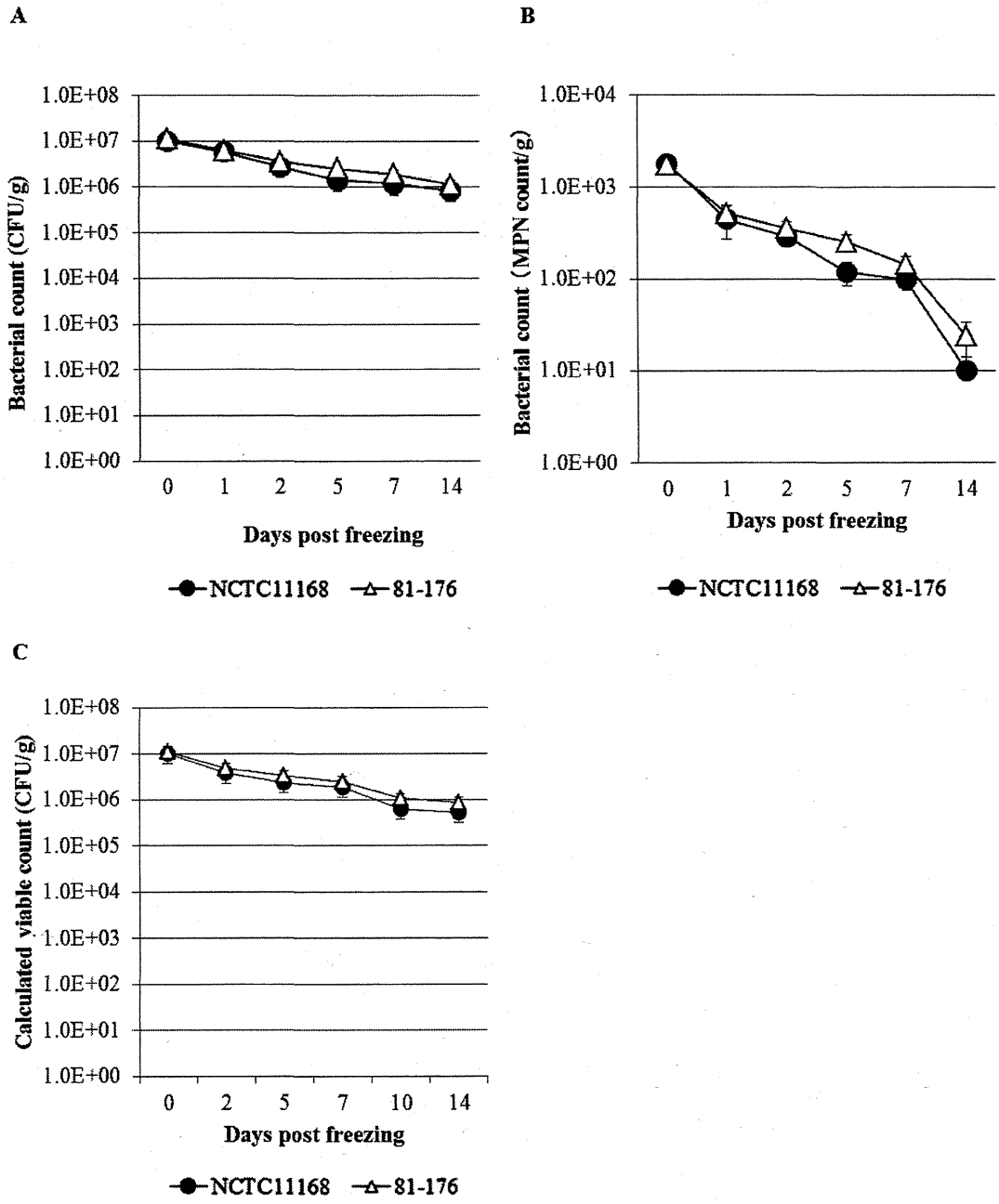

Fig. 1. Survival of *C. jejuni* NCTC11168 and 81-176 strains in minced chicken meats under freezing condition (−20℃). Sections A and B represent plate count data and section C represents viability of the spiked *C. jejuni* strains based on the EMA-PCR.

の菌数は低い値であった(Table 1). 大腸菌群数については，チルド処理群が2.80-4.51対数個/g（平均値3.79対数個/g)，急速冷凍処理群では1.92-4.43対数個/g（平均値3.14対数個/g)，腸内細菌科菌群数については，チルド処理群が2.34-4.36対数個/g（平均値3.59対数個/g)，急速冷凍処理群が2.08-4.30対数個/g（平均値3.01対数個/g）を示した(Table 1). 一般生菌数と同様に，大腸菌群数および腸内細菌科菌群数として最も高値を示した部位はモモであり，最も低値を示した部位はササミであった(Table 1). 急速冷凍処理群・チルド処理群の間において，指標菌数の有意差を認めた部位は砂肝のみであった（太字，Table 1). 指標菌の別では，腸内細菌科菌群数は他指標菌に比べ，冷凍処理による低減効果が低い傾向にあった(Table 1).

5. 市販輸入冷凍・国産チルド鶏モモ肉におけるカンピロバクター汚染率の比較

2014年5〜8月に都内で市販される輸入冷凍鶏モモ肉検体および国産チルド鶏モモ肉検体を対象にカンピロバクター汚染率を定性的に比較した. 輸入冷凍検体では，2.2%（1/45検体）の陽性率にとどまり，陽性検体から

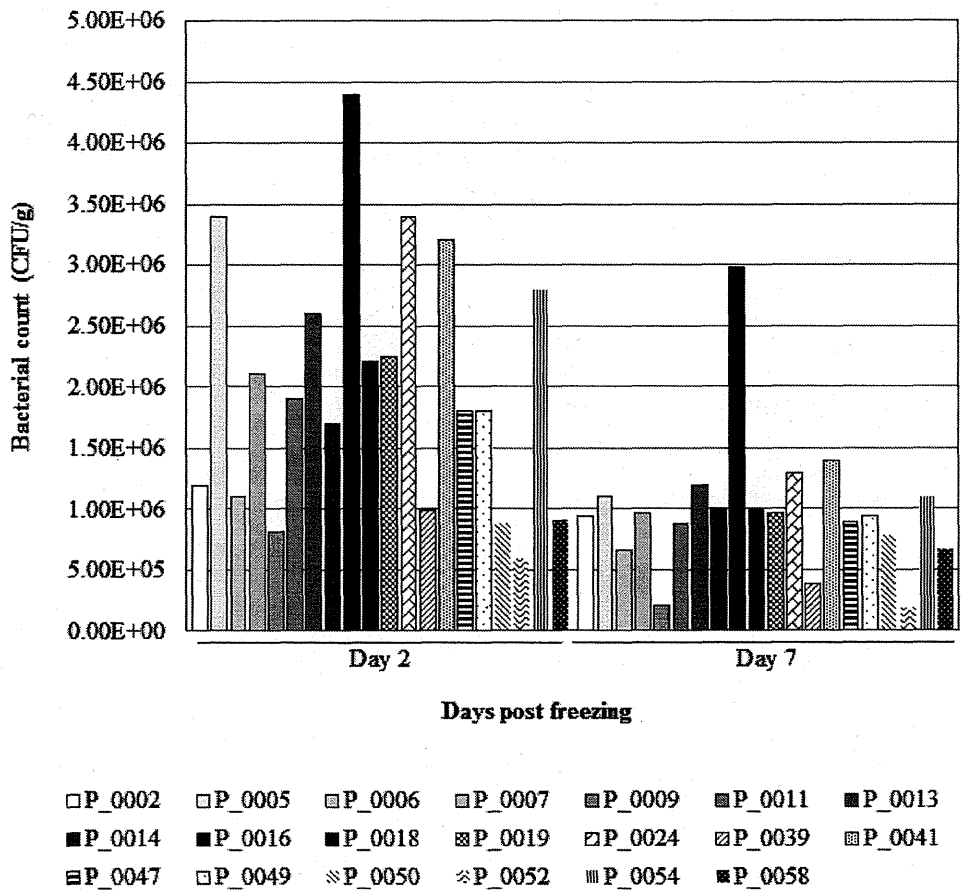

Fig. 2. Strain-to-strain diversity for the survival in minced chicken meats under freezing condition for days 2 and 7. A total of 20 *C. jejuni* strains originated from poultry were used for the comparative analysis. The details of each strain are described in ref. 1.

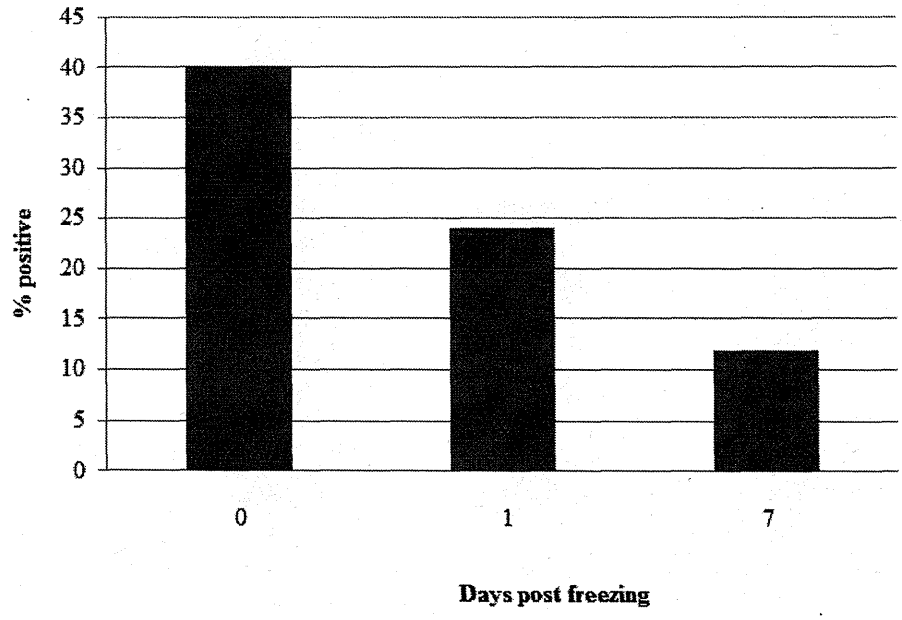

Fig. 3. Detection of *C. jejuni/coli* from naturally-contaminated minced chicken meats in treatment with freezing at −20℃ for 0 (non-frozen control), 1 and 7 days. Each groups are consisted of 50 samples, respectively. The group of day 0 (non-frozen control) exhibits 40% positivity for *C. jejuni/coli*.

Table 1. Numbers of *Campylobacter jejuni/coli* and indicator bacteria detected from chicken meat and offal treated with or without freezing

Sample	Treatment*1	No. *C. jejuni/coli* (MPN count/g)	Indicator bacterial count (CFU/g)		
			SPC*2	Coliforms	Enterobacteriaceae
Thigh	Chilled	11.00	6.0.E+04	2.6.E+04	1.8.E+04
	Frozen	11.00	7.8.E+04	2.7.E+04	2.0.E+04
Breast	Chilled	0.68	2.4.E+04	1.0.E+04	9.3.E+03
	Frozen	0.11	7.1.E+03	4.0.E+03	2.4.E+03
Tender	Chilled	0.27	6.0.E+03	6.3.E+02	2.2.E+02
	Frozen	0.19	6.0.E+02	8.3.E+01	1.2.E+02
Liver	Chilled	11.00	2.8.E+04	3.2.E+04	2.3.E+04
	Frozen	3.10	2.8.E+03	1.5.E+03	1.7.E+03
Gizzard	Chilled	11.00	**4.6.E+03***3	**1.8.E+03**	1.1.E+03
	Frozen	0.16	**5.8.E+02**	**3.7.E+02**	1.2.E+02

*1 Chilled, <10℃; Frozen, crust freezing at <−15℃.
*2 SPC, standard bacterial count.
*3 Bold means represent those means statistically differed between chilled- and frozen-samples.

Table 2. Prevalence of *C. jejuni/coli* in imported frozen or domestically-produced chilled chicken thighs

Country/Prefecture	No. samples tested	No. samples positive for *C. jejuni/coli* (% positive)	Isolate
Imported frozen chicken thighs			
Brazil	25	1 (4.0)	*C. coli*
Thailand	10	0 (0.0)	
USA	10	0 (0.0)	
Sub-total	45	1 (2.2)	*C. coli*
Domestically-produced chilled chicken thighs			
Gunma	15	4 (26.7)	*C. jejuni*
Saitama	15	3 (20.0)	*C. jejuni*
Iwate	15	5 (33.3)	*C. jejuni*
Sub-total	45	12 (26.7)	*C. jejuni*
Total	90	13 (14.4)	—

はC. coliのみが分離された．一方，国産チルド検体では，26.7%（12/45検体）の陽性率を示し，陽性検体からはいずれもC. jejuniが分離された(Table 2).

考　察

本研究では，鶏肉中のカンピロバクター汚染がヒト食中毒に強い疫学的関連性を示す状況を鑑み，同食鳥肉の流通段階における制御手法の一案として，冷凍処理を取り上げ，その有効性に関する諸検討を行った．

鶏肉中のカンピロバクター汚染に関しては，わが国と同様に米国においても，公衆衛生上，最も危害性の高い食品・病原体の組み合わせとして位置づけられている[3]．英国では鶏肉中のカンピロバクター汚染低減に有用と目される手法の許容性に関する消費者アンケート結果として，農場での衛生管理の徹底やワクチネーションとともに，冷凍処理がおおむね許容されることが報告されている[13]．冷凍処理に伴う鶏肉内カンピロバクター汚染低減に関する過去の知見として，数日間の冷凍では，0.91–1.44対数個の減少が[6, 19, 20]，3週間の冷凍では1.77–2.18対数個の減少が認められている[6, 20]．本研究で実施した，国産鶏挽肉を用いた添加回収試験においても，それらと同等の汚染低減効果が示され，その有効性が改めて検証された．また，カンピロバクターは，菌株間の遺伝的多様性に富み，一部の表現形質の差異としても顕れることが知られている[18]．本研究の成績により，カンピロバクターは菌株間で冷凍抵抗性に差異を示すことが明らかになった．一方，抵抗性に多様性を示す菌株のいずれも一定条件での冷凍処理を通じ，確実に生存菌数を低減させることも示された．

わが国で消費される鶏肉のおよそ3分の1は輸入品で占められているが[16]，過去には輸入冷凍鶏肉が国産チルド鶏肉に比べ，相対的に低い汚染率を示すことが報告されている[17]．輸入鶏肉の多くが船舶により冷凍状態で輸送される実態を鑑みると，本研究において示された，輸入鶏肉と国産チルド鶏肉検体間での陽性率の顕著な差異は，より長期的な冷凍処理が本菌の鶏肉内汚染制御に有効に機能していることを示唆しているといえよう．一方で，これを実用的に運用するためには，長期的な冷凍保存は望ましいものとは言えず，温度・時間の条件をさらに検討していく必要性が挙げられる．冷凍処理

には，低温ストレスのほか，冷凍・解凍を通じた浸透圧ストレス等の外的要因が介在している．菌株間で認められた冷凍抵抗性の多様性を裏づける科学的根拠はこれまで明らかにされていないが，本菌の低温下における生存には，ポリヌクレオチド・ホスホリラーゼ活性が必要であることが報告されている[7]．当該酵素活性を担う遺伝子配列の菌株間多様性に関する検討は，本研究において認められた菌株間での冷凍感受性の差異との関連性を探索するうえで有用かもしれない．

また，冷凍処理にかかわるその他の欠点としては，肉質低下に伴う経済的な損失のほか，ドリップの発生に伴う交差汚染も懸念される．国内の食鳥処理加工場の一部では，表面のみを急速冷凍させる「Crust-freezing法」も導入されており，実際に本研究において，急速冷凍処理群がチルド処理群に比べて，モモ検体を除き，低いカンピロバクター汚染菌数を示したことは，上述の欠点を最小限にとどめたうえで，本菌汚染を制御する手法としての応用が期待される．国内での馬肉の流通にあたっては，平成23年6月に，厚生労働省より，−20℃（中心温度）で48時間以上，−30℃（中心温度）で36時間以上，−40℃（中心温度）で18時間以上および急速冷凍装置を用いた場合には，−30℃（中心温度）18時間以上を保持する冷凍方法，ならびに液体窒素に浸す場合にあっては，1時間以上保持する方法の導入が寄生虫制御を目的として，通知された[10]．本研究で明らかにされた，表面急速冷凍法に伴うカンピロバクター汚染低減にかかわる知見を踏まえ，当該手法が検体にもたらす温度制御に関する知見を集積することで，より具体的かつ科学的な条件設定が可能になると思われる．

500〜800個ともいわれる本菌の最少発症菌数[12]を鑑みると，食鳥肉の生食は，少なからず感染リスクを伴うことは否定できない．こうした喫食形態にかかわる事項は，消費者の意識向上によるところが大きく，今後もリスクコミュニケーション等の啓発活動を持続的に行う必要性がある．一方で，多様化する食習慣を考慮するうえでは，生食による感染リスクを低減する手法の開発・導入も今後進めていく必要性があり，本研究の成績は，一つの科学的指標となりうるものと考えられる．

要　約

鶏肉におけるカンピロバクター・ジェジュニ／コリ汚染を流通段階で制御するための一手法として，冷凍処理の有効性を検証した．NCTC11168および81-176株を用いた添加回収試験の結果，鶏挽肉における生存性は，−20℃での2週間の冷凍処理により，最大で約1.9-2.3対数個の減少を認めた．40%の自然汚染率をあらわす鶏挽肉を同上温度での冷凍処理に供したところ，汚染率は1日後には半減し，一週間後にはさらにおよそ半減した．急速冷凍処理(Crust freezing)を行った食鳥部分肉（ムネ・ササミ・レバー・砂肝）は，チルド処理群に比べて，相対的に低いカンピロバクター汚染菌数を示した．しかし，モモ肉ではその差異は認められなかった．輸入冷凍鶏肉の本菌汚染率は，2.2%（1/45検体）と，国産チルド鶏肉検体の陽性率（26.7%，12/45検体）に比べて顕著に低い値を示した．以上の成績より，冷凍処理は，鶏肉におけるカンピロバクター汚染を低減する一手法であることが示された．

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Original article

Ex vivo proteomics of *Campylobacter jejuni* 81-176 reveal that FabG affects fatty acid composition to alter bacterial growth fitness in the chicken gut

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Abstract

Campylobacter jejuni is one of the leading causes of foodborne gastrointestinal illness worldwide. Here we performed ex vivo proteomic analysis of *C. jejuni* 81-176 in chicken, a main reservoir for human infection. At 0, 1 and 4 weeks post-infection (p.i.) with the GFP-expressing 81-176 strain, inocula were recovered from chicken ceca by cell sorting using flow cytometry. iTRAQ-coupled 2D-LC–MS/MS analyses that detected 55 *C. jejuni* proteins, among which either 3 (FabG, HydB, CJJ81176_0876) or 7 (MscS, CetB, FlhF, PurH, PglJ, LpxC, Icd) proteins exhibited >1.4-fold-increased expression at 1 or 4 week(s) p.i. compared with those at 0 weeks p.i., respectively. Deletion of the *fabG* gene clearly decreased the proportion of bacterial unsaturated fatty acids (UFAs) and chicken colonization. The UFA proportion of the parental strain was not altered when grown at 42 °C. These findings suggest that FabG might play a pivotal role in UFA production, linked to bacterial adaptation in the poultry host. To our knowledge, this is the first example of ex vivo *C. jejuni* proteomics, in which fatty acid metabolism might affect bacterial adaptation to the chicken host.

Keywords: *Campylobacter jejuni*; LC–MS; Chicken colonization; Fatty acid biosynthesis

1. Introduction

Campylobacter jejuni is one of the leading causes of foodborne illness in developed countries worldwide, with estimates from 2005 to 2011 of 14 cases of illness per 100,000 individuals annually in the US due to *Campylobacter* infection [1]. Epidemiological studies provide mounting evidence that chickens are one of the main sources of human campylobacteriosis [2–4]. Thus, control of *Campylobacter* infection on the farm is critical for prevention of human infection. However, it remains unclear how and why this microorganism establishes commensal colonization of the chicken gut. After oral challenge, *C. jejuni* immediately starts to colonize primarily in the cecum and lower small intestine of the chicken, reaching a plateau after 6 weeks of age [5]. To elucidate the molecular mechanisms underlying colonization, Hendrixson and DiRita (2004) first screened bacterial genes required for chicken colonization, identifying genes that were mainly responsible for flagellar motility/chemotaxis and lipo-oligosaccharide (LOS) biosynthesis [6]. A subsequent study identified a series of putative adhesins, including FlpA and Peb1A, as prerequisites for chicken colonization [7]. Those studies provide clear evidence for the essential role of the

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identified genes in establishing colonization, and most of the studies utilized a genetic background approach with the chicken model and colonization periods of 1–2 weeks. Thus, we could not exclude the possibility that the identified genes might indirectly affect colonization properties and might function only during the establishment stage of colonization.

More recently, Hu and co-workers used in vivo-induced antigen technology (IVIAT) to identify genes expressed in vivo in humans [8] and chickens [9]. Their results revealed differential gene expression between the two hosts. Because the immunoreactive targets might represent prolonged and/or predominant genetic expression, *C. jejuni* may respond differentially to host environments by altering expression of genes required for a series of functions, thereby leading to host adaptation. Thus, proteomic dynamics could provide informative data for improving our understanding of the molecular mechanisms underlying commensal colonization of the chicken by *C. jejuni*.

Here we report the first ex vivo proteomic analysis of *C. jejuni* infection during colonization of the chicken, by cell sorting using flow cytometry in combination with iTRAQ LC–MS/MS approaches. Among the detected proteins, we focused on the functional roles of fatty acid biosynthesis-related protein FabG, which exhibited increased expression during both early and late stages of colonization. The potential contribution of other proteins differentially expressed during colonization is also discussed.

2. Materials and methods

2.1. Bacterial strains and media

C. jejuni strain 81-176 harboring the GFP-expressing, kanamycin (Km)-resistant plasmid pWM1007 (81-176GFP) [10] was used for the colonization assay and as the parental strain for mutant construction. The *fabG* mutant (fabG–) was constructed essentially as described [11,12]. In brief, a 500-bp fragment upstream of the 5′ end and a 500-bp fragment downstream of the 3′ end of the *fabG* locus were amplified from the 81-176 strain via PCR using either fabG-s and fabG-BI-as or fabG-BI-s and fabG-as primers (Table S1). After *Bam*HI digestion, the two fragments were ligated and cloned into a pGEM-easy vector (Promega, Madison, WI, USA). A *cat* gene from plasmid pRY109 [12] was then inserted into the *Bam*HI site in the plasmid, and this allelic exchange plasmid (pGEM-fabG-Cm, Table 1) was introduced into the genome of strain 81-176 through natural transformation. Successful transformants were selected on Mueller-Hinton agar (MHA) (Becton–Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% horse blood and chloramphenicol (20 μg ml^{-1}). Allelic replacement was confirmed via nucleotide sequencing using primers fabG-conf-s and fabG-conf-as (Table S1). The *fabG* locus and the upstream region predicted by the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) to contain −35 and −10 promoter binding sites were amplified via PCR using fabG-compl-s and fabG-compl-as primers (Table S1). The resultant amplicons were cloned into the *Xba*I/*Eco*RI sites of the pRY108 plasmid [12], yielding pRY108-fabG-Km (Table 1). This plasmid was introduced into the *fabG* mutant through natural transformation, and the transformants were recovered on MHA containing Km (10 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}). Complementation was confirmed via PCR using the fabG-compl-conf-s and fabG-compl-conf-as primers (Table S1). The *flaA* mutant [13] was used as a non-motile mutant for the cell adhesion assay. All of the *C. jejuni* strains were routinely grown at 37 °C in Mueller-Hinton broth (MHB) or on MHA (Becton–Dickinson) with the appropriate antibiotics under microaerobic conditions (5% CO_2, 10% O_2, 85% N_2) in a multi-gas incubator (Astec-Bio, Fukuoka, Japan). *Escherichia coli* DH5α was maintained at 37 °C in Luria–Bertani (LB) broth or LB agar plates.

Table 1
Bacterial strains and plasmids used in this study.

Name	Nomenclature	Reference/ source
Bacteria strain		
81-176GFP	*C. jejuni* 81-176 harboring pWM1007	[10]
fabG–	81-176GFP fabG mutant, (Km/Cm)R	This study
flaA–	81-176GFP flaA mutant, CmR	[13]
DH5α	*E. coli* strain for DNA manipulation	Sigma–Aldrich
fabG–/+	*fabG*– strain complemented with fabG	This study
Plasmid		
pRY109	CmR shuttle vector	[12]
pGEM-fabG-Cm	pGEM::fabG– cat for recombination	This study
pRY108-fabG-Km	pRY108::fabG for complementation	This study

2.2. Animals

Specific pathogen-free (SPF) 14-day-old female white leghorn chickens were obtained from Nissei Bio (Yamanashi, Japan). During the 3 days of acclimation prior to use, we confirmed that all birds were negative for *Campylobacter* by PCR testing [14] using fecal swabs. All animals were fed sterile water and pelleted food (Nissei Bio) *ad libitum*. The animal experiments were performed according to animal care and use guidelines of the National Institute of Health Sciences, Japan.

2.3. Colonization experiments

The chickens were orally challenged with 500 μl of MHB containing approximately 2.8×10^7 81-176GFP cells grown in MHB at 37 °C with shaking (120 rpm) for 18 h. The animals (n = 5 each) were euthanized at 1-week intervals for up to 4 weeks p.i. and post-mortem ceca (>2 g contents) were aseptically collected from each bird. One gram of each sample was homogenized in 9 ml of sterile phosphate-buffered saline (PBS) (ThermoFisher Scientific, Carlsbad, CA) and then spread onto mCCDA agar containing Km

(200 μg/ml) to count viable cells. Simultaneously, 1 g of the remaining samples at 1 week p.i. or 4 weeks p.i. was pooled per group (5 samples each) and suspended in 50 ml of ice-cold 4% paraformaldehyde (PFA)/PBS for 30 min. After infiltration of the homogenates through 100 μm-pore and subsequently 40 μm-pore Steriflip filter units (Merck-Millipore, Billerica, MA), bacterial cells were separated from the filtrates using the buoyant density gradient centrifugation method [14].

2.4. Flow cytometry and cell sorting of GFP(+) C. jejuni

Flow cytometry was performed using a FACSCalibur (Becton Dickinson) equipped with an argon ion laser (488 nm) and bandpass filters at 530 ± 30 nm (Fluorescence 1, FL1) or 650 ± 13 nm (Fluorescence 2, FL2). The data were analyzed using CellQuest (Becton Dickinson). Each sample was acquired between 20 s and 30 s at a low rate, and the cell concentration was adjusted to maintain a count of 300–400 events/s. Unlabeled beads with a diameter of 1 μm (Life technologies) were used as a negative control (NC). To enumerate the GFP-positive population, we used 4% PFA-fixed 81-176GFP cells that were cultivated in MHB at 37 °C for 18 h as a positive control (PC) for gate setting.

For the sorting experiment, a FACSCant (Becton Dickinson) was used, and sorting criteria were defined by drawing gates in two bivariate dot plots representing forward scatter (FSC) vs. side scatter (SSC) and FL1 (GFP) vs. FL2 (PI). Using a 70-μm nozzle, GFP-positive gated cells were sorted in R mode to final cell numbers of 2×10^5 to 5×10^5 cells.

2.5. Protein digestion, iTRAQ labeling and strong cation exchange fractionation

Sorted cells were washed twice with PBS, followed by treatment with lysozyme, DNaseI, and RNaseA (Takara Bio, Shiga, Japan) in PBS. After washing four times with 30 mM Tris–HCl (pH 7.4), cell pellets were sonicated in lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, and 4% CHAPS, pH 8.0) to prepare protein extraction essentially as described [15]. The extracts were purified by ultrafiltration using concentrators and a Spin 5K MWCO 4 ml package (Agilent Technologies, Santa Clara, CA) and precipitated in trichloroacetic acid. The pellet was then dissolved in 50 mM triethylammonium bicarbonate. After quantification of protein concentrations using the BCA Protein assay (ThermoFischer Scientific), the samples (40 μg each) were reduced, alkylated and digested with trypsin. Each digest was then labeled accordingly using iTRAQ reagents (control, 115; 1 week p.i., 116; 4 weeks p.i., 117) (AB SCIEX).

2.6. 2D-LC–MS/MS analysis

The 2D-peptide fractionation was performed using a DiNa Direct Nano-flow LC system (KYA Technologies, Tokyo, Japan) with a strong cation exchange (SCX) column (HiQ sil SCX, 0.5-mm inside diameter (i.d.) × 35 mm), a reverse-phase (RP) trap column (HiQ Sil C18-3, 0.8-mm i.d. × 3 mm) and an RP analytical column (HiQ Sil C18-3 Gradient, 0.15-mm i.d. × 50 mm). Peptides that were trapped on the SCX column were eluted via an injection of ammonium formate buffer (pH 3.0, containing 2% acetonitrile (ACN)) at 6 different concentrations (10, 50, 100, 200, 300, and 500 mM). The eluent was subjected directly to the trap column and sequentially to the analytical column using a gradient of 0–100% solvent B in solvent A for 170 min (solvent A: 0.1% trifluoroacetic acid (TFA), 2% ACN; solvent B: 0.1% TFA, 70% ACN) at a flow rate of 200 nl/min. From 15 min to 158 min, one spot was eluted every 30 s, mixed with matrix (4 mg/ml of α-cyano-4-hydroxycinnamic acid (CHCA)/70% ACN/0.1% TFA) and analyzed accordingly using a 4800 MALDI-TOF/TOF analyzer (AB Sciex).

2.7. Data analysis

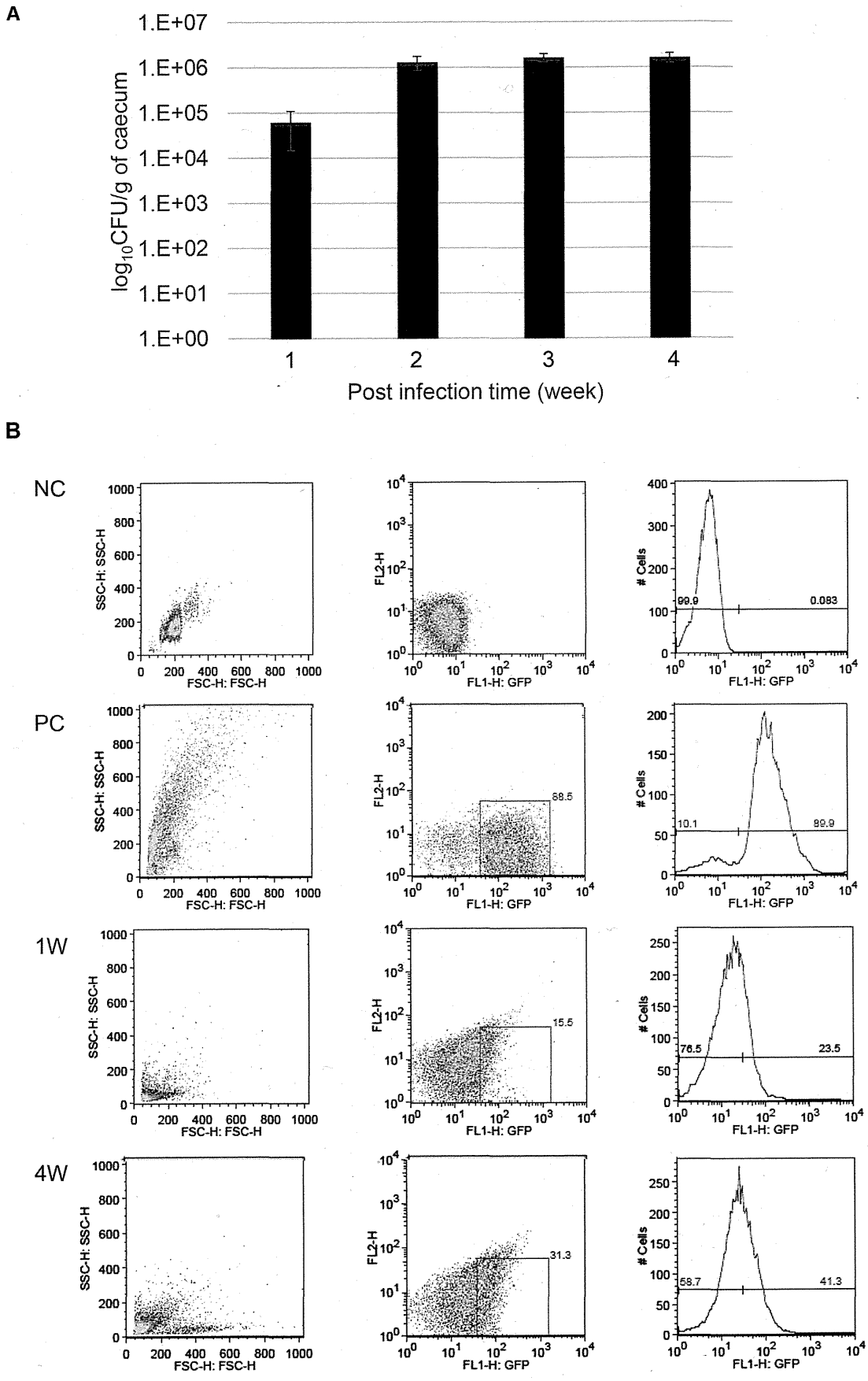
Relative quantification and protein identification were performed using ProteinPilot ver. 4 (ABSciex). Each MS/MS spectrum was searched against the genome sequence of *C. jejuni* 81-176 in the NCBI database (accession No. CP000538). Cysteine modification, phosphorylation and amidation were allowed for inclusion during a database search in the ProteinPilot program. The identified proteins were grouped by the Paragon algorithm (ABSciex) to minimize redundancy. The proteins that remained after a 1.4-fold cutoff for changed status for all iTRAQ ratios were selected for further analysis.

2.8. Chicken colonization assay

Chickens aged 17 days were orally inoculated with $1.4–2.2 \times 10^7$ CFUs of 81-176GFP, fabG–, or fabG–/+ strains that were cultured in MHB at 37 °C. After 1 and 4 weeks, 1 g of cecal sample was aseptically removed from 5 birds per group and homogenized in 9 ml PBS. The homogenate and its serial dilutions were spread onto mCCDA agar containing Km (200 μg/ml) to count viable bacterial cells.

2.9. Cell adhesion and growth assays

The cell adhesion properties of the bacterial strains to INT407 cells and their in vitro growth properties in MHB at 37 °C or 42 °C were examined as described previously [16]. Briefly, INT407 cells were seeded into 24-well culture plates (TPP) (3.0×10^5 cells well^{-1}) and incubated in RPMI1640 medium (Life Technologies, Carlsbad, CA, USA) for 20 h at 37 °C in a humidified CO_2 incubator. The cells were then rinsed and inoculated with *C. jejuni* at a multiplicity of infection (m.o.i.) of 100. At 60 min and 120 min post-infection, cells were washed three times with PBS to remove non-adherent bacteria, followed by cell detachment using 0.1% saponin in PBS. Serial dilutions of the suspensions were plated onto MH agar to determine the numbers of viable, cell-associated bacteria.


A
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
log10CFU/g of caecum
1
2
3
4
Post infection time (week)
B
NC
PC
1W
4W
SSC-H: SSC-H
FSC-H: FSC-H
FL2-H
FL1-H: GFP
Cells
99.9
0.083
68.5
10.1
99.9
15.5
76.5
23.5
31.3
58.7
41.3

2.10. Fatty acid methyl ester (FAME) assay

Bacterial cells grown on MHA for 22 h at either 37 °C or 42 °C were harvested, washed three times with PBS and freeze-dried. The pellets were then used to extract and measure fatty acid methyl ester (FAME) using the Sherlock Microbial ID system (MIDI Inc., Newark, DE, USA) accordingly. Each sample was tested twice, and mean values were used for a comparative analysis.

2.11. Statistics

Data for the plate counts and growth curve are presented as the means ± standard deviation (SD) of three independent experimental sets. 2D-LC–MS/MS and FAME data each represent means of two tests. For the in silico prediction of protein–protein interaction network mapping, STRING ver. 9.1 (http://string-db.org) and KEGG pathway database (http://www.genome.jp/kegg/pathway.html) were used accordingly.

3. Results and discussion

3.1. Ex vivo proteomic profiles of C. jejuni in the chicken cecum tract

Chickens were orally inoculated with *C. jejuni* 81-176GFP (Table 1), followed by feeding for up to 4 weeks. At 1-week intervals p.i., the numbers of inoculum in the chicken cecum were monitored, confirming an increase for up to 2 weeks ($1.3 \times 10^6 \pm 4.4 \times 10^5$ CFU/g), which then reached a plateau until 4 weeks p.i. (between 1.2×10^6 and 2.3×10^6 CFU/g) (Fig. 1A). We then selected samples at 1 week and 4 weeks p.i. as initial or stable colonizers (we defined "colonization" as residing stably or increasingly in chicken cecum compared with those at day 0), respectively. A flow cytometry-cell sorting approach in combination with LC–MS/MS (Fig. S1) was used to collect GFP-positive cells from the cecum samples, which resulted in the sorting of 15.5% and 31.3% of total bacterial cells from the samples at 1 week or 4 weeks p.i., respectively (Fig. 1B). A total of 55 proteins were finally detected as *C. jejuni* 81-176 proteins from 2343 peptide spectra (Table S2). We then attempted to identify proteins exhibiting increased expression in chicken ceca either at 1 week or 4 weeks p.i., to show bacterial proteins that positively associate with host colonization. Using a cutoff value of >0.95, 21 proteins were screened out (red color, Table S2), among which 10 proteins exhibited a >1.4-fold increase in expression at either 1 or 4 weeks p.i. (or at both time points) compared with the control (0 weeks p.i.) (bold in Table S2); these proteins included 3 (FabG, HydB, CJJ81176_0876) and 7 (MscS, CetB, FlhF, PurH, PglJ, LpxC and Icd) proteins that exhibited increased expression either at 1 week or 4 weeks p.i. (Table 2). Their (putative) functional roles in bacterial colonization are described below.

3.1.1. Flagellar motility and chemotaxis

Bacterial motility/chemotaxis are prerequisites for colonization [16]. We detected one flagellar biosynthetic protein FlhF that displayed increasing expression (1.82-fold) at 4 weeks p.i. compared with the control. This protein functions as a GTPase that affects the appropriate biosynthesis of flagella and expression of σ^{54}-dependent genes [17]. More recently, FlhF was identified as one of the predominant immunoreactive proteins in chickens [18], supporting its apparent expression in vivo. The reason for its reduced expression at 1 week p.i. (0.84-fold) has remained unclear; however, its increased expression at a later stage suggests its contribution to continuous colonization.

3.1.2. Energy/respiratory metabolism

HydB (quinone-reactive Ni/Fe-hydrogenase B) exhibited increased expression at both 1 week p.i. (3.6-fold) and 4 weeks p.i. (3.47-fold) compared with the control. Mutation of the *hydB* gene does not alter motility, but it impairs adherence to chicken intestinal epithelia [19] as well as chicken colonization [20]. In the closely related bacteria *Helicobacter pylori*, the homologous gene cluster *hydABCDE* was found to be transcriptionally regulated by iron in a Fur (ferric uptake regulator)-dependent manner [21]. Since hydrogen can be used as an electron donor by this pathogen through the activity of HydABCD [22], increased expression of the HydB might indicate elevated respiratory activity of this pathogen during colonization.

CetB (bipartite energy taxis response protein) also exhibited a 1.88-fold increase in expression at 4 weeks, but not at 1 week p.i. (0.82-fold) compared with the control. This metabolic system is required not only for growth, but also for bacterial chemotaxis/motility via the CheA system [23,24]. CetB senses extracellular changes through the PAS domain [25], and the increased CetB expression might indicate an altered host environment at later stages. In such a scenario, *C. jejuni* might utilize this sensing machinery for prolonged colonization.

PurH putatively functions as an aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase that converts AICAR to form 5-formamide-4-imidazolecarboxamide ribotide (FAICAR) in the biogenesis of purines [26]. *H. pylori* is likely to require the production of purines for growth [27]. The increased expression of PurH at 4W p.i. thus suggested a functional role of purine synthesis in bacterial

Fig. 1. Dynamics of *C. jejuni* colonization in chickens. (A) Numbers of *C. jejuni* 81-176GFP strain in chicken cecum enumerated by plate counts on mCCDA agar at 1-week intervals post oral infection. (B) Flow cytometry analysis of *C. jejuni* 81-176GFP from the chicken cecum at 1 week (1W) and 4 weeks (4W) p.i. Controls (PC or NC) represent 4% PFA-fixed 81-176GFP cells or unlabeled beads (1 μm), respectively. The proportion (%) of GFP-positive cells is shown in the upper middle panel of the grid line. The left panels show a dendrogram of FSC/SSC prior to gate setting. The right panels show a dendrogram of the GFP-positive population by GFP (FL1)/% cells. The pink colored frame corresponds to the sorted fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of *C. jejuni* 81-176 proteins that exhibited increased expression during bacterial colonization in chicken cecum.

Summary of protein										Relative value[b]			
										(116:115)		(117:115)	
No. peptide	Unused	Total	%Cov	%Cov(50)	%Cov(95)	Accession no.	Gene name	Gerne ID[a]	Protein name	Ratio	Pval	Ratio	Pval
Increased expression at an early course of colonization													
10	2.00	2.00	36.84	6.48	6.48	gi|87250211	*fabG*	CJJ81176_0461 (Cj0435)	3-Oxoacyl-(acyl-carrier-protein) reductase	4.41	<0.03	2.91	<0.03
13	1.86	1.86	41.16	6.31	2.10	gi|87249757	*hydB*	CJJ81176_1282 (Cj1266c)	Quinone-reactive Ni/Fe-hydrogenase, large subunit	3.62	<0.03	3.47	<0.03
18	1.45	1.10	19.31	4.87	4.87	gi|87249519	–	CJJ81176_0876 (Cj0860)	Putative integral membrane protein	1.40	<0.03	0.94	<0.03
Increased expression at later course of colonization													
5	2.03	2.03	35.18	3.44	1.15	gi|87249278	*mscS*	CJJ81176_1025 (Cj1007c)	Mechanosensitive ion channel family protein	0.50	<0.03	2.04	<0.03
3	2.10	2.10	28.79	5.45	5.45	gi|87249158	*cetB*	CJJ81176_1204 (Cj1189c)	Bipartite energy taxis response protein CetB	0.82	<0.03	1.88	<0.03
17	1.49	1.49	31.61	4.13	4.13	gi|87249038	*flhF*	CJJ81176_0102 (Cj0064c)	Flagellar biosynthetic protein FlhF	0.84	<0.03	1.82	<0.03
14	1.65	1.65	29.32	4.98	4.98	gi|87248903	*purH*	CJJ81176_0976 (Cj0953c)	Bifunctional purine biosynthesis protein PurH	0.45	<0.03	1.55	<0.03
16	1.55	1.55	31.68	4.55	4.55	gi|87249129	*pglJ*	CJJ81176_1144 (Cj1127c)	GalNAc transferase (PglJ)	0.73	<0.03	1.42	<0.03
12	1.88	1.88	26.82	2.88	2.88	gi|87250208	*lpxC*	CJJ81176_0167 (Cj0132)	UDP-3-0-acyl N-acetylglucosamine deacetylase	0.68	<0.03	1.41	<0.03
15	1.55	0.58	27.38	5.05	5.05	gi|167005187	*icd*	CJJ81176_0556 (Cj0531)	Isocitrate dehydrogenase, NADP-dependent	0.80	<0.03	1.41	<0.03

[a] Gene ID orthologued in strains 81-176 or NCTC11168 (in the parenthesis), respectively.
[b] Relative values of 116:115 (samples at 1 week p.i. vs. 0 week p.i.) and 117:115 (samples at 4 weeks p.i. vs. 0 week p.i.) are shown.

colonization in chicken gut, although further analysis would be necessary to confirm this idea.

Icd (isocitrate dehydrogenase) converts isocitrate to α-ketoglutarate, which participates in the more biosynthetic intermediate-generating half of the TCA cycle. CJJ81176_0556 is putative NADP-ICD based on its amino acid sequences, the catalysis of which generates NADPH, which is an important source of reducing power. NADPH also plays a role in cellular defense against oxidative damage and detoxification of reactive oxygen species [28]. Its increased expression might thus suggest the necessity for anti-oxidation responses in this pathogen to adapt the chicken gut environment.

3.1.3. Transport and membrane proteins

CJJ81176_0876 (putative integral membrane protein) showed increased expression only at 1 week p.i. This protein contains two EamA domains, and therefore, it might function as a transporter for drugs, metabolites or nucleotide sugars [29]. Current knowledge regarding a series of metabolic requirements in host adaptation and initiation of colonization [22] is also suggestive of its potential function as a transporter.

CJJ81176_1025 encodes a putative mechanosensitive ion channel family protein, MscS, which senses changes in lateral tension in the bilayer of the cytoplasmic membrane that are generated by rapid water flow into the cell [30]. Because osmolarity in the chicken intestine is generally higher with greater variations than that in humans [31], our data suggest that the increased expression of MscS might support bacterial osmo-adaptation, leading to stable colonization. A more detailed functional role for this protein in bacterial host adaptation will be assessed in our future studies.

Table 3
Fatty acid composition of *C. jejuni* 81-176GFP and fabG-strains.

Fatty acid[a]	Relative abundance (%)		
	81-176GFP (37 °C)	fabG– (37 °C)	81-176GFP (42 °C)
C12:00	1.31	1.31	1.29
C13:00	–	0.13	–
C14:00	23.02	31.78	23.29
C15:00	0.29	0.75	0.24
C14:0 3OH	3.31	2.59	2.49
C16:1 ω7c	16.34	14.34	14.28
C16:1 ω5c	0.29	0.27	0.32
C16:00	22.42	24.36	22.69
C17:00	0.41	1.04	0.45
C17:00 ω5c	0.00	0.00	1.33
C18:1 ω7c	18.43	10.79	19.28
C19 CYCLO 9,10	0.88	–	0.88
C19 CYCLO 11,12	6.21	6.32	6.29
Total saturated fatty acids	57.85	68.28	57.62
Total unsaturated fatty acids	42.15	31.72	42.38
Total cyclopropane fatty acids	7.09	6.32	7.17

[a] Unsaturated fatty acids are in bold.

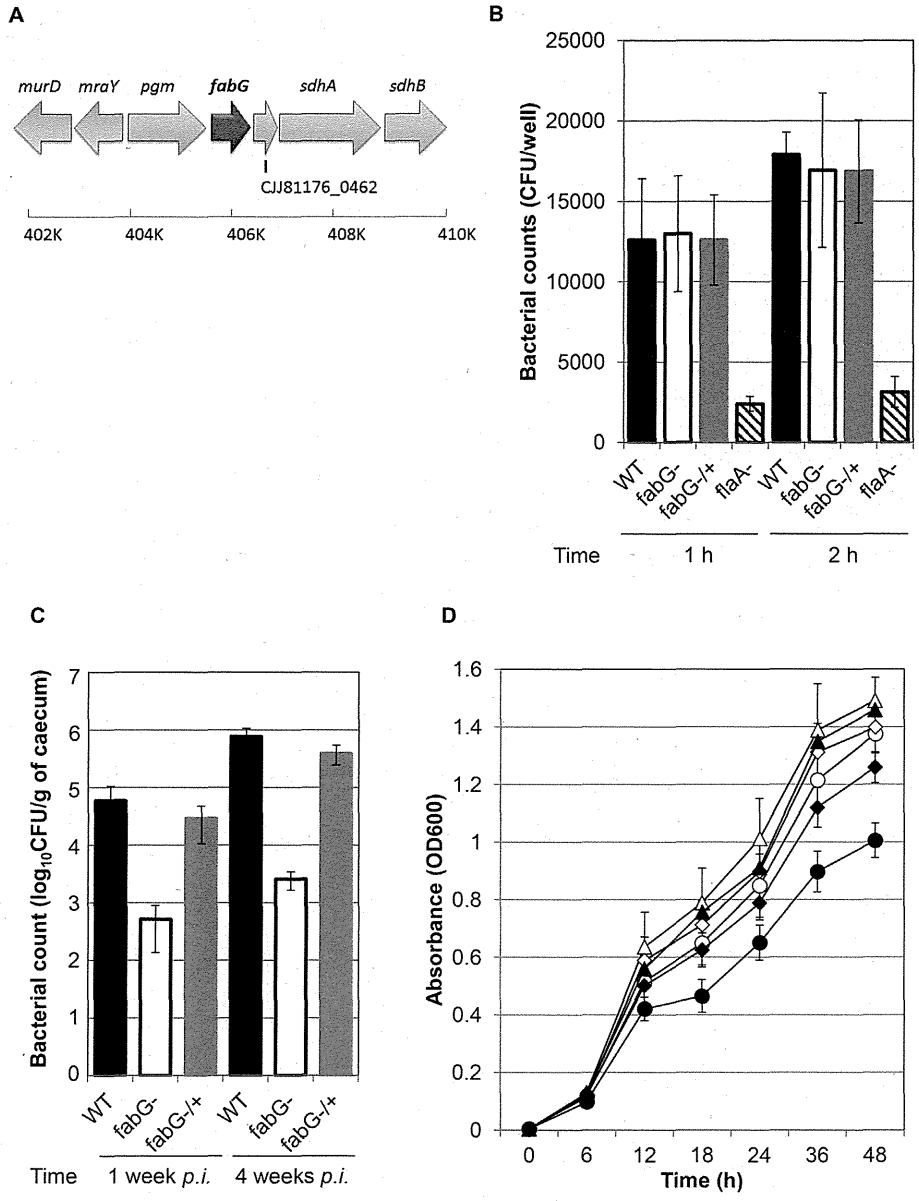


Fig. 2. Characterization of the *fabG* mutant. (A) Schematic illustration of *C. jejuni* 81-176 *fabG* and neighbor genes on the bacterial genome. (B) Cell adhesion of *C. jejuni* strains 81-176GFP (closed bars), fabG– (open bars), fabG–/+ (gray bars), and flaA– (slash bars) to INT407 cells was examined. Error bars represent standard deviations from three independent experiments. (C) Chicken colonization by *C. jejuni* 81-176GFP or fabG-strains at 1 and 4 weeks p.i. Bars represent standard means from each mean of five birds in each group. Asterisks represent $p > 0.05$ compared with the means of 81-176GFP-infected birds by Student *t*-test. (D) The growth kinetics of *C. jejuni* strains 81-176GFP (triangles), fabG– (circles) and fabG–/+ (diamonds) in MHB at 37 °C (open symbols) or 42 °C (closed symbols), respectively. Error bars represent standard deviation from three independent experiments.

3.1.4. Surface structure

PglJ is a glycosyltransferase that acts on undecaprenyl-linked substrates at various stages of the N-linked glycosylation process in the bacterial membrane [32]. Its increased expression during later stages of infection may be involved in the glycosylation that occurs on the membrane, thereby affecting bacterial hydrophobicity to alter persistent colonization.

LpxC (UDP-3-0-acyl N-acetylglucosamine deacetylase), which catalyzes the committed step of lipopolysaccharides (LPS) [33], was also reported to exhibit altered expression during colonization (1.41-fold at 4 weeks p.i.). In agreement with our data, *H. pylori* also caused upregulation of the homologous gene in mice [34].

We also identified FabG (putative 3-oxoacyl-(acyl-carrier-protein) reductase) as being expressed greater at both 1 week p.i. (4.41-fold) and 4 weeks p.i. (2.91-fold) than that of the control. FabG was previously identified as an essential gene in *C. jejuni*, together with other fatty acid biosynthesis/elongation genes [35], suggesting that the fatty acid biosynthetic system might be a key player in commensal colonization. However, little is known regarding the molecular function(s) of *C. jejuni* fatty acids and their biosynthesis-associated enzymes, except that a β-hydroxy-

acyl-acyl carrier protein (ACP) dehydratase (FabZ) catalyzes an essential step in type II fatty acid synthase (FAS II) [36], a principle route for production of membrane phospholipid acyl chains in bacteria [37,38].

3.2. C. jejuni *FabG affects fatty acid composition to achieve chicken colonization*

Given the potential link between the expression of FabG and chicken colonization, we constructed the *fabG* mutant (fabG−, Table 1) from the 81-176GFP strain to examine its biological role in colonization.

In silico analysis using STRING predicted interactions of FabG to AcpP (CJJ81176_0468), acetyl-CoA carboxylase β and several fatty acid biosynthetic enzymes like FabDFHZ (Fig. S2A). According to KEGG analyses, it is likely that FabG transforms acetoacetyl-[Acp] into (R)-3-hydroxyl-butanoyl-[Acp], which is the third biochemical reaction (after FabD and FabH/FabF) from substrate malonyl-CoA to contribute to biosynthesis of the short fatty acid chain in *C. jejuni* 81-176 (Fig. S2B). It also contributes to fatty acid elongation; FabZ and FabI are the other two enzymes involved in the metabolic pathway. This metabolic pathway is requested (through the transient expression of Fab enzymes) for recovery after a shock, indicating the essential role of these enzymes in fatty acid membrane integrity [38]. We thus compared the fatty acid compositions in 81-176GFP and fabG-strains cultured at 37 °C using the FAME assay. The results revealed that the fabG-strain exhibited about a 1.3-fold-decreased proportion of unsaturated fatty acids (UFA) (C16:1 ω7c, C16:1 ω5c, C18:1 ω7c, C19 cyclo 9,10 and C19 cyclo 11,12) compared with the parental strain (from 42.2% to 31.7%, of particular yields of C18:1 ω7c decreased from 18.4% to 10.8%) when grown at 37 °C (Table 3). The UFA proportion in the fatty acid composition of the wild type strain was not altered when cultivated at 42 °C (42.4%) compared with that at 37 °C (Table 3). The in vitro assay further demonstrated that the fabG-strain and its complemented strain (fabG−/+ strain) did not show altered adherence properties on INT407 cells (Fig. 2B), suggesting a non-essential role for FabG in cell adhesion of this pathogen. Nevertheless, fabG-exhibited either 114-fold- or 284-fold-decreased chicken colonization at 1 and 4 weeks p.i. compared with the parental strain (Fig. 2C). The complementation of the *fabG* gene restored the colonization property, which exhibited no significant differences from those of the WT strain (Fig. 2C). The decreased colonization of the fabG-strain was explained at least in part by the observation that the growth rate of the fabG-strain at 42 °C (almost identical to chicken body temperature) was lower than that at 37 °C, while WT and fabG−/+ strains exhibited almost identical growth kinetics at both temperatures (Fig. 2D). A similar influence of the *fabG* mutation in the growth kinetics of *Lactococcus lactis* has been reported [39], and the link between the degree of UFA and the growth kinetics of *H. pylori* [40] also supports our idea. It remains unclear how and whether each fatty acid components affects bacterial growth fitness. Nevertheless, our data provide the first evidence that FabG affects fatty acid composition, thereby altering bacterial growth kinetics. A previous report demonstrating a corresponding increase in the UFA/SFA ratio of *L. lactis* with increased growth temperature [41] provides an idea that changes in the composition of the bacterial cell membrane in response to growth temperature might affect a degree of fluidity, leading to host adaptation of this pathogen.

In principle, ex vivo proteomics necessitate retrieving cells from the cecum followed by separation from other bacterial species, which potentially affect subsequent regulation of proteins, thereby unifying the pattern. This cannot exclude the possibility that our data include certain biases. Nevertheless, we demonstrate herein the ex vivo proteomic dynamics of *C. jejuni* in the chicken. The observed proteomic dynamics might be accompanied by altered host adaptation mechanisms that are orchestrated by this bacteria. Further study focusing on the (bio)chemical functionality of fatty acid metabolism would provide a better understanding of the molecular dynamics of this pathogen, which is associated with its commensalism in the chicken.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.10.001.

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