Metabolic programming determines the pathogenicity of *Campylobacter jejuni*

Koji Hosomi

National Institutes of Biomedical Innovation, Health and Nutrition

Noritoshi Hatanaka

Osaka Metropolitan University

Atsushi Hinenoya

Osaka Metropolitan University

Yoko Tojima

National Institutes of Biomedical Innovation, Health and Nutrition

Mari Furuta

National Institutes of Biomedical Innovation, Health and Nutrition

Takahiro Nagatake

Meiji University

Azusa Saika

National Institutes of Biomedical Innovation, Health and Nutrition

Soichiro Kawai

National Institutes of Biomedical Innovation, Health and Nutrition

Ken Yoshii

National Institutes of Biomedical Innovation, Health and Nutrition

Saki Kondo

National Institutes of Biomedical Innovation, Health and Nutrition

Shinji Yamasaki

Osaka Metropolitan University

Jun Kunisawa (✉ kunisawa@nibiohn.go.jp)

National Institutes of Biomedical Innovation, Health and Nutrition   https://orcid.org/0000-0003-4901-1125

Article

Abstract

Many pathogens acquire pathogenicity through a specialized capacity for metabolic adaptation, but unique adaptations in the metabolism of *Campylobacter jejuni* remain to be investigated. Here, we found that cultivation of *C. jejuni* under different conditions resulted in changes to the metabolic pathways associated with their altered pathogenicity in mice, such as highly frequent colonization and subsequent severe inflammation in the intestine. The metabolic changes involved the aspartate transferase–mediated pathway with serine as a substrate, which was required for aerobic energy generation. Our findings propose a novel pathway for controlling the pathogenicity of *C. jejuni* with promising preventive and therapeutic strategies for targeting bacterial metabolism.

Introduction

Enteritic bacterial infections are a global public health problem with high mortality and morbidity, particularly among children in developing countries [1]. Their threat is increasing in concert with a rise in antibiotic resistance, and therefore a new antimicrobial treatment needs to be developed. Among various pathogens, *Campylobacter* infection is one of the leading causes of diarrheal mortality in children younger than 5 years, with approximately 30,000 deaths in 2015 [1]. Even in developed countries, *Campylobacter* infections in the United States of America (USA) and Japan during 2018 were reported to be 9,723 and 1,995 cases, respectively [2,3]. Furthermore, molecular mimicry between the sialylated lipooligosaccharide (LOS) structures of *Campylobacter* and ganglioside epitopes on human nerves leads to cross-reactive immune responses that are thought to trigger severe neuronal disorders such as Guillain–Barré syndrome and Miller Fisher syndrome [4,5]. Therefore, *Campylobacter* infection is not only a short-term risk as a foodborne illness but may also necessitate long-term care for which effective treatments are required.

Host and gut commensal bacteria maintain intestinal environmental homeostasis and create a defense system against enteritic pathogens [6,7]. As part of the defense mechanism against pathogens, commensal bacteria occupy major nutrient niches and produce inhibitory fermentation products that directly and indirectly limit pathogen expansion in the intestine [7]. To fight this system, an enteritic pathogen dynamically reprograms its metabolism during infection, which allows it to overcome colonization resistance, and then expand and induce inflammation [6]. For example, *Citrobacter rodentium*, a murine enteritic pathogen, changes its metabolism to switch from carbohydrate to amino acid utilization in the intestine so that pathogen colonization can be promoted [8]. Thus, bacterial metabolism is now considered to be a new target for controlling infections caused by pathogens.

Campylobacter jejuni is a gram-negative, microaerophilic spiral-shaped bacterium, and this organism most commonly causes disease symptoms of inflammatory diarrhea, fever, cramps, and nausea in human [9,10]. Once a host orally ingests contaminated water or food, *C. jejuni* predominantly colonizes the mucous layers on the epithelium in the lower intestinal tract, the niches of which are presumably ideal for

colonization given that they naturally harbor a plentiful supply of nutrients and carbon sources that support *C. jejuni* metabolism and robust growth. *C. jejuni* has evolved specialized metabolic pathways that are adapted to environmental conditions (such as nutrients, temperature, and oxygen tension) in the host intestine, particularly in its natural host, the avian intestine [9]. Unlike most other bacteria, *C. jejuni* lacks the ability to use carbohydrates as a carbon source and prefers to utilize amino acids such as serine, aspartate, and glutamate, which are the most common amino acids found in chick excreta [11]. Indeed, serine catabolism is required for colonization of the chicken intestine [12,13]. Interestingly, different strains of *C. jejuni* exhibit genetical diversity in their ability to utilize amino acids, and the diversity in *C. jejuni* strains correlates with clinical presentations [14]. However, the exact requirements for the metabolic pathway leading to pathogenicity of *C. jejuni*, especially that causing inflammation, remain elusive.

Here, we show that metabolic changes of *C. jejuni* cultured under different conditions result in significant changes in their ability to colonize the intestine and in the subsequent inflammation in a mouse infectious model and that these changes required aspartate transferase–mediated metabolic reprogramming.

Results

C. jejuni shows different pathogenicity in different culture conditions

To see the effects of culture condition on the pathogenicity of *C. jejuni*, we cultured *C. jejuni* in different conditions and examined their colonization and intestinal inflammation in mice. In this study, we selected blood agar, a widely used culture medium for *Campylobacter*, and Bolton broth, a medium suitable for microaerophilic bacteria such as *Campylobacter* because it has improved aerotolerance and osmotic balance. When mice were administered *C. jejuni* cultured on blood agar, the number of fecal CFU gradually increased from day 1 to day 2 after infection (Fig. 1A). In contrast, mice administered *C. jejuni* cultured in Bolton broth showed a greater increase in fecal CFU (Fig. 1A). Then, we measured the neutrophil myeloperoxidase (MPO) level as a marker of intestinal inflammation in the feces [15]. Mice administered with *C. jejuni* cultured on blood agar showed little fecal MPO throughout the experimental period, whereas fecal MPO dramatically increased on days 2 and 3 after infection in the mice given *C. jejuni* cultured in Bolton broth (Fig. 1B). Consistently, at 3 days post-infection, flow cytometric analysis revealed an increase in neutrophils in the colon of mice dosed with *C. jejuni* cultured in Bolton broth (Fig. 1C, S1). Histological analysis further indicated neutrophil accumulation in the mucosal layer, as well as damage to the epithelial cell layer of the colon (Fig. 1D, 1E). In summary, mouse intestine was colonized frequently and with greater intestinal inflammation (indicated by neutrophil accumulation) by *C. jejuni* that had been cultured in Bolton broth than when cultured on blood agar, suggesting that the difference in growth environment affects the pathogenicity of *C. jejuni*.

One of the major differences between Bolton broth and blood agar is whether the medium state is liquid or solid; therefore, we next examined the pathogenicity of *C. jejuni* when cultured in Bolton broth (liquid) and on Bolton agar (solid). Mice administered *C. jejuni* cultured on Bolton agar showed fewer intestinal

colonized bacteria and little fecal MPO in comparison with mice dosed with *C. jejuni* cultured in Bolton broth (Fig. 2A, 2B). Thus, the medium state appears to be a determinant in the pathogenicity of *C. jejuni* in mice.

Different culture conditions had little effect on the virulence factors of C. jejuni

It is known that *C. jejuni* expresses various virulence factors, including motility, cell adhesion/invasion, and cytotoxicity, which determine its pathogenesis [16–19]. In comparison with blood agar, Bolton broth culture did not increase the expression levels of the following virulence genes: major cell adhesion molecules, *cadF*, *peb1A*, and *peb4*; flagellar secreted proteins, *flaC*, *ciaB*, and *ciaC* (but not *fspA*) and flagellar structural components, *flgE*, *flgK*, *flgL*, *flaA*, *flaB*, and *fliD*, which are major factors associated with motility and cell invasion; and other virulence-associated factors, *porA* and *htrA* (Fig. S2A).

C. jejuni cultured in Bolton broth showed increased expression of known cytotoxic toxin subunits *cdtA* and *cdtC* [20] in comparison with that cultured on blood agar (Fig. S2A). However, in a cytolethal distending toxin (CDT)−mediated cell-cycle arrest assay, *C. jejuni* cultured in Bolton broth showed rather weaker activity to arrest the cell cycle at the G_2/M stage on HeLa cells than *C. jejuni* cultured on Bolton agar or blood agar (Fig. S3), possibly because the cytotoxicity appears to depend on decreased *cdtB* expression rather than increased *cdtA* and *cdtC* expression (Fig. S2A).

The expression of capsular polysaccharide (CPS) and LOS gene clusters was increased in *C. jejuni* cultured in Bolton broth over that seen on blood agar cultures [21,22] (Fig. S2B). Indeed, alcian blue staining [23] certainly showed that the content of CPS/LOS was slightly increased in *C. jejuni* cultured in Bolton broth compared with that cultured on Bolton agar and blood agar (Fig. S4A). However, the TNF-α production levels from macrophages, which are innate immune cells that sense bacterial infection and produce inflammatory cytokines such as TNF-α via CPS/LOS-mediated innate immune signals [24,25], were comparable regardless of the bacterial culture condition (Fig. S4B), suggesting that the increase in CPS/LOS did not explain the difference in inflammatory activity of *C. jejuni*. Taken together, these results show that, at least with regards to known virulence factors, no significant increase in pathogenicity was observed in Bolton broth culture.

Transcriptome and morphological characteristics of C. jejuni cultured in Bolton broth

To seek the critical molecules in *C. jejuni* that determined their pathogenicity, we compared gene expression between *C. jejuni* cultured in Bolton broth and on blood agar by RNAseq. A volcano plot revealed dramatic changes in gene expression (Fig. 3A). Of the total 1,653 genes of *C. jejuni* (Genbank accession no. CP000538.1), the expression of 118 genes increased more than 6-fold in the culture of Bolton broth compared with blood agar. Gene ontology−based informatic analysis using TargetMine [26] revealed that 118 genes found 3 enriched GO terms (p < 0.01, Benjamini Hochberg); namely, the generation of precursor metabolites and energy (GO:0006091), carbohydrate metabolic process (GO:0005975), and developmental maturation (GO:0021700) (Fig. 3B).

Given that our gene expression analysis suggested anatomical structural changes in *C. jejuni* cultured in Bolton broth, we next observed the shape of *C. jejuni* by using scanning electron microscopy. *C. jejuni* cultured on blood agar showed a number of coccus-shaped cells, consistent with a previous study [27]. In contrast, when cultured in Bolton broth, *C. jejuni* cells were almost all spirals (Fig. 3C). *C. jejuni* cultured on Bolton agar showed an intermediate phenotype, with some coccoid cells observed (Fig. 3C). Thus, the different culture conditions, including the medium state and other factors, affect the morphology of *C. jejuni*.

Altered metabolic profile in C. jejuni cultured in Bolton broth

Because our gene expression analysis suggested there were metabolic function changes (Fig. 3A, 3B) and because this metabolic adaptation to environmental condition is accompanied by morphological changes [28], we next evaluated the energy metabolic flux of *C. jejuni*. The results of flux analysis showed that *C. jejuni* cultured in Bolton broth had an increased oxygen consumption rate (OCR) compared with *C. jejuni* cultured on blood agar (Fig. 4A). Then we used Bolton agar to investigate whether the difference between agar and broth has an effect on OCR. Compared with *C. jejuni* cultured in Bolton broth, the OCR of *C. jejuni* cultured on Bolton agar decreased to a level similar to that of *C. jejuni* cultured on blood agar (Fig. 4A). Thus, the difference in culture condition, especially the difference between agar and broth, affected the energy metabolic flux of *C. jejuni*.

Next, to understand the metabolic characteristics of *C. jejuni*, we compared the metabolic flux of *C. jejuni* with that of two other enteric pathogens, *Escherichia coli* and *Salmonella enterica*, when these bacteria were cultured in Bolton broth. Both *E. coli* and *S. enterica* showed higher extracellular acidification rate (ECAR) levels in addition to a high OCR, whereas a low ECAR was detected in *C. jejuni*, even when cultured in Bolton broth (Fig. S5). Thus, *C. jejuni* likely has poorer metabolic activity than other pathogens such as *E. coli* and *S. enterica* and, because the organism lacks the glycolytic pathway, it depends on the tricarboxylic acid (TCA) cycle to generate its energy, suggesting that *C. jejuni* may change its pathogenicity via a unique metabolic adaptation.

These findings prompted us to perform a metabolome analysis to reveal the energy metabolism of *C. jejuni* in more detail. Metabolite profiles were categorized into 3 groups based on the differences in the culture conditions (Bolton broth, Bolton agar, and blood agar) (Fig. S6). Of note, major metabolites increased specifically in *C. jejuni* cultured in Bolton broth, including aspartate, glutamate, proline, succinate, malate, isocitrate, citrate, and tryptophan (Fig. 4B, S6), and most of them appeared to be associated with central carbohydrate metabolism, especially with the malate−aspartate shuttle in the TCA cycle (Fig. 4C) [29].

This result was also supported by the following genomic data. The Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis [30] indicates the presence in the complete TCA cycle in *C. jejuni* (Fig. S7) of aspartate aminotransferase, an enzyme encoded by the *aspC* gene (CJJ81176_0783) that converts oxaloacetate into aspartate as a crucial reaction (EC 2.6.1.1) in the malate−aspartate shuttle

(Fig. S8), as well as the presence of the aspartate−glutamate transporter Peb1ABC (Fig. S9). In contrast, *S. enterica* (KEGG organism code: stm) and *E. coli* (KEGG organism code: eoi) possess the TCA cycle and *aspC* gene (EC 2.6.1.1) but lack the aspartate−glutamate transporter Peb1ABC (Fig. S10). Thus, it is likely that, unlike other pathogens, *C. jejuni* cultured in Bolton broth preferentially uses the malate−aspartate pathway to generate aerobic energy.

Aspartate aminotransferase−mediated metabolism is associated with *C. jejuni* pathogenicity

Given these findings, we next examined whether these metabolic changes were prerequisite for the pathogenicity of *C. jejuni*. To test this, we investigated the effects of inhibiting aspartate aminotransferase with a specific inhibitor, aminooxyacetate (AOA). Treatment with AOA reduced the OCR of *C. jejuni* cultured in Bolton broth in a dose-dependent manner (Fig. 4D). Thus, our results suggest that aspartate aminotransferase−mediated metabolism was promoted in *C. jejuni* cultured in Bolton broth.

We further attempted to understand the role of the aminotransferase-mediated metabolism in *C. jejuni* pathogenicity in mice. Consistent with the OCR, supplementation of AOA suppressed the growth of *C. jejuni* in a dose-dependent manner (Fig. 4E). Furthermore, fewer *C. jejuni* colonies and less MPO were detected at all measurement points in fecal samples from mice administered AOA-pretreated *C. jejuni* (Fig. 4F, 4G). Thus, our results suggested that the increased aspartate aminotransferase−mediated metabolism caused by Bolton broth culture played a critical role in *C. jejuni* growth, colonization, and subsequent induction of intestinal inflammation.

Serine as a crucial substrate in the energy metabolism of *C. jejuni* and its pathogenicity

Because it lacks the glycolytic pathway, *C. jejuni* predominantly relies on amino acids as the carbon source to fuel the TCA cycle for energy generation (Fig. S5) [9]. Therefore, to reveal which metabolites promote *C. jejuni* metabolism such as oxygen consumption, we performed a metabolome analysis of *C. jejuni*-cultured supernatant. Serine and asparagine were drastically decreased in the *C. jejuni*-cultured supernatant in comparison with the uncultured fresh medium (Fig. 5A). Although aspartate, glutamine, glutamate, and proline are amino acids capable of being used for catabolism to support growth of *C. jejuni* [9], they were not decreased in *C. jejuni*−cultured supernatant (Fig. S11), suggesting that the amino acids serine and asparagine are preferentially used by *C. jejuni* when cultured in Bolton broth.

Next, we examined the effects of serine and asparagine on the metabolic change and pathogenicity of *C. jejuni*. Supplementation with serine or asparagine increased the OCR in a dose-dependent manner in *C. jejuni* cultured in minimum essential medium (MEM), which is a nutrient-restricted medium that lacks non-essential amino acids such as serine and asparagine (Fig. 5B). Serine appeared to be more effective to increase the both the OCR and the growth of *C. jejuni* than asparagine (Fig. 5B, 5C). Consistent with this result, treatment with AOA reduced the OCR of *C. jejuni* cultured in MEM supplemented with serine (Fig. S12). Finally, mice given *C. jejuni* cultured in MEM supplemented with serine showed a higher number of intestinal bacterial colonies and increased fecal MPO in comparison with mice dosed with *C.*

jejuni cultured in MEM (Fig. 5D, 5E). Thus, serine appears to be utilized as a crucial substrate for energy metabolism of C. jejuni and is subsequently required for its pathogenicity in mice.

Discussion

We have revealed the crucial role of metabolic reprogramming in the pathogenicity of C. jejuni in mice. In comparison with that grown on blood agar or Bolton agar, C. jejuni cultured in Bolton broth colonized the mouse intestine more frequently and subsequently induced intestinal inflammation yet simultaneously increased serine-mediated energy metabolism resulting in high oxygen consumption. Inhibition with AOA showed that all the phenotypes were dependent on aspartate aminotransferase, suggesting that the aspartate aminotransferase−mediated metabolic change with serine as a substrate is closely linked to C. jejuni pathogenicity. Collectively, our findings suggest that C. jejuni reprograms its metabolism to adapt to the environment of the host intestine and that this metabolic adaptation is required for its pathogenicity and subsequent intestinal inflammation; therefore, the findings provide a novel strategy for developing preventive and therapeutic approaches, including the establishment of a mouse infectious model.

Regarding the relationship between bacterial metabolism and the pathogenicity of C. jejuni, it is interesting that AOA completely suppressed intestinal colonization of C. jejuni and inflammation in mice. AOA is a chemical compound that inhibits pyridoxal phosphate−dependent enzymes, including 4-aminobutyrate aminotransferase for generating gamma-aminobutyric acid and aspartate aminotransferase for the malate−aspartate shuttle, resulting in neuronal and metabolic dysfunction in mammalians [31]. Mammalian aspartate aminotransferase is commonly known as a clinical biomarker for liver health and catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate in the malate−aspartate shuttle, which is an NADH shuttle that generates energy by coupling glycolysis with mitochondrial metabolism [29]. In genomic information on the C. jejuni strain 81−176, the aspC gene (CJJ81176_0783) is annotated as encoding aspartate aminotransferase, which we speculate the target inhibited by AOA. Indeed, it has been demonstrated that aspartate aminotransferase is involved in the growth of C. jejuni in studies using gene mutants of the aspB gene (Cj0762c), which encodes aspartate aminotransferase (EC:2.6.1.1) in the C. jejuni strain NCTC 11168 [32]. Although it is unclear whether the malate−aspartate shuttle functions in C. jejuni bacteria in the same way as it does in mammalian cells, these findings taken together suggest a crucial role for aspartate aminotransferase−mediated energy metabolism in the growth and pathogenicity of C. jejuni.

Unlike most other bacteria, C. jejuni lacks the ability to use carbohydrates as a carbon source for energy metabolism because it lacks the appropriate transporters to take up sugars like glucose or galactose, and also lacks several key enzymes within the glycolytic pathway [9,11]. Indeed, C. jejuni showed little ECAR in this study. Therefore, C. jejuni predominantly relies on amino acids as its carbon source for energy metabolism. In C. jejuni, l-serine is preferentially catabolized to pyruvate in the TCA cycle and utilized for bacterial growth through the action of the transporter SdaC and l-serine dehydratase SdaA [13]. In addition, because sdaC or sdaA mutations impaired colonization in avian gut, l-serine is crucially important for the

growth of *C. jejuni* and intestinal colonization [13]. Although most strains of *C. jejuni*, including strain 81–176 used in this study, do not possess any identifiable asparagine transporters, asparagine is converted to aspartate by asparaginase AnsB in periplasm and transported into cytoplasm by the Peb1 transport system to be used in the TCA cycle [14]. When an *ansB* mutant of *C. jejuni* is intraperitoneally injected to *myd88*$^{-/-}$ *nramp1*$^{-/-}$ mice, periplasmic AnsB is required for liver colonization [14]. Consistent with these previous reports, our findings demonstrated that l-serine and l-asparagine are utilized for energy metabolism in *C. jejuni* and are therefore linked to its pathogenicity, such as colonization and intestinal inflammation in mice.

l-serine is also converted to phosphoenolpyruvate (PEP) via pyruvate and oxaloacetate as a substrate for gluconeogenesis required for CPS and LOS biogenesis and protein glycosylation [9]. In addition, the presence of l-serine may be sensed by *C. jejuni* to induce transcriptional changes, such as upregulation of nucleotide sugars, biosynthesis of amino acids, and downregulation of flagellar assembly components [33]. These previous reports are consistent with the findings in this study, such as expression of virulence factors of *C. jejuni* cultured in Bolton broth. Taken together, the data suggest that bacterial metabolism is associated with the regulation of virulence factors, and this will be an important research topic in the future.

When considering why the metabolic activity of *C. jejuni* decreased when grown on agar, spatial changes in the metabolic profile of *C. jejuni* colonies on agar is a possible reason. A 3D agent-based model that describes the establishment of simple bacterial colonies expanding by the physical force of their growth demonstrated that radial colony expansion is limited by mechanical forces [34]. Nutrient penetration instead governs vertical colony growth through thin layers of vertically oriented cells lifting up their ancestors from below [34]. We speculate that the nutritional environment around a bacterial cell greatly changes depending on the medium state, broth (liquid) or agar (solid), even when both states contain the same components, with a subsequent change in the metabolic function of the bacterial cell. This speculation is supported by our morphological observation and a previous study [27]. Scanning electron microscopy of a single colony of *C. jejuni* showed that cells with characteristic morphological forms predominate at different locations within the colony [27]. Spiral forms predominate at the edge, while coccoid forms predominate in the center, suggesting that these forms represent actively growing and inactive cells, respectively [27]. Similar morphological differences were found in different culture conditions in this study.

Regarding the establishment of a novel murine model for *Campylobacter* enteropathy, several models have been proposed [35–37], but they do not recapitulate host natural conditions because they require genetical and environmental manipulations of the mice. Therefore, a novel murine model that evaluates *C. jejuni* enteropathy is required to understand the pathogenesis and development of vaccines and therapeutics for *C. jejuni* [18,19]. In this regard, the strategy of modifying the bacterial culture condition is innovative in that it can induce intestinal inflammation as a host natural condition without genetical and environmental manipulations of mice. Fecal MPO, measured as an intestinal inflammation marker in this

study, is a common and available biomarker for both human and animal model studies [15,38]. An added advantage is that the dose of *C. jejuni* in this model is lower than that in other models that require large *C. jejuni* doses [18]. Although the model should be further improved to induce severe inflammation that causes bloody diarrhea, which is sometimes observed in human, we expect this model to provide the fundamental knowledge needed for innovative technological change to establish a rodent evaluation method for vaccines and therapeutic drugs in the medical research field.

In summary, culture conditions had a great influence on the metabolic function of *C. jejuni*, and the bacterial energy metabolism, as indicated by oxygen consumption, was upregulated in a liquid Bolton broth culture. These metabolic changes accelerated the growth of *C. jejuni* and subsequently promoted more frequent intestinal colonization in mice, resulting in the induction of intestinal inflammation. Thus, bacterial metabolic reprogramming is a promising target for the control of *Campylobacter* infection. Since the results obtained from this study is depending on a *C. jejuni* strain, further studies using various *C. jejuni* strains are required if our finding is widely acceptable in the pathogenesis of *C. jejuni*.

Methods

Study approval

All experiments involving mice were approved by the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health and Nutrition (Approval no. DS27-48R10) and of Osaka Metropolitan University (Approval no. BS22-Shi-73 and BS22-Jitsu-89) and were conducted in accordance with their guidelines.

Bacterial Culture

C. jejuni strain 81–176 was cultured slightly aerobically at 37°C by using Anaeropack (Mitsubishi Gas Chemical). Colombia 5% Horse Blood agar plates (Biomerieux, Tokyo, Japan), Bolton broth (OXOID), Bolton broth supplemented with 1.5% agar (Bolton agar plate), and MEM (Nacalai Tesque) were used for the experiments. l-Serine (Fujifilm), l-asparagine (Fujifilm), and AOA (Sigma-Aldrich, St. Louis, Missouri, USA) were added to the culture medium. *E. coli* O111 (IID561) was obtained from the Pathogenic Microbes Repository Unit (University of Tokyo, Tokyo, Japan) and cultured aerobically at 37°C in Bolton broth (OXOID). *S.* Enteritidis (RIMD1933006) was obtained from the Pathogenic Microbes Repository Unit (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and cultured aerobically at 37°C in Bolton broth (OXOID).

Infection Model

Male BALB/c mice (age, 7 weeks) were purchased from CLEA Japan, Inc (Tokyo, Japan). Mice were maintained with drinking water containing 0.5 g/l vancomycin (Nacalai Tesque) for 3 days and normal

water (without vancomycin) for 1 day, then *C. jejuni* suspended in 500 μl of Brucella broth was orally administered to the mice at 1.0×10^6 cells, which was estimated from the absorbance of *C. jejuni*-cultured medium at OD_{600} = 1 as 5.0×10^8 cells. Fecal samples were collected and the number of *C. jejuni* colonies was counted by using *Campylobacter* blood-free selective agar base (OXOID) supplemented with CCDA supplement (OXOID).

MPO ELISA

Fecal samples were collected, immediately frozen in liquid nitrogen, and stored at − 80°C. Samples were suspended at 100 mg/ml in 1 mM Tris (pH 7.5), 200 mM NaCl, 5 mM EDTA buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich), and PhosStop phosphatase inhibitor (1 tablet/10 ml) (Roche) and mixed by vortex for 10 min. After centrifugation at $1,500 \times g$ for 15 min at 4°C, MPO was measured in the supernatant using an MPO ELISA kit (Hycult Biotech Inc., PA, USA) in accordance with the manufacturer's instructions.

Flow Cytometric Analysis

Flow cytometry was performed as described previously, with some modification [39−41]. Small intestine and colon were opened longitudinally and washed vigorously in PBS on ice. The intestinal samples were cut into ~ 2-cm sections and incubated in 2 (for small intestine) or 4 (for colon) mg/ml collagenase (Wako) in RPMI1640 medium (Sigma-Aldrich) containing 2% (vol/vol) newborn calf serum (Equitech-Bio, Kerrville, Texas, USA) for 15 min at 37°C with stirring. The cell suspensions were filtered through cell strainers (pore size, 100 μm; BD Biosciences, Franklin Lakes, New Jersey, USA). This treatment with collagenase was repeated again to prepare cell samples.

For flow cytometric analysis, intestinal lymphocytes were obtained from the cell samples by purification with Percoll (GE Healthcare, Tokyo, Japan) as a cell layer between 40% and 75% solution after gradient-centrifugation ($820 \times g$, 20 min, 20°C). These lymphocyte samples were stained with an anti-CD16/32 monoclonal antibody (TruStain fcX; Biolegend, San Diego, California, USA) and 7-AAD (Biolegend) to avoid non-specific staining and detect dead cells, respectively. The cells were further stained with the fluorescently labeled antibodies BV421-anti-CD45 (Biolegend, clone 30-F11), FITC-anti-Ly6G (Biolegend, clone 1A8), and APC-Cy7-anti-CD11b (Biolegend, clone M1/70). Samples were analyzed by using MACSQuant (Miltenyi Biotech, Bergish Gladbach, Germany), and data analysis was performed by using FlowJo 9.9 (Tree Star, Ashland, Oregon, USA).

Histologic Analysis

Frozen tissue was analyzed histologically as described previously with some modification [42]. Tissue samples were washed with PBS on ice and frozen in Tissue-TeK OCT compound (Sakura Finetek, Tokyo, Japan) in liquid nitrogen. Frozen tissue sections (6 μm) were prepared by using a cryostat (model

CM3050 S) and were fixed for 30 min at 4°C in prechilled 95% ethanol (Nacalai Tesque) followed by 1 min at room temperature in prechilled 100% acetone (Nacalai Tesque).

For hematoxylin and eosin (HE) staining, tissue sections were washed with running water for 10 min, stained with Mayer hematoxylin solution (Wako) for 10 min, and washed with running water for 30 min. Tissue sections were then stained with 1% eosin Y solution (Wako) for 1 min, washed with running water for 10 s, and dehydrated through increasing concentrations of ethanol (Nacalai Tesque) for 1 min at each concentration, 70–100%. Tissue sections underwent final dehydration in xylene (Nacalai Tesque) for 3 min and were mounted in Permount (Falma, Tokyo, Japan).

For immune-histological analysis, tissue sections were washed with PBS for 10 min and then blocked in 2% (vol/vol) newborn calf serum in PBS for 30 min at room temperature in an incubation chamber (Cosmo Bio, Tokyo, Japan). Tissue sections were incubated with AF647-labeled anti-Ly6G monoclonal antibody (Biolegend, clone 1A8; 1:100) and PE-labeled anti-EpCAM monoclonal antibody (Biolegend, clone G8.8; 1:100) in 2% (vol/vol) newborn calf serum in PBS for 16 h at 4°C in the incubation chamber, washed once for 5 min each in 0.1% (vol/vol) Tween-20 (Nacalai Tesque) with PBS and in PBS only, and then stained with DAPI (AAT Bioquest, Sunnyvale, California, USA; 1 μM) for 10 min at room temperature in the incubation chamber. Finally, tissue sections were washed twice with PBS, mounted in Fluoromount (Diagnostic BioSystems, Pleasanton, California, USA), and examined under a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

Whole Transcriptome RNA-Seq

C. jejuni was cultured on blood agar or in Bolton broth for 48 h and bacterial cells were collected by centrifugation at 10,000 × *g* for 5 min at 4°C. Total RNA was isolated from the cells using NucleoSpin RNA (Takara Bio Inc.) in accordance with the manufacturer's instructions. RNA samples were sent to Takara Bio Inc. for RNA-Seq analysis. Briefly, RNA was quantified using NanoDrop (Thermo Fisher Scientific) and the fragment size distribution of RNA was assessed on Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, California, USA). After ribosomal RNA depletion by using a Ribo-Zero Magnetic kit for gram-negative bacteria (Illumina), a cDNA library was constructed using Agilent XT-Auto System (Agilent Technologies) with TruSeq Stranded mRNA Library Prep (Illumina) and IDT for Illumina - TruSeq RNA UD Indexes (Illumina) in accordance with Ribo-Zero rRNA Removal kit reference guide v02 and TruSeq Standard mRNA reference guide v00. We then performed 150-bp paired-end sequencing by using NovaSeq 6000 (Illumina) with NovaSeq 6000 S4 Reagent kit and NovaSeq Xp 4-Line kit in accordance with NovaSeq 6000 sequencing system guide v11 and bcl2fastq2 conversion software v2.20 software guide, dated February 2019. RNA-Seq data analysis was performed using STAR and Genedata Profiler Genome (Genedata) to map the RNA-Seq reads to the *C. jejuni* strain 81–176 reference genome (Genbank accession no. CP000538.1) and to compute the gene expression level (as a transcript per million value) per genomics element.

Analysis Of Cell Cycle Arrest By CDT

A CDT-mediated cell cycle arrest assay was performed as previously described [43]. Briefly, *C. jejuni* was cultured for 48 h and bacterial cells were harvested and suspended in PBS. Then, the bacterial concentration was adjusted to OD_{600} = 10 and the suspension was sonicated 3 times for 1 min duration with storing for 1 min on ice using a handy sonicator (UR-20P; Tomy Seiko Co., Ltd, Tokyo, Japan). Bacterial sonic lysate was centrifuged at 12,000 × g for 10 min at 4°C, supernatant was collected, filtered through a 0.22 µm pore-size PVDF filter membrane (Merck KgaA, Darmstadt, Germany) and used for the cytotoxicity assay.

About 2.5×10^5 HeLa cells in 4 mL of a culture medium containing MEM (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 5% heat inactivated fetal bovine serum, 1% GlutaMax, and 1% antibiotic cocktail of streptomycin and penicillin (Life Technologies, Carlsbad, California, USA) were seeded in a 25 cm^2 flask and incubated with 400 µL of bacterial sonic lysate that had been diluted 1,000 times with PBS at 37°C under 5% CO_2 in air. After 48 h incubation, cells were collected by trypsin/EDTA and fixed with 70% ethanol on ice for 1 h. After that, cells were stained with 50 µg/mL of propidium iodide in PBS that contained 0.25 mg/mL of Rnase A (Sigma-Aldrich) at 4°C for 30 min in the dark. DNA content of cells was analyzed by flow cytometry analysis using CytoFLEX S (Beckman Coulter, Brea, California, USA).

Alcian Blue Staining

C. jejuni was cultured on blood agar, in Bolton broth, or on Bolton agar for 48 h and collected by centrifugation at 10,000 × g for 5 min at room temperature (25°C). Electrophoresis of the bacterial cells was performed using NuPAGE electrophoresis system (Invitrogen) with 4−12% discontinuous Bis-Tris gel and MES buffer. SeeBlue Plus2 prestained standard (Invitrogen) was used as a molecular weight size marker. Total protein was stained with Coomassie brilliant blue. For detection of LOS and CPS, after electrophoresis the gels were treated with 40% ethanol-5% acetate buffer for 15 min at room temperature (25°C) and then stained with 10% Alcian blue in 40% ethanol-5% acetate buffer at room temperature (25°C) in the dark overnight. The stained gels were washed with 40% ethanol-5% acetate buffer at room temperature (25°C) in the dark overnight. The image was captured using ImageQuant LAS 4000 (Fujifilm) and quantitative data analysis was performed using Multi Gayge v3.1 Imaging Software (Fujifilm) to calculate QL value.

Coculture of bone marrow−derived macrophages and C. jejuni

Bone marrow−derived macrophages were prepared as described previously [44]. Briefly, bone marrow cells were aseptically isolated from mouse femora and cultured (5×10^5 cells/ml) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 ng/ml macrophage colony−stimulating factor (Peprotech, Rocky Hill, NJ, USA) in a temperature-responsive 60-mm culture dish

(RepCell, CellSeed, Tokyo, Japan) at 37°C under 5% CO_2 in air. After 6 days incubation, macrophages were harvested and redistributed (5×10^5 cells/ml) onto a 96-well plate. After incubation with heat-killed (70°C, 30 min) *C. jejuni* at several different doses for 48 h, TNF-α was measured in the culture supernatant by using a Cytometric Bead Array Mouse Inflammation kit (BD Bioscience).

Scanning Electron Microscopy

C. jejuni was cultured on blood agar, in Bolton broth, or on Bolton agar for 48 h, and bacterial cells were collected by centrifugation at 10,000 × *g* for 5 min at 4°C and fixed with 2% glutaraldehyde in 0.1 M phosphate buffer. The samples were sent to Hanaichi UltraStructure Research Institute and observed by using scanning electron microscopy (JSM-7500F, JEOL Ltd., Tokyo, Japan).

Flux Analysis

C. jejuni was cultured in several different culture conditions and collected by centrifugation at 10,000 × *g* for 5 min at room temperature (25°C). *E. coli* and *S. enterica* were cultured in Bolton broth and collected by centrifugation at 10,000 × *g* for 5 min at room temperature (25°C). The bacterial cells were suspended in PBS at 5.0×10^7 cells/ml, which was a quantity estimated from the absorbance of bacterial cultured medium taking OD600 = 1 as 5.0×10^8 cells. 100 μl of the bacterial cell suspension was seeded in a 15-μg/ml Cell-Tak-coated Seahorse 24-well plate (Corning), and the plate was centrifuged at 1,400 x g for 10 min at room temperature (25°C), and then 400 μl of Brucella broth (BD) prewarmed to 37°C was added. After being pre-equilibrated for 1 h, the OCR was measured by using a Seahorse Bioscience XF24 Extracellular Flux Analyzer (Agilent Technologies). Xfe Wave software (Agilent Technologies) was used to analyze the results.

Liquid Chromatography–mass Spectrometry

Hydrophilic metabolites were extracted as previously described with a slight modification [45]. Bacterial cells were collected and washed with PBS twice and suspended in PBS. The cell suspension and bacterial cultured medium were diluted with water (Wako) to make 200 μl, mixed with 400 μl of methanol (Wako) containing methionine sulfone (Wako) as an internal standard, and then 400 μl of chloroform (Nacalai Tesque) was added. After centrifugation at 20,000 × *g* at 4°C for 15 min, 200 μl of supernatant was centrifugally filtered through a 5-kDa cutoff filter (Human Metabolome Technologies, Inc.). The filtrates were lyophilized, resuspended in water (Wako), and analyzed by liquid chromatography–mass spectrometry (LC-MS/MS). The LC-MS/MS analysis was performed as previously described [46] using a Nexera system (Shimadzu, Kyoto, Japan) equipped with two LC-40D pumps, a DGU-405 degasser, an SIL-40C autosampler, a CTO-40C column oven, and a CBM-40 control module, coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu). A pentafluorophenylpropyl column (Discovery HS F5, 150 mm × 2.1 mm, 3 μm; Sigma-Aldrich) was used to separate the metabolites. Instrument control and

data analysis were performed using the software LabSolutions LCMS with LC/MSMS method package for primary metabolites ver. 2 (Shimadzu).

To measure the total protein content, after centrifugation of the bacterial cell suspension treated with ethanol and chloroform as described above, the pellet was dried at 80°C, washed with frozen acetone, and resuspended with 0.1N NaOH. Protein concentration was measured using a BCA protein assay kit (Life Technologies) in accordance with the manufacturer's instructions.

Statistical analysis

Statistical significance was evaluated by one-way ANOVA for comparison of multiple groups and the Mann–Whitney U-test for two groups by using Prism 7 (GraphPad Software, La Jolla, California, USA). A P value less than 0.05 was considered to be significant (*P< 0.05, ** P< 0.01, n.s. not significant). Heatmap was created by using the R packages 'corrplot' (https://github.com/taiyun/corrplot) and 'superheat'[47].

Declarations

Author contribution

K.H. and J.K. designed the study and wrote the manuscript, K.H., Y.T., and M.F. performed animal experiments, K.H., N.H., A.H., and S.Y. performed genomic and bacterial analyses, K.H., T.N., and A.S. performed metabolome and immunological analyses. K.H. and S.K. performed biochemical analysis.

Disclosure

The authors declare no competing financial interests.

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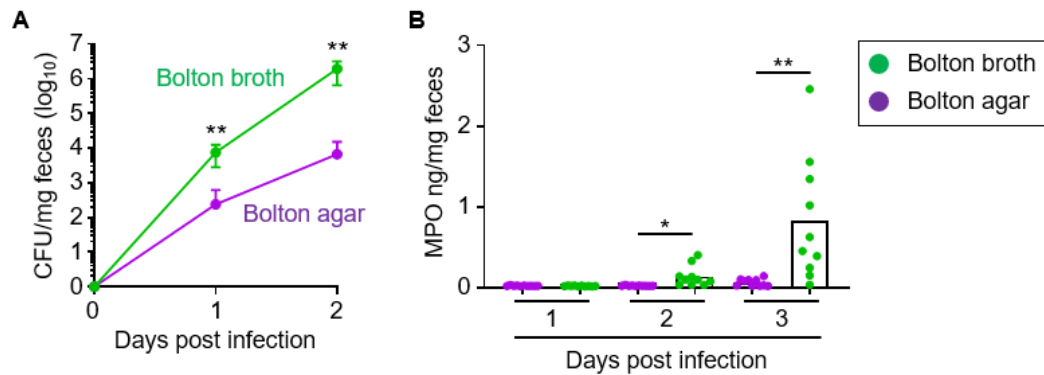
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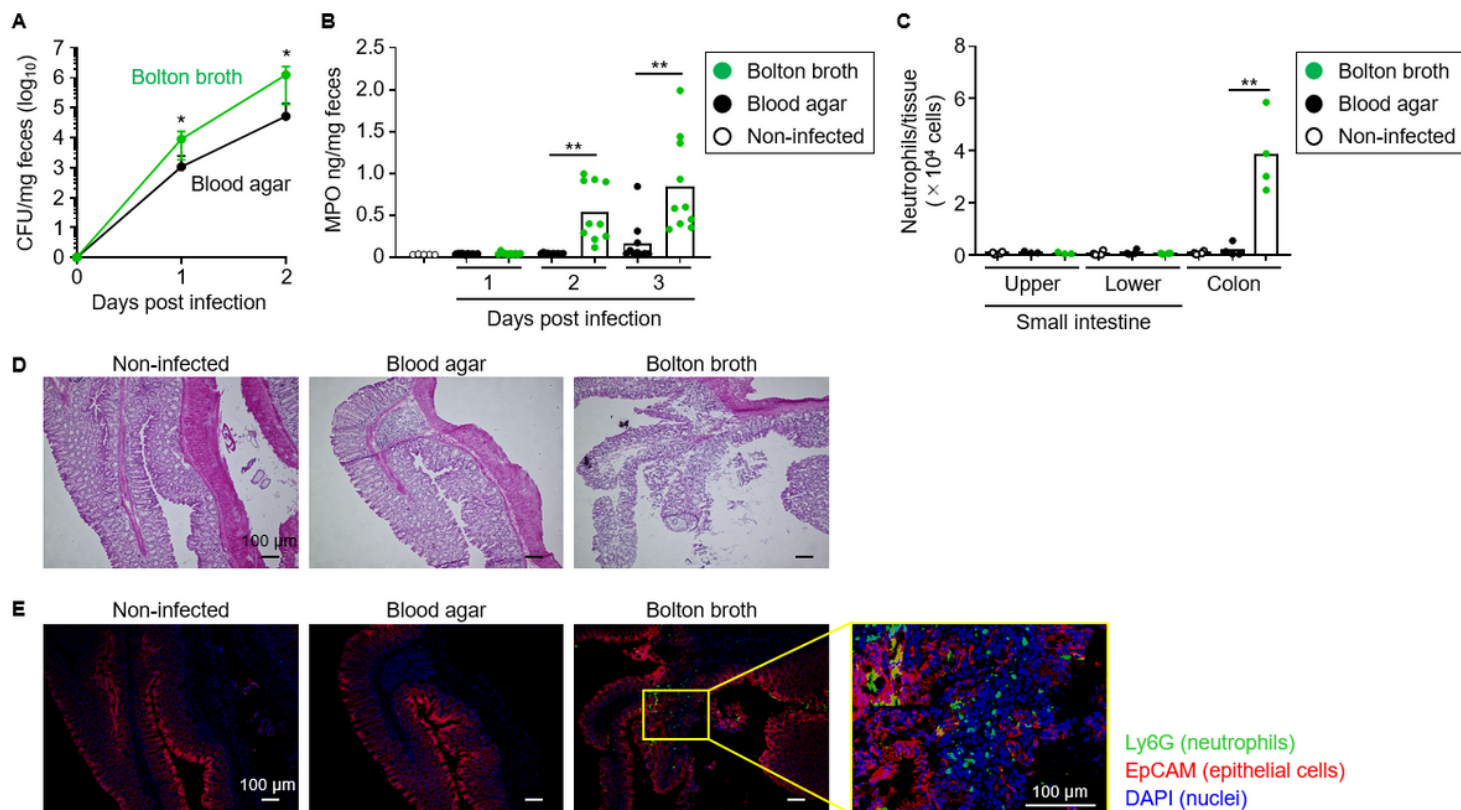
Figure 1

Figure 1

Induction of intestinal inflammation in mice by *C. jejuni* cultured in Bolton broth

(A) *C. jejuni* colonization in mice (n=10, mean ± 1 SD). Before infection (day 0) and on days 1 and 2 after infection of *C. jejuni* cultured in Bolton broth or on blood agar, fecal samples were collected, and the number of *C. jejuni* colonies was counted by using *Campylobacter* blood-free selective agar base supplemented with CCDA supplement. Data are combined from 2 independent experiments. *P< 0.05 (two-tailed Mann−Whitney *U*-test). (B) Neutrophil myeloperoxidase (MPO) in feces (n=10, mean). At days 1, 2, and 3 after infection, fecal samples were collected and MPO was measured. Data are combined from 2 independent experiments. **P< 0.01 (two-tailed Mann−Whitney *U*-test). (C) Number of neutrophils in the intestine at day 3 post-infection (n=4, mean). Neutrophils were defined as CD45$^+$CD11b$^+$Ly6G^{high} cells, as shown in Fig. S1, and counted in the small intestine (upper and lower sites) and colon by using flow cytometry. Data are combined from 2 independent experiments. **P< 0.01 (one-way ANOVA). (D) Hematoxylin and eosin (HE) staining of colon at day 3 post-infection. (E) Immunohistologic analysis of

colon at day 3 after infection. Neutrophils, epithelial cells, and nuclei were visualized by using Ly6G monoclonal antibody (green), EpCAM monoclonal antibody (red), and DAPI (blue) staining, respectively.
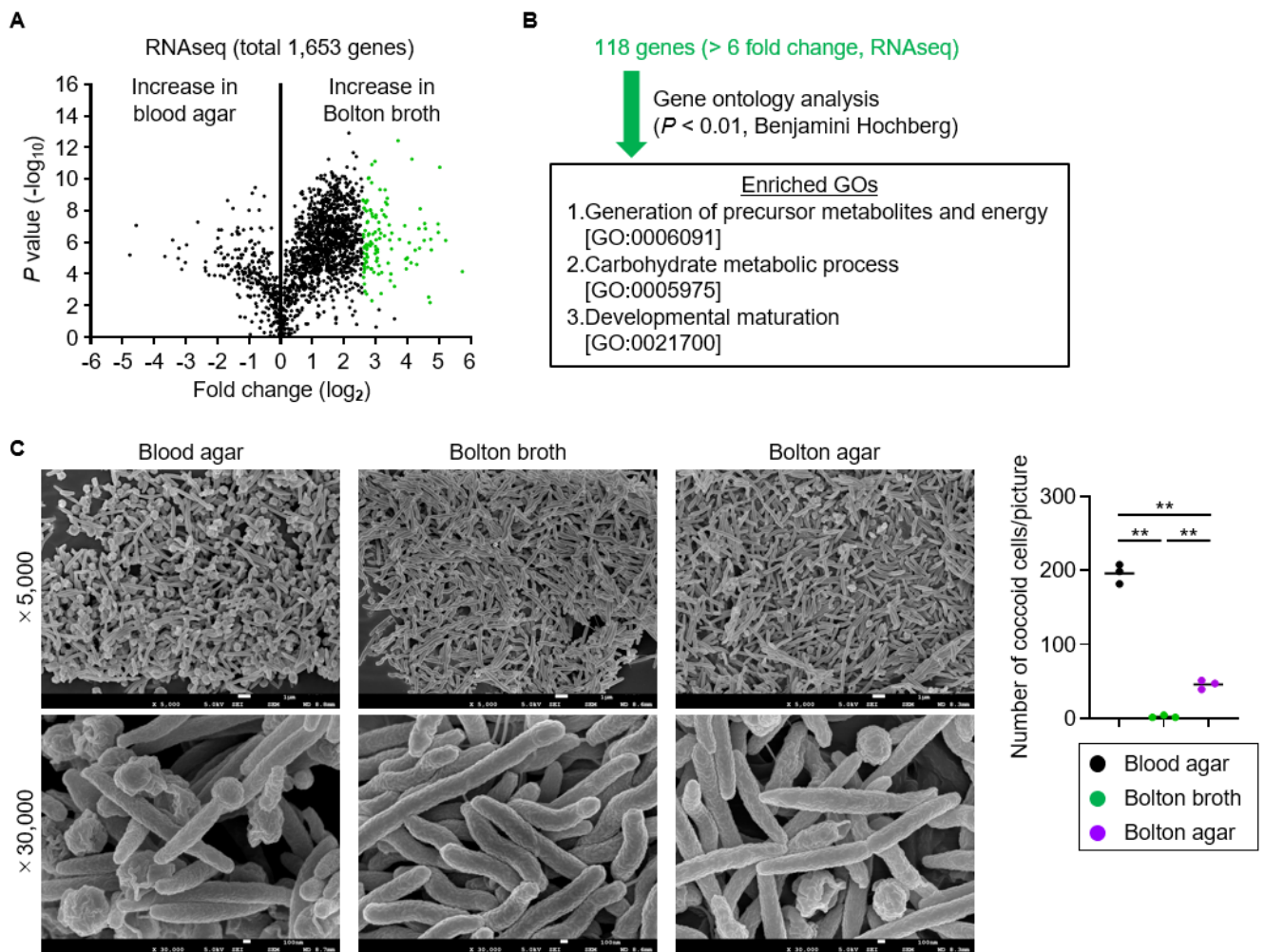

Figure 2

Figure 2

The medium state influences the pathogenicity of *C. jejuni*

(A) *C. jejuni* colonization in mice (n=10, mean ± 1 SD). Before infection (day 0) and on days 1 and 2 post infection of *C. jejuni* cultured in Bolton broth or on Bolton agar, fecal samples were collected, and the number of *C. jejuni* colonies was counted by using *Campylobacter* blood-free selective agar base supplemented with CCDA supplement. Data are combined from 2 independent experiments. $**P< 0.01$ (two-tailed Mann–Whitney U-test). (B) Neutrophil myeloperoxidase (MPO) in feces (n=10, mean). At days 1, 2, and 3 after infection, fecal samples were collected and MPO was measured. Data are combined from 2 independent experiments. $*P< 0.05$; $**P< 0.01$ (one-way ANOVA).
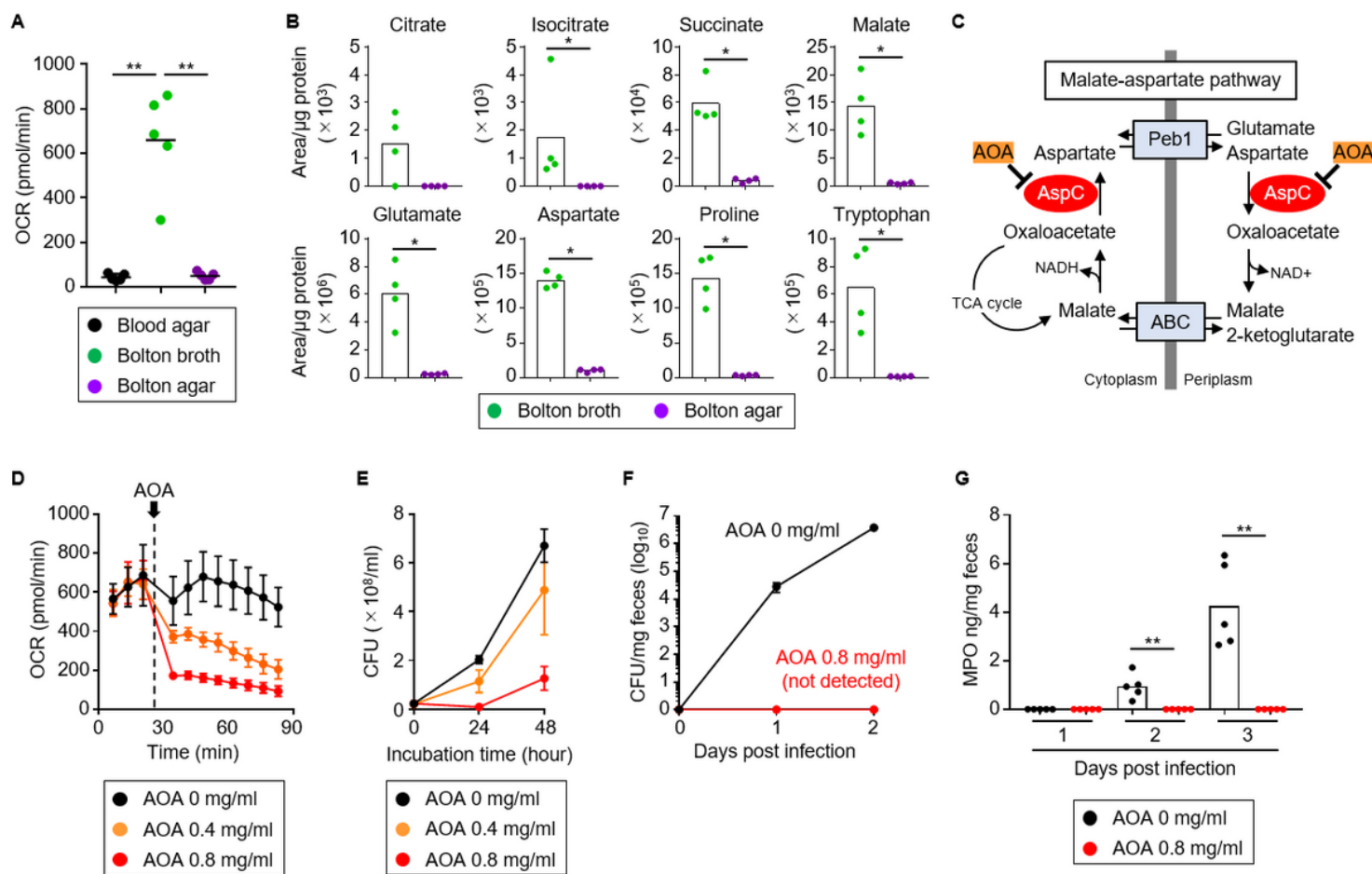
Figure 3

Figure 3

Gene expression analysis and anatomical structure of *C. jejuni*

(A) Volcano plot of 1,653 genes of *C. jejuni* cultured in Bolton broth or on blood agar (n=4). The green dots represent 118 genes that increased in expression more than 6-fold in the culture of Bolton broth compared with blood agar. (B) Gene ontology analysis using TargetMine [26] for the 118 genes. (C) Morphological observation of *C. jejuni* cultured in Bolton broth, on blood agar, and on Bolton agar by using scanning electron microscopy (n=3, mean). **$P < 0.01$ (one-way ANOVA).

Figure 4

Figure 4

Metabolic changes of *C. jejuni* and its pathogenicity

(A) Oxygen consumption of *C. jejuni* (n=5, mean). The oxygen consumption rate (OCR) of *C. jejuni* cultured on blood agar, in Bolton broth, or on Bolton agar was measured. Data are representative of 2 independent experiments. **$P < 0.01$ (one-way ANOVA). (B) Liquid chromatography-tandem mass spectroscopy analysis of *C. jejuni* (n=4, mean). The 8 metabolites forming a single cluster (aspartate, glutamate, proline, succinate, malate, isocitrate, citrate, and tryptophan) were selected from Fig. S6. Data are representative of 2 independent experiments. *$P < 0.05$ (two-tailed Mann−Whitney *U*-test). (C) Schematic diagram of malate−aspartate shuttle in the tricarboxylic acid (TCA) cycle. (D) Inhibition of oxygen consumption of *C. jejuni* by the aspartate aminotransferase inhibitor aminooxyacetate (AOA) (n=5, mean ± 1 SD). OCR was monitored in *C. jejuni* cultured in Bolton broth upon the addition of AOA. Data are representative of 2 independent experiments. (E) Inhibition of *C. jejuni* growth by AOA (n=4, mean ± 1 SD). *C. jejuni* was cultured in Bolton broth in the presence or absence of AOA, and the number of *C. jejuni* colonies was counted. Data are representative of 2 independent experiments. (F) Inhibition of *C. jejuni* colonization in mice by AOA (n=5, mean ± 1 SD). *C. jejuni* was cultured in Bolton broth in the

presence or absence of AOA and the bacterial cells were administered into mice. Before infection (day 0) and on days 1 and 2 post-infection, fecal samples were collected, and the number of *C. jejuni* colonies was counted by using *Campylobacter* blood-free selective agar base supplemented with CCDA supplement. Data are representative of 2 independent experiments. (G) Inhibition of *C. jejuni*–induced MPO elevation in fecal samples in mice by AOA (n=5, mean). *C. jejuni* was cultured in Bolton broth in the presence or absence of AOA and the bacterial cells were administered into mice. At days 1, 2, and 3 post-infection, fecal samples were collected and MPO was measured. Data are representative of 2 independent experiments. **$P < 0.01$ (two-tailed Mann–Whitney U-test).
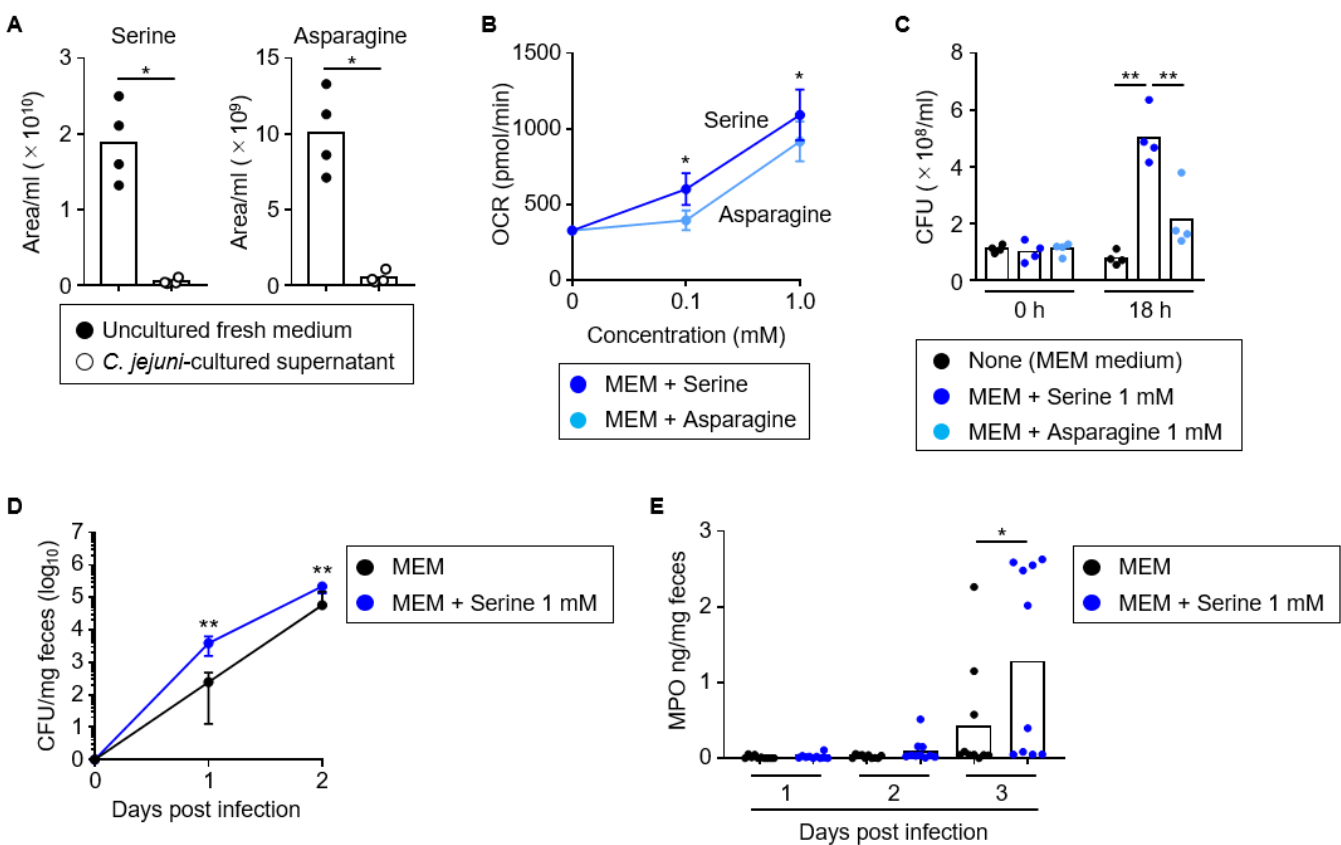

Figure 5

Figure 5

Crucial role of serine in the metabolism and pathogenicity of *C. jejuni*

(A) Utilization of serine and asparagine by *C. jejuni* (n=4, mean). Serine and asparagine were measured in the supernatant of *C. jejuni* cultured in Bolton broth or uncultured fresh medium by using liquid chromatography–mass spectrometry. Data are representative of 2 independent experiments. *$P < 0.05$ (two-tailed Mann–Whitney U-test). (B) Increased oxygen consumption in *C. jejuni* by serine or asparagine (n=5, mean ± 1 SD). The oxygen consumption rate (OCR) in *C. jejuni* cultured in minimum essential

medium (MEM) in the presence or absence of serine or asparagine was measured. Data are representative of 2 independent experiments. *$P < 0.05$ (two-tailed Mann−Whitney U-test). (C) Increased growth of *C. jejuni* by serine or asparagine (n=4, mean). *C. jejuni* was cultured in MEM in the presence or absence of serine or asparagine and the number of *C. jejuni* colonies was counted. Data are representative of 2 independent experiments. **$P < 0.01$ (one-way ANOVA). (D) Promoted *C. jejuni* colonization in mice by serine (n=10, mean ± 1 SD). *C. jejuni* was cultured in MEM in the presence or absence of serine and the bacterial cells were administered into mice. Before infection (day 0) and on days 1 and 2 after infection, fecal samples were collected, and the number of *C. jejuni* colonies was counted by using *Campylobacter* blood-free selective agar base supplemented with CCDA supplement. Data are combined from 2 independent experiments. **$P < 0.01$ (two-tailed Mann−Whitney U-test). (E) Elevation of *C. jejuni*-induced MPO in fecal samples in mice by serine (n=10, mean). *C. jejuni* was cultured in MEM in the presence or absence of serine and the bacterial cells were administered into mice. At days 1, 2, and 3 post infection, fecal samples were collected and MPO was measured. Data are combined from 2 independent experiments. *$P < 0.05$ (two-tailed Mann−Whitney U-test).

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