C. rodentium* to mice and analysis for non-canonical inflammasome activation.

(B) Body weight monitoring of C3H/HeJ mice that received *C. rodentium*. Data are means \pm SD (Mock: $n = 3$, Infection: $n = 6$). ** $p < 0.01$.

(C) Survival curve of C3H/HeJ mice that received *C. rodentium* (Mock: $n = 3$, Infection: $n = 6$). * $p < 0.05$.

(D) Representative images of gross colon specimens from C3H/HeJ mice that received *C. rodentium* at 8–10 dpi.

(E) Body weight changes in C57BL/6J mice that received PBS (Mock), Cr-SubABwt, or Cr-SubABmt, expressed as relative changes from 0 dpi. Data are averages (\pm SD) of 5 mice per group. ** $p < 0.01$ (figure reprinted with permission from Tsutsuki et al., 2022).

(F) Representative images of gross colon specimens from C57BL/6J mice that received PBS (Mock), Cr-SubABwt, or Cr-SubABmt at 15–16 dpi (figure reprinted with permission from Tsutsuki et al., 2022).

for at least 1 year. On the other hand, mouse feces must be fresh for the CFU assay. Do not freeze feces as freezing kills fecal bacteria.

Note: The disposable feeding needle is made of a plastic material and is 38 mm long, straight but flexible. Flexibility of the tip reduces the chance of traumatic injury to the esophagus. Stainless steel feeding needles are also available (Wang et al., 2020).

△ CRITICAL: If mice lost 20% of their body weight in a few days, or more than 25% of their body weight in a week, euthanize them as a humane endpoint and score the mice as dead. Construct survival curves by using the Kaplan-Meier method, and analyze the statistical

significance via the log-rank (Mantel-Cox) test with GraphPad Prism 7.0 (GraphPad Software) (Figures 3B and 3C). See [problem 3](#) in [troubleshooting](#).

Collection of intestines and feces from euthanized mice

⌚ Timing: 3 h

This section describes the procedures to collect intestines from infected mice and to prepare tissue homogenates. In order to measure fecal CFUs, feces must also be collected at this step.

4. Euthanize mice and collect intestines.
 - a. Euthanize the mice with isoflurane or cervical dislocation.

Note: In this article, we euthanized C3H/HeJ mice that received *C. rodentium* at 8–10 dpi. In our previous article, we euthanized C57BL/6J mice that received Cr-VF transformants (e.g., Cr-SubABwt) in a time-dependent manner at 4, 6, and 11 dpi for assay. In addition, for pathological analysis of the intestine, we euthanized Cr-SubABwt-infected mice at 14–15 dpi as the endpoint, and we euthanized mock (PBS)-infected and Cr-SubABmt-infected mice at 16 dpi (Figures 3E and 3F).

Note: Bacterial CFU in feces increased gradually until about 11 dpi. This increase pattern is consistent with other reports (Wang et al., 2020).

- b. Collect intestines and place them in a clean Petri dish containing chilled PBS. Wash the outside of the intestines with PBS and transfer the intestines to a clean Petri dish.
- c. After removing the excess PBS, measure the length of the intestines (from the cecum to the rectum) and take pictures on a clean white paper (Figures 3D and 3F).
- d. Cut open the intestines longitudinally. Wash them by swirling in a clean Petri dish with fresh chilled PBS. Wipe off excess PBS with a paper towel and cut the intestines (from distal colon region) into segments. See [problem 4](#) in [troubleshooting](#).

Note: In the protocol with the C57BL/6J mice that received Cr-SubAB transformants, we assessed fecal CFUs (Tsutsuki et al., 2022). After cutting open the intestines longitudinally, collect feces and put them into a sterilized 1.5-mL tube, measure the fecal weights, and prepare fecal homogenates for determination of CFUs of Cr-SubAB transformants in the feces. (See the section below “[determination of the CFUs in feces of *C. rodentium* transformants carrying the plasmid encoding VF and *amp*^R genes \(Cr-VF\)](#).”).

5. Prepare tissue homogenates.
 - a. Weigh tissue segments and add 5–10 × volume of RIPA buffer containing protease inhibitor. If the tissue weight is 50 mg, add 500 μL of the buffer. Homogenize the segments with the Polytron homogenizer (Kinematica) on ice for 3 min (at intervals of 30 s ON/30 s OFF).
 - b. Sonicate samples with the Bioruptor UCD-250 (Tosho Electric) for 2 min (at high power, at intervals of 10 s ON/10 s OFF). Centrifuge at 15,000 × g for 15 min at 4°C.
6. Collect supernatants as protein samples for inflammasome activation analysis.
 - a. Measure protein concentrations by using BCA methods (Protein Assay BCA Kit, FUJIFILM Wako Pure Chemical Corp.).
 - b. Subject the samples to the enzyme-linked immunosorbent assay (ELISA) for IL-18, SDS-PAGE, and Western blotting for inflammasome-related protein expression and activation according to the section “[analysis of non-canonical inflammasome activation in the mouse intestine](#)” below (Figure 4).

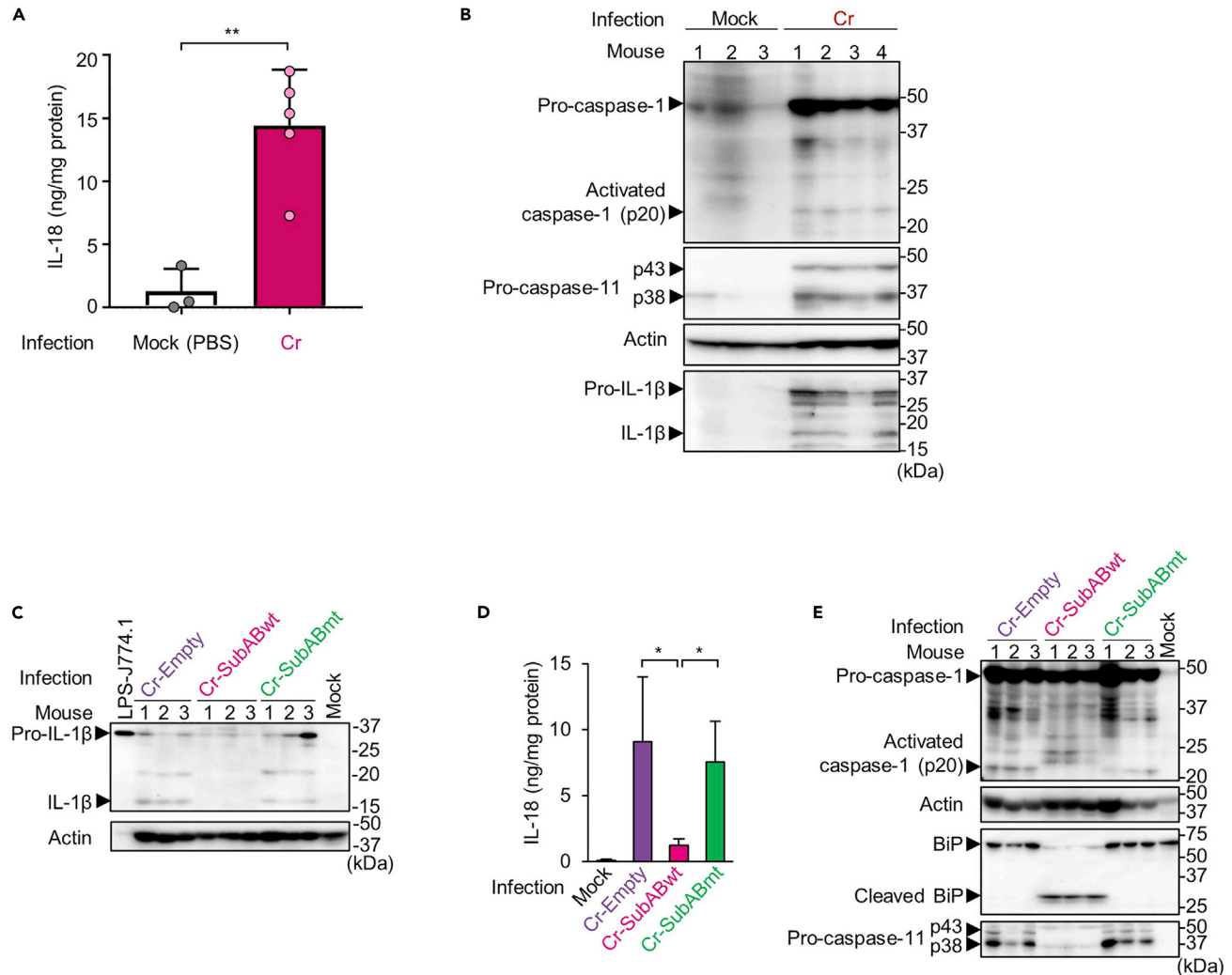


Figure 4. Evaluation of non-canonical inflammasome activation in mice intestine

(A) Intestinal IL-18 production in C3H/HeJ mice infected with *C. rodentium* (Cr) at 8–10 dpi. Intestinal proteins were analyzed by using a mouse IL-18 ELISA kit. Data are means \pm SD (Mock: n = 3, Infection: n = 5). **p < 0.01.

(B) Representative Western blotting images of caspase-1 activation (upper panel), caspase-11 expression (middle panel), and IL-1 β production (lower panel). Intestinal proteins from C3H/HeJ mice were analyzed by means of Western blotting by using anti-caspase-1, anti-caspase-11, and anti-IL-1 β antibodies. Actin served as the loading control.

(C) Representative Western blotting images of IL-1 β production (upper panel). Actin served as the loading control (lower panel). Intestinal proteins from C57BL/6J mice that received Cr-SubAB transformants (Cr-SubABwt or Cr-SubABmt) were analyzed by means of Western blotting by using anti-IL-1 β antibody (figure reprinted with permission from [Tsutsuki et al., 2022](#)).

(D) Intestinal IL-18 production. Intestinal proteins from C57BL/6J mice were analyzed by means of a mouse IL-18 ELISA kit. Data are averages (\pm SD) of 3 mice per group. *p < 0.05 (figure reprinted with permission from [Tsutsuki et al., 2022](#)).

(E) Representative Western blotting images of caspase-1 activation (upper panel), BiP cleavage (middle panel), and caspase-11 expression (lower panel). Actin served as the loading control. Intestinal proteins from C57BL/6J mice that received Cr-SubAB transformants were analyzed by means of Western blotting by using anti-caspase-1, anti-BiP, and anti-caspase-11 antibodies (figure reprinted with permission from [Tsutsuki et al., 2022](#)).

Note: Solubilized samples can be used for IL-18 analysis by ELISA for at least 1 month and for caspase-1 and caspase-11 analysis by Western blotting for at least 1 year.

Analysis of non-canonical inflammasome activation in the mouse intestine

© Timing: 2 days

This section describes the ELISA procedure for IL-18, and the procedure for SDS-PAGE and Western blotting to analyze IL-1 β production and caspase-1 activation.

7. ELISA for IL-18.

Note: IL-18 is a member of the IL-1 cytokine family. Production of the activated form (mature IL-18) via processing by caspase-1 is inflammasome-dependent. In this article, mouse intestinal IL-18 levels were determined by using ELISA according to manufacturers' instructions. A mouse IL-18 ELISA kit is commercially available from different manufacturers (e.g., MBL Cat#7625, https://ruo.mbl.co.jp/bio/dtl/dtlfiles/7625_220301_ver9.pdf).

- Dilute each sample (1:20–1:200) with the Assay diluent included in the ELISA kit (e.g., corresponding to a protein concentration of 0.03–0.3 mg/mL).
- Transfer 100 μ L of each sample to mouse IL-18 antibody-coated microwells and allow a reaction to proceed for 1 h at room temperature (approx. 25°C).
- Prepare Wash solution by diluting Wash concentrate included in the kit to 1:10 with ddH₂O prior to use. Discard the well contents, and fill each well with a Wash solution (approx. 300 μ L). Repeat this washing step for a total of four times.
- After removing the Wash solution, fill the wells with 100 μ L of Conjugated antibody solution and incubate for another 1 h at room temperature.
- Wash the microplate again as in step c. After removing the Wash solution, fill the wells with 100 μ L of Substrate reagent and incubate for 30 min in the dark at room temperature.
- Add 100 μ L of Stop solution to each well. After the reaction stops, immediately (within 30 min) measure the absorbance at 450 nm with a microplate reader (e.g., iMark™ Microplate Absorbance Reader, Bio-Rad).
- Calculate the IL-18 concentration (pg/mL) of each sample as well as the standard. Plot a standard growth curve and calculate IL-18 with the results shown as ng per mg of protein (ng/mg protein) using the protein concentration determined by means of the BCA method.

△ CRITICAL: If sample concentration is outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.

8. SDS-PAGE and Western blotting for IL-1 β production and caspase-1 activation.

Note: Production of activated IL-1 β via processing by caspase-1 is inflammasome-dependent. Caspase-1 activation occurs by auto-processing of pro-caspase-1 (p50) to activated forms of caspase-1 (p10 and p20 subunits). In this article, intestinal levels of activated IL-1 β and caspase-1 (p20) were evaluated as biomarkers of inflammasome activation by Western blotting. The endoplasmic reticulum chaperone BiP, a SubAB substrate protein, was also analyzed for its cleavage type as a marker showing SubAB activity (Figure 4E).

Note: Western blotting (also known as immunoblotting) is generally used in research to identify and detect proteins. *STAR Protocols* provides excellent articles explaining Western blotting (Burckhardt et al., 2021; Ziman and Oviedo, 2021). See those articles for more details.

- Prepare 12% acrylamide gel (for the activated form of caspase-1, actin, and BiP) and 15% acrylamide gel (for the activated form of IL-1 β). Load appropriate amounts of protein samples to each well.
- Run electrophoresis with a constant current of 30 mA per gel for approximately 60–90 min until the blue dye in the sample buffer reaches approximately 5 mm from the bottom of the gel.
- Transfer the separated proteins onto a PVDF membrane (e.g., Immobilon-P PVDF membrane, Merck Millipore) under wet transfer conditions at 100 V for 1 h on ice.

- d. Block the membrane with 5% skim milk in TBST with gentle shaking for 1 h at room temperature (approx. 25°C). Wash the membrane with TBST with gentle shaking. Incubate it with primary antibody overnight (12–18 h) at 4°C.

Note: Use Can get signal (Toyobo) solution 1 (for primary antibody) and solution 2 (for secondary antibody) as antibody dilution buffer.

- e. Wash the membrane 5 times for 5 min with TBST and then incubate it with secondary antibody for 1 h at room temperature.
- f. Wash the membrane 5 times for 5 min with TBST. Prepare chemiluminescent reagent according to the manufacturer's protocol (e.g., Immobilon Western Chemiluminescent HRP Substrate, Merck Millipore, https://www.merckmillipore.com/JP/en/product/Immobilon-Western-Chemiluminescent-HRP-Substrate,MM_NF-WBKLS0050).
- g. Detect the band with an image analyzer (e.g., ChemiDoc™ XRS System with Image Lab Software for PC Version 6.0.1, Bio-Rad).

Note: When comparing caspase-1 activation quantitatively, quantify the band intensity of activated caspase-1 (p20) detected around 20 kDa (Arrowhead in Figures 4B and 4E) by densitometry. Also quantify the band intensity of actin as a loading control. Divide the p20 band intensity by the actin band intensity for each lane, and use the p20/actin value for evaluation.

△ **CRITICAL:** Mouse tissue homogenates usually contain endogenous immunoglobulin. Secondary antibody against mouse monoclonal antibody binds to the endogenous immunoglobulin and causes inaccurate blotting images. To reduce the signals from the immunoglobulin light chain (25 kDa) and heavy chain (50 kDa), use a conformation-specific secondary antibody (e.g., Anti-mouse IgG Veriblot for IP secondary antibody (HRP) ab131368, Abcam). See problem 5 in troubleshooting.

Determination of the CFUs in feces of *C. rodentium* transformants carrying the plasmid encoding VF and *amp^R* genes (Cr-VF)

⌚ **Timing:** 2 days

This section describes the procedure to prepare fecal homogenates and the calculation of intestinal CFUs of Cr-VF with the *amp^R* gene.

9. Prepare fecal homogenates.
 - a. After euthanasia of C57BL/6J mice that received Cr-SubAB transformants, collect intestines into clean Petri dishes.
 - b. Cut open the intestines longitudinally. Collect feces randomly into sterilized 1.5-mL tubes and weigh the feces.
 - c. Add 50 µL of PBS for every 10 mg of feces (20% w/v in PBS). Dissolve the feces by vigorous vortexing. Prepare serial dilutions of fecal slurries as shown in Figure 5A.
 - d. Plate the serial dilutions onto MacConkey agar plates containing ampicillin (100 µg/mL) and incubate the plates overnight at 37°C.

Note: Mouse feces must be fresh for the CFU assay. Do not freeze feces as freezing kills fecal bacteria. Diluted samples should preferably be spread on agar plates immediately after preparation.

Note: After this incubation, Cr transformants such as Cr-SubAB form purple (or reddish purple) colonies on MacConkey agar plates containing ampicillin; they are resistant to ampicillin

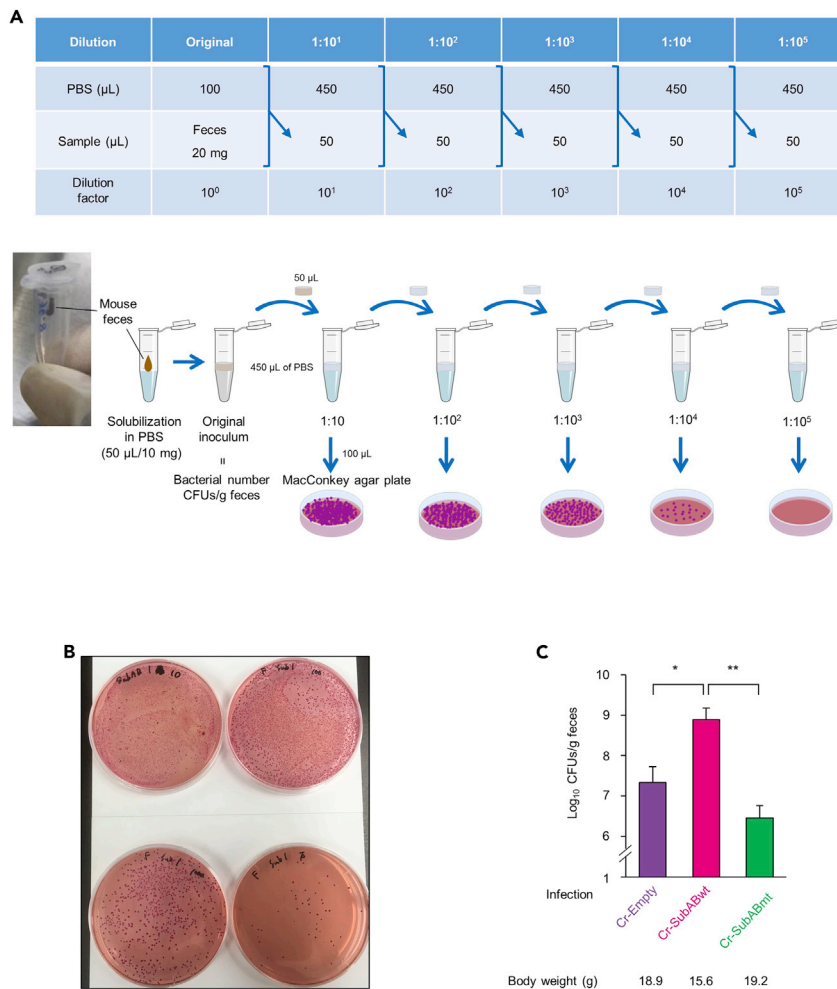


Figure 5. Fecal CFU assay for *C. rodentium* colonization

(A) Serial dilution procedure for the mouse fecal CFU assay.

(B) Colony formation of fecal Cr transformants on MacConkey agar plates containing ampicillin. Purple (or reddish purple) colonies formed on agar plates inoculated with diluted fecal homogenates from Cr-SubABwt-infected C57BL/6J mice.

(C) Fecal bacterial CFUs of mice that received Cr-SubAB transformants (figure reprinted with permission from [Tsutsuki et al., 2022](#)). Mouse feces were collected at 11 dpi from intestines of three mice (three feces per mouse). The number of viable bacteria was determined by CFU analysis of the plates; results are given as log₁₀ CFUs per gram of feces. Data are means ± SD (n = 9 per group). *p < 0.05; **p < 0.01.

because of the *amp^R* gene on the pET23b plasmid. This color change is due to lactose metabolism (Figure 5B). See problem 6 in troubleshooting.

10. Calculate intestinal CFUs of Cr-VF (e.g., Cr-SubABwt) with the *amp^R* gene.
 - a. Count the colonies on the agar plates and determine the number of viable bacteria by means of the CFU method (Figure 5).
 - b. Calculate intestinal bacterial CFUs with the results shown as CFUs per gram of feces (CFUs/g). CFUs/g = (CFUs in the diluted solution) × 10 × (dilution factor) × 5.

Note: For example, for the samples shown in Figure 5C, the CFUs of Cr-empty = 430 (approx. average of CFUs in 1:10³ dilution) × 10 × 10³ (dilution factor) × 5 = 2.15 × 10⁷ CFUs/g; the CFUs of Cr-SubABwt = 1558.9 (approx. average of CFUs in 1:10⁴ dilution) × 10 × 10⁴ (dilution

factor) $\times 5 = 77.9 \times 10^7$ CFUs/g; the CFUs of Cr-SubABmt = 570 (approx. average of CFUs in 10^2 dilution) $\times 10 \times 10^2$ (dilution factor) $\times 5 = 0.29 \times 10^7$ CFUs/g.

EXPECTED OUTCOMES

Non-canonical inflammasome activation caused by bacterial infection is an important event as a host defense against bacterial infection (Liu et al., 2012). According to our protocol, C3H/HeJ mice lost significant body weight after 7 days of *C. rodentium* infection (Figure 3B) and showed intestinal hyperplasia with reduced colon length (Figure 3D). These symptoms are due to the pathogenicity of *C. rodentium*, but certain papers reported that the inflammasome and IL-18 are not responsible for these symptoms (Liu et al., 2012). Production of IL-18 and IL-1 β and activation of the inflammasome-related caspases (caspase-1 and -11) in the intestinal tissue were observed in infected mice (Figures 4A and 4B). This protocol may also be useful for the analysis of other inflammasome components, such as gasdermin D, which has been reported to be closely related to caspase-11. The protocol using C3H/HeJ mice is expected to be an effective experimental model for the development and evaluation of drugs that suppress the inflammatory response such as the inflammasome activation and production of IL-1 family members.

C57BL/6J mice did not lose weight when infected with 5×10^9 CFUs of *C. rodentium* (Mallick et al., 2012). When we investigated the inhibitory effect of SubAB on the inflammasome, we also administered the same bacterial CFUs of the transformants carrying empty vector (Cr-empty) as a negative control, but no weight loss was seen on day 11 of infection (Figure 5C). This mouse strain was less susceptible to *C. rodentium* infection than was C3H/HeJ, so the extent of weight loss did not change. The protocol using C57BL/6J mice may be suitable for use in the functional analysis of negative regulators of inflammasome activation because the infection is exacerbated by reduced elimination of bacteria and induced weight loss.

Mice that received Cr-SubABwt, which inhibits inflammasome activation, demonstrated a significant weight loss after 7 days, intestinal hyperplasia, and diarrhea at 14–15 dpi (Figure 3F). The counts of viable bacteria in the feces of mice that received Cr-SubABwt were significantly higher than those in the other groups (Figure 5C), which suggests a correlation between increased bacterial survival and weight loss. As in our recent report (Tsutsuki et al., 2022), protocols using C57BL/6J mice are expected to facilitate assessment of *E. coli* VFs that disrupt host defense mechanisms. We hope that this protocol will continue to be useful in many studies as one of the *in vivo* evaluation methods for non-canonical inflammasome activation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Student's *t*-test was used to determine significant differences when only two treatment groups were being compared. Survival curves of mice were constructed by using the Kaplan-Meier method, and statistical significance was analyzed via the log-rank (Mantel-Cox) test with GraphPad Prism 7.0 (GraphPad Software). All data are given as means \pm standard deviation (SD). Data for each experiment were acquired from at least three experiments. A *p*-value of less than 0.05 was said to be statistically significant.

LIMITATIONS

Our protocol introduced here may be useful as an *in vivo* evaluation system for non-canonical inflammasomes activation induced by infection with intestinal pathogenic *E. coli*, and we believe that it may be applicable to various research fields including bacteriology and immunology. Although this protocol can be applied to animal experiments with not only *C. rodentium* but also other infectious intestinal bacteria, most pathogenic *E. coli* bacteria that are clinically isolated from patients do not naturally infect most laboratory animal species including mice. Specialized mice, such as sterile mice and mice whose intestinal flora has been altered by administration of antibiotics, should be used. The limitation of our protocol is that *C. rodentium* is a mouse pathogen and may not

completely reflect the pathology of human enteropathogenic and enterohemorrhagic *E. coli* (EPEC/EHEC) infection. Therefore, comparing the results obtained from the human-derived bacterial infection model with the results obtained from the *C. rodentium* infection model is necessary.

The murine oral infection model of *C. rodentium* has been used extensively as a natural infection model for enteropathogenic *E. coli* including EPEC and EHEC. We expect that our procedure will be useful for analyzing VFs of *E. coli* (e.g., effectors, colonization factors) and their regulators, as in the functional analysis of toxins that we recently reported (Tsutsuki et al., 2022). In the present study, the epithelial and mucosal inflammasome could not be analyzed. However, if inflammasome sensor proteins nucleotide-binding domain leucine-rich repeat containing (NLR) family (e.g., NLRP3, NLRC4, NLRP6, and NLRP9b) in intestinal tissue can be analyzed, our infection model may become a useful method for evaluating epithelial and mucosal inflammasome in intestine.

TROUBLESHOOTING

Problem 1

The concentration of bacteria is too low (step 7c-Note in “before you begin”).

Potential solution

Enlarge and concentrate the culture scale. Centrifuge to remove the supernatant, reduce the amount of PBS added, and adjust to the desired concentration.

Problem 2

Mice die immediately after oral administration of either PBS or *C. rodentium* (step 2e in “step-by-step method details”).

Potential solution

During administration, hold the mouse firmly, but not too tightly, by the neck skin. Place the abdomen facing up, and gently grab around the mouse’s neck.

Problem 3

Infected mice lost 20% in body weight in a few days, or more than 25% in a week (step 3c-CRITICAL in “step-by-step method details”).

Potential solution

Euthanize according to humanitarian endpoints and count as dead mice.

Problem 4

Hyperplasia is not suppressed by administration of anti-inflammatory agents (step 4d in “step-by-step method details”).

Potential solution

Such symptoms are due to the pathogenicity of *C. rodentium*, but IL-1 β and IL-18 are reportedly not responsible for intestinal hyperplasia (Liu et al., 2012). Administration of antibacterial drugs, however, may reduce the pathogenicity of *C. rodentium* and the viable cell count and may lead to improvement of symptoms. Therefore, we believe that hyperplasia would be not suppressed even if an anti-inflammatory agent without antibacterial effect is administered.

Problem 5

Strong signals from the immunoglobulin light chain (25 kDa) and heavy chain (50 kDa) interfere with signals from the proteins of interest (step 8g-CRITICAL in “step-by-step method details”).

Potential solution

If available, probe the immunoblot with an antibody derived from a different species. As an alternative, use a conformation-specific secondary antibody that binds to native immunoglobulin but not to denatured immunoglobulin (as described above).

Problem 6

Bacteria other than *C. rodentium* grow on the plate (step 9d-Note in “step-by-step method details”).

Potential solution

Antibiotics contained in agar plates block the growth of bacteria other than Cr transformants. However, drug-resistant bacteria other than Cr transformants may form colonies. In that case, do not count colonies that do not show purple (or reddish purple) or colonies with different characteristics.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hiroyasu Tsutsuki (tsutsuki@kumamoto-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

H.T. and T.S., experimental design, inflammasome assay, data analysis, writing the paper; T.Z. and T.T., bacterial studies (preparation of bacterial standard growth curve), ELISA, writing the paper; K.Y. and H.T., molecular biology (preparation of SubABwt and SubABmt constructs), data analysis, editing the paper; H.T., T.Z., T.T., and T.S., animal studies, histology data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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