ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

doi:10.1111/j.1365-2052.2010.02168.x

Caecal transcriptome analysis of colonized and non-colonized chickens within two genetic lines that differ in caecal colonization by *Campylobacter jejuni*

X. Y. Li*,[1,2], C. L. Swaggerty[†,1], M. H. Kogut[†], H. I. Chiang*, Y. Wang*, K. J. Genovese[†], H. He[†], I. Y. Pevzner[‡] and H. J. Zhou*

*Department of Poultry Science, Texas A&M University, College Station, TX 77843, USA. [†]Southern Plains Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service, College Station, TX 77845, USA. [‡]Cobb-Vantress, Inc., Siloam Springs, AR 72761, USA

Summary

Campylobacter jejuni is one of the most common causes of human bacterial enteritis worldwide. The molecular mechanisms of the host responses of chickens to *C. jejuni* colonization are not well understood. We have previously found differences in *C. jejuni* colonization at 7-days post-inoculation (pi) between two genetic broiler lines. However, within each line, not all birds were colonized by *C. jejuni* (27.5% colonized in line A, and 70% in line B). Therefore, the objective of the present experiments was to further define the differences in host gene expression between colonized and non-colonized chickens within each genetic line. RNA isolated from ceca of colonized and non-colonized birds within each line was applied to a chicken 44K Agilent microarray for the pair comparison. There were differences in the mechanisms of host resistant to *C. jejuni* colonization between line A and line B. Ten times more differentially expressed genes were observed between colonized and non-colonized chickens within line B than those within line A. Our study supports the fact that the *MAPK* pathway is important in host response to *C. jejuni* colonization in line B, but not in line A. The data indicate that inhibition of small GTPase-mediated signal transduction could enhance the resistance of chickens to *C. jejuni* colonization and that the tumour necrosis factor receptor superfamily genes play important roles in determining *C. jejuni* non-colonization in broilers.

Keywords: broiler, *C. jejuni*, ceca, colonization, transcriptome.

Introduction

As a food-borne pathogen, *Campylobacter jejuni* (*C. jejuni*) is a major cause of human diarrhoea that causes more than 90% of all human campylobacteriosis cases in the United States and other developed countries (Gu *et al.* 2009). The clinical symptoms of campylobacteriosis are variable in humans. The basis of this diversity in the manifestation of campylobacteriosis is poorly understood, but it may be caused by differences in the *Campylobacter* strains and/or differences in host susceptibility. Chickens are a major reservoir of *C. jejuni*, with a major source of human campylobacteriosis being chicken meat contamination due to improper handling (Altekruse *et al.* 1999; Friedman *et al.* 2004). In general, there are no obvious clinical signs of *C. jejuni* colonization in chickens, and production traits are usually not affected (Dhillon *et al.* 2006). Significant reduction of *C. jejuni* in chicken gut will greatly decrease the contamination of poultry meat and consequently improve the overall safety of the food supply by reducing the number of food poisoning cases.

Host genetics plays an indispensable role in response to *C. jejuni* colonization of chickens. Distinct susceptibility

Address for correspondence

Huaijun Zhou, Department of Poultry Science, Texas A&M University, College Station, TX 77843, USA.
E-mail: hjzhou@poultry.tamu.edu;

Michael H. Kogut, Southern Plains Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service, College Station, TX 77845, USA.
E-mail: michael.kogut@ars.usda.gov

[1]Equal contribution.
[2]Current address: College of Animal Science and Technology, Shandong Agricultural University, Taian,Shandong 271018, China.

Accepted for publication 4 October 2010

responses of different lines of chickens to *C. jejuni* colonization have been reported (Stern *et al.* 1990; Boyd *et al.* 2005; Li *et al.* 2008b). We have demonstrated significant differences in *C. jejuni* colonization between two genetic lines of chickens (A and B) on day 7 post-inoculation (pi), with line A being more resistant to *C. jejuni* colonization than line B (Li *et al.* 2008b). The molecular mechanisms of susceptibility to *C. jejuni* inoculation between these two genetic lines have been elucidated through gene expression profiling of cecae (Li *et al.*, 2010).

Interestingly, we have also found a differential susceptibility pattern of birds within each line (Li *et al.* 2008b). We hypothesize that these differences in susceptibility patterns within lines are because of distinct gene expression responses of the birds at the local caecal level. Therefore, the objective of this study was to profile the host genes expressed by the colonized and non-colonized birds within each line by measuring mRNA levels using a chicken 44K Agilent microarray.

Materials and methods

Bacterial challenge and sample collection

Two broiler lines, A and B, were obtained from a commercial breeding company. The bacterial inoculation, sample collection and bacterial enumeration were performed as described previously (Li *et al.* 2008b). In brief, *C. jejuni* strain 5088 was enriched in Bolton broth (Oxoid) at 42 °C for 40 h. In each line, 80 one-day-old chickens were orally inoculated with 0.5 ml of inoculants with a dose of 1.8×10^5 colony forming units (cfu) per chicken. On day 7 pi, caecal contents were collected for colonization quantification, and ceca (including part of the caecal tonsil) were aseptically removed and immersed into 10 volumes of RNAlater (Ambion) for the isolation of total RNA. All animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University and the United States Department of Agriculture.

Experimental design and microarray hybridization

Based on the number of bacteria in caecal contents on day 7 pi, 22 of 80 (27.5%) chickens were colonized by *C. jejuni* in line A, and 56 of 80 (70%) were colonized in line B. Twenty caecal samples from each of the caecal colonized and non-colonized birds from each line (thus a total of 40 birds from each line) were used for RNA isolation.

For total RNA isolation, a 15- to 20-mg section of caecum was removed from the RNAlater-stabilized sample, cut into pieces and placed in a 2-ml centrifuge tube containing 600 µl Qiagen RNeasy Mini Kit lysis buffer (Qiagen). The PRO200 homogenizer (PRO Scientific) was used to homogenize the lysate. Total RNA was isolated from each homogenized sample and treated with a TURBO DNA*free*™ Kit (Ambion) according to the manufacturer's protocol. Four biological pools (five individuals per pool with equal amounts of total RNA) from each group were made, with individuals selected at random. Eight pair comparisons between colonized (C) and non-colonized (N) groups within each line were used to conduct the microarray experiment with dye swap, in which each sample was labelled with Cy5 and Cy3, respectively. The details of labelling, hybridization and washing procedures have been described previously (Li *et al.* 2008a). Briefly, 400 ng of total RNA from each pooled sample was labelled with either Cy3 or Cy5 and hybridized with another pooled sample labelled with the other dye. The post-hybridization washing was performed according to the manufacturer's recommendation (Agilent). Slides were scanned using a GenePix Personal 4100A scanner at 5-µm resolution (Molecular Devices).

Microarray data analysis

The signal intensity of each probe was filtered by negative controls in the array and signal-to-noise ratio (SNR) before normalization. Data normalization was performed using locally weighted regression or smoothing scatter plots (LOWESS) (Cleveland 1974; Yang *et al.* 2002) by R 2.8.1 (http://www.r-project.org). The normalized natural log intensities were analysed using a mixed model by SAS (SAS), with fixed effects of group (C or N), line (A or B), interaction of line × group, and dye (Cy5 or Cy3), and with random effects of slide and array. The comparison of AC/AN (i.e. line A colonized chickens/line A non-colonized chickens) and BC/BN (line B colonized chickens/line B non-colonized chickens) was performed. A P value <0.01 was considered as significant in the present study. Minimum information about a microarray experiment (MIAME) for this experiment has been deposited in NCBI's Gene Expression Omnibus (GEO) (Barrett *et al.* 2007). The accession numbers are: platform: GPL6413; series: GSE10257.

Those differentially expressed genes within each line were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (Dennis *et al.* 2003; Huang *et al.* 2008) for Gene Ontology (GO) biological process (BP) functional annotation analysis. In brief, lists of those differentially expressed genes in each comparison were uploaded to the DAVID database as a gene list, and *Gallus gallus* whole genome was used as the background. The default setting was used.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to validate microarray results with primers (Table 1) as described previously (Li *et al.* 2008a). In brief, 1 ug of total RNA was reversely transcribed into cDNA using random hexamers and Thermoscript™ RT-PCR system (Invitrogen). The amplification was performed as 1 cycle of 95 °C for 10 min and 40 cycles of 59 °C for 15 s and 59 °C for

Table 1 Primer sets for qRT-PCR.

Gene	Accession No.	Primer sequence (5′–3′)	
CD5	Y12011	Forward	ACAGGAGGCTGATGAAGAGG
		Reverse	TGAGCGTAATCGTTGTCTCC
Mx1	Z23168	Forward	GCAGAAGGCATCAGCAAAG
		Reverse	GCTCAGGCGTTTACTTGCTC
TNFR8	AJ276964	Forward	ACTGAAGTGACGCAGAGCAA
		Reverse	GCATTGACAGCTCCTCTTCC
VAV3	AY046915	Forward	ATCAACATTCCTGACTTGGT
		Reverse	GATATGGCTGTTTCCACTTG
TBC1D23	AJ851485	Forward	CCGCCGAGCAGCTGGGAAAA
		Reverse	AGGATGCGAGGCTGTCCCCC
RABGAP1	CR407064	Forward	CCGTGTACAGCTGCCCAAGAGG
		Reverse	TCCTGCTGAGCTTGCTGCTCT
ARFGAP1	CR407511	Forward	TGCCAGGGAAGAGTGGGGTGG
		Reverse	TGGCAAGCAGCACTCAGCGT
B-*actin*	L08165	Forward	ACGTCTCACTGGATTTCGAGCAGG
		Reverse	TGCATCCTGTCAGCAATGCCAG

1 min, using SYBR Green Master Mix and an ABI Prism 7900HT system (Applied Biosystems). The chicken *β-actin* gene was used as the internal standard to correct the input of cDNA. Triplicate qRT-PCRs were performed for each cDNA, and the average Ct was used for further analysis. The relative fold change between two groups within lines was calculated using the $2^{-\Delta\ \Delta Ct}$ method (Livak & Schmittgen 2001).

Results

Global assessment of transcriptome profiling

Signal intensity of each probe was filtered against negative controls in the microarray and then normalized. In total, 27 955 probes were detected as expressed (SNR >3) and were used for the following analysis. Gene expression differences between colonized and non-colonized chickens within a given line [i.e. line A = A colonized (AC) and A non-colonized (AN); line B = B colonized (BC) and B non-colonized (BN)] were analysed.

Gene expression differences within line A

In the comparison of AC/AN, 564 genes were found to be differentially expressed, with a false discovery rate (FDR) of 0.605 (Fig. 1a). Of these 564 genes, 277 had a higher expression in AC than AN, with 32 of these genes having a fold change of >2.00.

Gene expression differences within line B

The comparison of BC/BN showed 6105 genes differentially expressed with a FDR of 0.025. Of the 6105 differentially expressed genes, 2248 had a higher expression in BC than in BN (Fig. 1b), with 324 of the genes having a greater than 2.00-fold change. More than 3000 genes had a lower

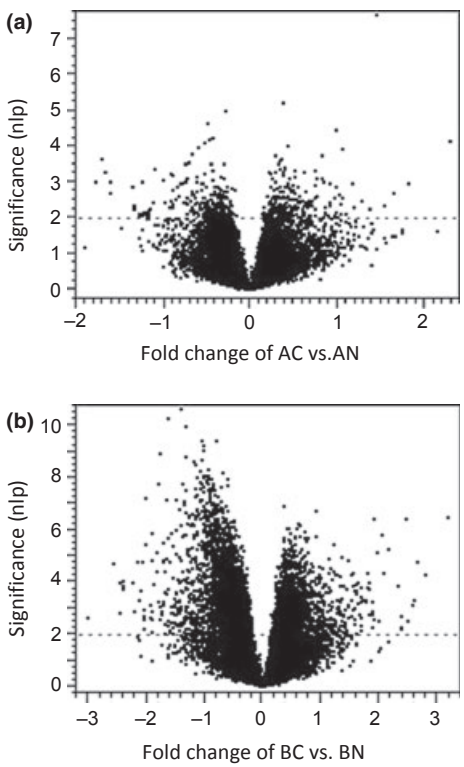

Figure 1 Volcano plots of differentially expressed genes between colonized and non-colonized chickens in line (a and b). nlp represents negative log10 of *P* value. Fold change was log2 transformed. Positive values mean that gene expression was higher in colonized (c) chickens than in non-colonized (N) chickens. a: AC/AN. b: BC/BN.

expression in BC than in BN, with 597 genes having a fold change of >2.00.

In the comparison of differentially expressed genes between AC/AN and BC/BN, 128 genes were shared between these two comparisons, 16 of which were in the same direction of the regulation (seven had higher expression in group C than group N, and nine had lower expression in group C than group N in both lines A and B) (Table S1). There were 436 and 5977 differentially expressed genes that were only observed in the comparison of AC/AN and BC/BN, respectively. These were referred to as line-specific genes (i.e. line A specific and line B specific) (Fig. 2).

Functional annotation analysis

Functional GO BP annotation was analysed based on the differentially expressed genes between two groups within a given line (i.e. AC/AN, BC/BN).

Within line A

In the comparison of AC/AN, only small GTPase-mediated signal transduction was significantly enriched, with a fold enrichment of 3.58. Decreases in gene expression from this signal transduction could promote resistance to *C. jejuni* colonization. No GO BP terms were enriched in the genes which had lower expression in AC than in AN.

Within line B

For the comparison of BC/BN, 54 enriched GO BP terms were identified. Thirty were from genes which had higher expression in BC than in BN, 30 were related to genes which had lower expression in BC than in BN, and six of those terms were shared (Table 2) between BC and BN. For genes which had higher expression in BC than in BN, the enriched functional terms could be roughly grouped into five clusters: (i) metabolic processes including carboxylic acid metabolic processes, fatty acid metabolic processes, monocarboxylic acid metabolic processes, nitrogen compound metabolic processes and organic acid metabolic processes; (ii) immune

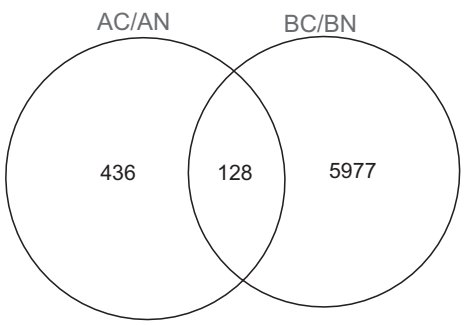

Figure 2 Overlapping differentially expressed genes between AC/AN and BC/BN.

Table 2 Fold enrichment of GO BP terms for differentially expressed genes in the comparison of BC/BN.

Term	hBC/BN	lBC/BN
Apoptosis	1.95	
ATP biosynthetic process		3.26
ATP metabolic process		3.02
Carboxylic acid metabolic process	2.24	
Cell projection organization and biogenesis		2.44
Cellular component organization and biogenesis	1.43	1.50
Cellular localization	1.64	1.52
Cellular morphogenesis during differentiation		2.81
Coenzyme biosynthetic process		2.33
Coenzyme metabolic process		2.30
Cofactor metabolic process		2.05
Electron transport	1.66	
Enzyme linked receptor protein signalling pathway		2.27
Establishment of protein localization		1.69
Fatty acid metabolic process	3.27	
Generation of precursor metabolites and energy	1.74	
Intracellular protein transport		2.13
Mitotic cell cycle		3.42
Monocarboxylic acid metabolic process	2.80	
Neurite morphogenesis		3.13
Nitrogen compound metabolic process	2.14	
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.30	1.24
Organelle organization and biogenesis	1.47	
Organic acid metabolic process	2.24	
Oxidative phosphorylation		2.38
Positive regulation of cellular metabolic process		2.12
Positive regulation of developmental process		4.36
Positive regulation of transcription		2.44
Protein localization		1.68
Protein metabolic process	1.23	1.15
Protein modification process	1.31	1.26
Protein transport		1.75
Protein-chromophore linkage	5.08	
Purine nucleotide biosynthetic process		2.18
Ras protein signal transduction	2.08	
Regulation of catalytic activity	2.28	
Regulation of cell differentiation		2.45
Regulation of GTPase activity	2.98	
Regulation of hydrolase activity	2.64	
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process		1.25
Regulation of Ras protein signal transduction	2.13	
Regulation of small GTPase-mediated signal transduction	2.06	

Table 2 (Continued).

Term	hBC/BN	lBC/BN
Response to DNA damage stimulus	2.54	
Response to endogenous stimulus	2.45	
Response to radiation	3.81	
Response to stress	1.93	
Rhythmic process	4.48	
Ribonucleoprotein complex biogenesis and assembly	2.69	
RNA biosynthetic process		1.31
RNA metabolic process	1.31	1.22
tRNA metabolic process	2.88	
Ubiquitin cycle		2.11
Vasculature development		2.58
Vesicle-mediated transport	2.09	

Note: hBC/BN represents genes had higher expression in BC than in BN.
lBC/BN represents genes had lower expression in BC than in BN.

response including apoptosis, response to DNA damage stimulus, response to endogenous stimulus, response to radiation and response to stress; (iii) transport (electron transport and vesicle-mediated transport); (iv) signal transduction (Ras protein signal transduction, small GTPase-mediated signal transduction); and (v) rhythmic process. For genes which had lower expression in BC than in BN, the enriched terms could be clustered into three groups: (i) metabolic processes including ATP biosynthetic processes, ATP metabolic processes, coenzyme metabolic processes, cofactor metabolic processes, positive regulation of cellular metabolic processes, purine nucleotide biosynthetic processes, and RNA metabolic processes; (ii) development and morphogenesis including cellular morphogenesis during differentiation, meurite morphogenesis, regulation of cell differentiation, and vasculature development; and (iii) macromolecular localization and transport (establishment of protein localization, intracellular protein transport, and protein localization).

Immune-related genes

There are 426 immune-related genes with 542 probes in the current microarray. There were 17 immune-related genes significantly and differentially expressed in the comparison of AC/AN with a fold difference ≥1.2 (Table 3) and 60 immune-related genes in BC/BN (Table 4) ($P < 0.01$) with a fold difference ≥1.2. To classify immune-related genes, all 17 genes in line A and 60 in line B that were differentially expressed immune-related genes within each line were uploaded to the DAVID program to run functional classification analysis.

Within line A, 4 of 17 immune-related genes had higher expression in AC than in AN, including *caspase 1* (*CASP1*), *prostaglandin-endoperoxide synthase 2* (*PTGS2*), *serine/threonine-protein kinase Chk2*, and *Granzyme-like molecule* (*GZMA*).

Table 3 Fold difference of immune-related genes in the comparison of AC/AN

Accession	Gene description	
		lAC/AN
BU125345	*Complement 8A*	2.4
AJ276964	*Tumour necrosis factor receptor superfamily, member 8*	2.0
CR523798	*T-cell receptor beta chain V2,D,J,C regions*	1.9
AJ720954	*Complement component receptor 2 (CR2)*	1.9
U04611	*T-cell receptor alpha*	1.8
Y12011	*CD5*	1.6
CR352482	*Tumour necrosis factor receptor superfamily, member 11b*	1.4
AF320331	*Interferon regulatory factor 4*	1.4
AJ719814	*CD79b molecule*	1.4
Y12012	*CD4*	1.4
CR733120	*CD83*	1.3
BX935885	*Small inducible cytokine A13 precursor*	1.3
AJ450706	*MAPK/ERK kinase kinase-1*	1.3
		hAC/AN
BX935020	*Caspase 1(CASP1)*	1.2
M64990	*Prostaglandin-endoperoxide synthase 2 (PTGS2)*	1.3
EF118300	*Serine/threonine-protein kinase Chk2*	1.8
AJ544060	*Granzyme-like molecule (GZMA)*	1.8

Note: hAC/AN represents genes with higher expression in AC than in AN.
lAC/AN represents genes with lower expression in AC than in AN.

The other 14 immune-related genes had lower expression in AC than in AN, with the highest fold change of 2.4 in the *complement 8A* gene (BU125345) (Table 3). Functional analysis results (Table S2) showed that these immune-related genes were enriched in two GO terms, these being receptor activity (*CD79B*, *tumour necrosis factor receptor superfamily, member 8* (*TNFRSF8*), *TNFRSF11B*, and *CD5*) and cell differentiation (*interferon regulatory factor* (*IRF4*), *PTGS2*, and *CASP1*).

Within line B, more differentially expressed immune-related genes were observed compared to line A. In total, 60 differentially expressed immune-related genes were detected, and 34 had higher expression in BC than in BN, with the highest fold difference of 2.7 being in *TNFRS19*. Of 26 immune-related genes which had lower expression in BC than in BN, the highest fold change of 3.2 was found in *interleukin 9* (*IL-9*) (Table 4). Functional analysis (Table S3) demonstrated that these immune-related genes were enriched in the following GO terms, including cell commu-

Table 4 Fold change of immune-related genes in the comparison of BC/BN

Accession	Gene description	
		lBC/BN
BX931122	*Interleukin 9 (IL9)*	3.2
BX931297	*Cytokine like protein 17*	3.1
Z23168	*MX1*	2.5
AJ450829	*Chemokine receptor(CRL1)*	2.4
AY831397	*IFN-beta*	2.0
AB032767	*CD9*	1.8
AB076264	*CDw137*	1.5
BU142537	*AP-1; JUN; jun oncogene; transcription factor AP-1*	1.5
AF139097	*Interleukin 15 (IL15)*	1.5
CR388992	*Carboxypeptidase E*	1.5
M64990	*Prostaglandin-endoperoxide synthase 2 (PTGS2)*	1.5
X75915	*Bone morphogenetic protein 4 (BMP4)*	1.5
AJ720845	*RAS guanyl releasing protein 3 (calcium and DAG-regulated)*	1.4
NM_001031045	*TGF beta 2*	1.4
AJ719686	*Insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3)*	1.4
CO759833	*CD48*	1.4
X07202	*ETS2*	1.3
AJ720932	*Death-associated protein*	1.3
AJ719298	*GTP-binding nuclear protein ran*	1.3
D14313	*Transcription factor 8*	1.3
AF296874	*TNF receptor superfamily, member 6*	1.3
D13225	*c-kit*	1.3
DQ010055	*Initiatorcaspase*	1.2
BX935400	*CKLF-like MARVEL transmembrane domain containing 8*	1.2
AJ720321	*Mitogen-activated protein kinase kinase kinase 7 interacting protein 1 (MAP3K7IP1),*	1.2
CR406144	*JNK-MAPK9; c-Jun N-terminal kinase*	1.2
		hBC/BN
AJ720236	*NCK adaptor protein 2*	1.2
AJ721122	*Mitogen-activated proteinkinase kinase 5 (MAP2K5)*	1.2
CR387429	*B-BTN1b*	1.2
AJ719869	*CD 47*	1.2
AJ720408	*Interleukin-1 receptor-associated kinase4 (IRAK4)*	1.2
AJ719947	*CD82*	1.2
U35037	*Insulin-like growth factor 2 receptor (IGF2R)*	1.2
D13719	*NFkB-1; Nuclear factor NF-kBp50/p105*	1.3
AJ720057	*Cytokine receptor-like factor 3*	1.3
AJ720431	*Signal transducer and activator of transcription 1 (STAT1)*	1.3
AJ720175	*CCR4-NOT transcription complex, subunit 8*	1.3
X56559	*Integrin, alpha 6*	1.3
BX930367	*PREDICTED: bactericidal/ permeability-increasing protein (BPI)*	1.4
BX932911	*Invariant chain*	1.4
ENSGALT 00000012062	*E-selectin precursor*	1.4
AJ851819	*Signal transducer and activator of transcription 3 (STAT3)*	1.4
AF082664	*INF-a/b-R1; IFNAR1; interferon receptor 1*	1.4
BG625680	*Putative E-selectin*	1.4
AJ450706	*MAPK/ERK kinase kinase-1*	1.4
L39767	*Interferon regulatory factor 8 (IRF8)*	1.4
L12032	*Dorsalin-1 precursor*	1.4
L08168	*P38; Proto-oncogene C-crk 38*	1.4
AJ719881	*PABPC1 poly(A) binding protein, cytoplasmic 1*	1.4
BU375600	*Single immunoglobulin and toll-interleukin 1 receptor domain*	1.5
AB108485	*Receptor (TNFRSF)-interacting serine-threonine kinase 1*	1.5
BU356288	*Rfp-y(class 1 alpha chain)*	1.6
L20625	*MCFS-1 receptor*	1.7
AY046915	*VAV3*	1.7
AJ851742	*TNFAIP3 interacting protein 2*	1.7
AF306851	*Immunoglobulin-like receptor A1*	2.0
AJ719290	*Serine/threonine kinase 17a*	2.1
AF498236	*SOCS2*	2.1
BU344261	*TBK1*	2.3
BX931334	*Tumour necrosis factor receptor superfamily, member 19*	2.7

Note: hBC/BN represents genes had higher expression in BC than in BN. lBC/BN represents genes had lower expression in BC than in BN.

nity (*VAV3*, *NFKB1*, *TGFB2*, *BMP4*, *SOCS2* and *ITGA6*), binding (*Mx1*, *NFKB1*, *IGF2BP3*, *ITGA6*, *IRF8*, *VAV3* and *TGFB2*), cytokine activity (*BMP4* and *IFNB*), response to stimulus (*IRF8*, *TGFB2* and *IFNB*) and leucocyte migration (*TGFB2* and *ITGA6*).

Validation of gene expression from microarray analysis by quantitative real-time PCR

qRT-PCR was performed to validate the microarray data. An aliquot of RNA from the same sample used for the

Table 5 Comparison of gene expression levels (fold changes) between microarray and qRT-PCR.

Comparison	Method	*Mx1*	*VAV3*	*CD5*	*TNFR8*	*TBC1D23*	*RABGAP1*	*ARFGAP1*
AN/AC	qRT-PCR			1.49	2.65*			
	Microarray			1.61*	1.99*			
BN/BC	qRT-PCR	1.71*	−1.35*			1.11	1.50*	1.48*
	Microarray	2.51*	−1.73*			1.22*	1.32*	1.23*

*Represents genes that were significantly differentially expressed in the comparison ($P < 0.05$ in qRT-PCR result, $P < 0.01$ in microarray).

microarray analysis was used for qRT-PCR validation. Three immune-related genes and four genes from small GTPase-mediated signal transduction (functional analysis indicated that this pathway was associated with *C. jejuni* colonization in the current study) were selected for the validation. All genes tested were consistent between microarray and qRT-PCR results in terms of regulation direction (upregulation or downregulation) (Table 5). Two genes, *CD5* and *TBC1D23*, were not differentially expressed in the qRT-PCR results.

Discussion

Functions associated with colonized and non-colonized chickens

In general, there are noteworthy differences in the mechanisms of host resistant to *C. jejuni* colonization between line A and line B. Our expression profile and GO enrichment analysis strongly support this conclusion. There were 10 times the number of genes differentially expressed in line B than line A (6,105 in BC/BN vs. 564 in AC/AN, Fig. 1). Further GO enrichment analysis supported the hypothesis that many enriched biological processes may contribute to the differences between colonized and non-colonized chickens in line B but not in line A.

Of particular interest, small GTPase-mediated signal transduction was enriched in both line A and line B, with higher expression in colonized birds than in non-colonized birds. This functional term primarily includes *VAV3*, *TBC1 domain-containing kinase* (*TBCK*), *TBC1 domain family member 23* (*TBC1D23*), *Rab GTPase-activating protein 1* (*RABGAP1*), *Ras guanine nucleotide exchange factors (GEF) domain family, member 1C* (*RASGEF1C*), *Rho guanine nucleotide exchange factor 4 isoform A* (*ARHGEF4*), *ADP-ribosylation faction GTPase-activating protein (GAP) 1* (*ARFGAP1*), and *Ral GEFs with PH domain and SH3-binding motif 2* (*RALGPS2*). Ras-like small G proteins have a ubiquitous role in cell biology; thus, they are common targets for virulence factors that are secreted by bacterial pathogens (Boquet 2000). Small G proteins regulate a myriad of cellular processes, including growth, differentiation, membrane trafficking, cytoskeletal organization and nuclear import. Many bacterial virulence factors can act on small Rho family GTPases, including GEFs and GAPs, which induce localized signalling to actin rearrangement at the sites of bacterial invasion (Cossart & Sansonetti 2004; Rottner *et al.* 2004). Actin cytoskeleton is crucial in the regulation of host–pathogen interactions (Rottner *et al.* 2004). In addition, Krause-Gruszczynska *et al.* (2007) have suggested that activation of small Rho family GTPases is essential in the signalling pathways involved in the *C. jejuni* invasion process *in vitro*. Furthermore, it has been reported that small GTPases are involved in *TLR* signalling pathways and are activated rapidly in chicken heterophils following *Salmonella* lipopolysaccharide (LPS) stimulation (Kogut *et al.* 2007). Therefore, in colonized (C) chickens, following *C. jejuni* inoculation, higher expressed *Rho GEF 4A* could activate the GDP–GTP exchange and then stimulate Rho, Rac family protein and actin cytoskeleton, which are necessary for bacterial invasion. This could finally activate small GTPase signal pathways, which promote bacterial colonization. In contrast, in non-colonized birds, without the activation of small GTPase family gene expression, birds will be more resistant to *C. jejuni* colonization. The work presented herein provides the first line of evidence *in vivo* that small GTPase signal pathways are associated with *C. jejuni* colonization in chickens. The validation of three genes (*Vav3*, *TBC1B23* and *RABGAP1*) in this signal pathway by qRT-PCR further confirms the evidence of the importance of GTPase-mediated signal transduction in *C. jejuni* colonization. It will be highly interesting to investigate the precise mechanisms of how the small GTPase family regulates genetic resistance to *C. jejuni* colonization in the chicken.

Broad enriched functional terms have been observed with respect to bacterial colonization in the chicken: carboxylic acid metabolic process, ubiquitin cycle, signal transduction and immune-related function such as apoptosis, response to DNA damage stimulus, endogenous stimulus, radiation and stress. In the current study, carboxylic acid metabolic process was significantly enriched, with genes more highly expressed in colonized birds than in non-colonized birds. Because of a lack of the glycolytic enzyme phosphofructokinase in *C. jejuni*, carbohydrates cannot be utilized as an energy source. Instead, *C. jejuni* uses the intermediates as energy from the tricarboxylic acid cycle (Dasti *et al.* 2010). Highly expressed genes from carboxylic acid metabolic process are expected to generate more intermediates of carboxylic acids in colonized birds, which might explain why colonized birds have more metabolites that are utilized for bacterial colonization than do non-colonized birds.

The mitotic cell cycle, regulation of cellular differentiation and ubiquitin cycle functional terms were enriched in line B. The findings herein demonstrate that cell cycle may contribute to the differences between colonized and non-colonized chickens in line B following *C. jejuni* inoculation. Ubiquitin-mediated proteolysis is one of the key mechanisms underlying cell cycle control (Nakayama & Nakayama 2006). In mammals, the cell cycle has been reported to be involved in many processes that affect the growth and colonization of pathogenic bacteria, including immune responses, maintenance of epithelial barrier functions, and cellular differentiation (Oswald *et al.* 2005). In non-colonized birds, highly expressed genes in ubiquitin and cell cycle may enhance immune response and improve epithelial barrier integrity, subsequently preventing bacterial colonization, while in colonized birds, repressing the cell cycle through ubiquitin cycle reduces the differentiation of Th cells and inhibits the host response to *C. jejuni* inoculation, therefore promoting bacterial colonization.

Immune-related genes involved in the response to *C. jejuni* colonization

Protein kinases and phosphatases play important roles in regulating protein phosphorylation and influencing the response of host cells to external pathogenic bacteria. It has been reported that *C. jejuni* invades human epithelial cells and triggers signal transduction cascades, resulting in bacterial internalization and pathogenesis (Wooldridge *et al.* 1996; Watson & Galan 2005). The mitogen-activated protein kinase (MAPK) cascades play important roles in linking a variety of extracellular signals to cellular events such as growth, differentiation, apoptosis and inflammatory response (Widmann *et al.* 1999). There are three major MAPK pathways: the mitogen-induced extracellular signal-regulated kinase (ERK) MAPKs were linked to cell proliferation and survival, whereas the stress-activated MAPKs, p38 and c-Jun N-terminal kinase (JNK), were associated with apoptosis (Boldt *et al.* 2002; Junttila *et al.* 2008; Zhang & Dong 2007). In addition, the ERK and p38 MAPKs are involved in *C. jejuni* invasion in intestinal epithelial cells (Watson & Galan 2005; Hu *et al.* 2006). Inhibition of *ERK MAPK* phosphorylation and *p38 MAPK* results in significant reduction of *C. jejuni* (Hu *et al.* 2006). *p38 MAPK* expression is increased in human Caco-2 cells after *C. jejuni* infection (MacCallum *et al.* 2005). Based on the KEGG *MAPK* signalling pathway, there were nine genes significantly regulated in BN/BC that participate in either the classical MAPK pathway, JNK and p38 MAPK pathway, or ERK pathway at different levels of regulation. In the classical MAPK pathway, the current study showed that higher expression of *RAS guanyl releasing protein 3* (*RasGRP*) was associated with non-colonization, while elevated *NFkB-1* expression resulted in increased colonization. In the JNK and p38 MAPK pathways, both *TGFB2* and *TNF receptor superfamily (TNFRSF), member 6 (FAS)* are genes encoding receptors that interact with pathogen stimuli. MAP3K7IP1 (TAB 1), JNK-MAPK9 (JNK), MAPK/ERK kinase kinase 1 (MEKK1) and proto-oncogene C-crk 38 (CrkII) are intermediate regulators in the signal pathway. Our study indicated that higher expression of both receptor genes and *TAB 1* and *JNK* are associated with non-colonization in birds, while higher expression of *MEKK1* and *CrkII* results in increased *C. jejuni* colonization. Although the exact mechanism of regulation is not fully understood, it seems that these two groups of genes are regulated in opposite directions. We speculate that the up-regulated expression of *TAB 1* and *JNK* might induce T-cell activation and T-helper cell differentiation, therefore enhancing immune response and preventing *C. jejuni* persistence in cecae, while increased expression of *MEKK1* and *CrkII* inhibits lymph cell activation and differentiation. Only one gene in the ERK5 pathway, *MAP2K5*, was differentially upregulated in colonized birds compared to non-colonized birds. Collectively, the results of the current study revealed that the JNK and p38 MAPK pathways might play an essential role in the regulation of *C. jejuni* colonization in chickens. As *MAPK* pathways involve complicated regulations by multiple mechanisms, microarray analysis from the current study would not be able to delineate these mechanisms. However, these regulated genes identified in the *MAPK* pathway have provided great targets for further investigation towards understanding how JNK and p38 MAPK pathways regulate *C. jejuni* colonization in birds.

The TNFRSF gene group plays an essential role in the development and regulation of the immune system (Silke & Brink 2010), and many members have key functions in the host response to bacterial infection (Aggarwal 2003). The TNF family ligands can bind to death domain-containing TNF receptors that can subsequently activate intracellular signalling pathways to promote apoptotic cell death (Bridgham & Johnson 2003). Several TNFRSF members, including *TNFRSF1B*, *5*, and *9*, have in a comprehensive microarray analysis been associated with host response to a variety of pathogens (Jenner & Young 2005). *TNF-α* is significantly upregulated following *C. jejuni* infection in human colonic epithelial cells (Zheng *et al.* 2008) and THP-1 cells (Tabbara *et al.* 2010). Interestingly, in the present study, no significant gene expression change of TNF was identified following *C. jejuni* inoculation. However, four *TNFRSF* (*6*, *8*, *11b* and *19*) genes were differentially regulated between colonized and non-colonized chickens within line A or line B or both. All *TNFRSF* genes had lower gene expression in colonized chickens than in non-colonized chickens except *TNFRSF19*. *TNFRSF8* had lower expression in AC than in AN. *TNFRSF8*, also known as *CD30*, leads to enhanced cell proliferation, cell growth arrest or apoptosis (Lee *et al.* 1996). Two additional TNFRSF members (*TNFRSF11b*, also known as *OPG*, and *TNFRSF6*, also known as *FAS*) also showed lower expression in colonized

than in non-colonized chickens in both lines A and B. This might be because lower expression of TNFRSFs could inhibit cell proliferation and apoptosis and limited immune response, resulting in more bacterial colonization in the gut. However, *TNFRSF19* had the opposite direction of expression (higher expression in BC than BN) compared with the three other TNFRSF genes. *TNFRSF19* is associated with embryo development (Pispa *et al.* 2003), and the function in regulating immune response still remains to be elucidated. Our study clearly shows that TNFRSF genes play a significant role in *C. jejuni* colonization in the chicken.

The results presented in this study represent local caecal transcriptomal differences between colonized and non-colonized chickens within each of two genetic broiler lines (A and B) following *C. jejuni* inoculation. The data indicate that inhibition of small GTPase-mediated signal transduction could enhance resistance to *C. jejuni* colonization, and that *TNFRSF* genes play important roles in determining *C. jejuni* non-colonization in broilers. There was a difference in the mechanisms of host resistance to *C. jejuni* colonization between line A and line B. Our study supports the hypothesis that the MAPK pathway is important in host response to *C. jejuni* colonization in line B, but not in line A. While this is an important step forward in our understanding of host response to *C. jejuni* colonization in chickens, it will be important to follow up these analyses with further studies to investigate innate immune response to *C. jejuni* colonization. This will add valuable information towards our understanding of the mechanisms underlying *C. jejuni* colonization in chickens.

Acknowledgements

This project was supported by National Research Initiative Competitive Grant no. 2007–35604–17903 from the USDA National Institute of Food and Agriculture Animal Genome program. The authors thank Norman J. Stern from the Poultry Microbiological Safety Research Unit, USDA, for kindly providing the bacterial strain. Mention of commercial productions is for the sole purpose of providing specific information and is not a recommendation or endorsement by the USDA.

References

Aggarwal B.B. (2003) Signalling pathways of the TNF superfamily: a double-edged sword. *Nature Reviews Immunology* **3,** 745–56.

Altekruse S.F., Stern N.J., Fields P.I. & Swerdlow D.L. (1999) *Campylobacter jejuni*–an emerging foodborne pathogen. *Emerging Infectious Diseases* **5,** 28–35.

Barrett T., Troup D.B., Wilhite S.E., Ledoux P., Rudnev D., Evangelista C., Kim I.F., Soboleva A., Tomashevsky M. & Edgar R. (2007) NCBI GEO: mining tens of millions of expression profiles–database and tools update. *Nucleic Acids Research* **35,** D760–5.

Boldt S., Weidle U.H. & Kolch W. (2002) The role of MAPK pathways in the action of chemotherapeutic drugs. *Carcinogenesis* **23,** 1831–8.

Boquet P. (2000) Small GTP binding proteins and bacterial virulence. *Microbes and Infection* **2,** 837–43.

Boyd Y., Herbert E.G., Marston K.L., Jones M.A. & Barrow P.A. (2005) Host genes affect intestinal colonisation of newly hatched chickens by *Campylobacter jejuni*. *Immunogenetics* **57,** 248–53.

Bridgham J.T. & Johnson A.L. (2003) Characterization of chicken TNFR superfamily decoy receptors, DcR3 and osteoprotegerin. *Biochemical and Biophysical Research Communications* **307,** 956–61.

Cleveland W.S. (1974) Robust locally weighted regression and smoothing scatterplots. *Journal of the American Statistical Association* **74,** 829–36.

Cossart P. & Sansonetti P.J. (2004) Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* **304,** 242–8.

Dasti J.I., Tareen A.M., Lugert R., Zautner A.E. & Gross U. (2010) *Campylobacter jejuni:* a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *International Journal of Medical Microbiology* **300,** 205–11.

Dennis G., Sherman B., Hosack D., Yang J., Gao W., Lane H.C. & Lempicki R. (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biology* **4,** 3.

Dhillon A.S., Shivaprasad H.L., Schaberg D., Wier F., Weber S. & Bandli D. (2006) *Campylobacter jejuni* infection in broiler chickens. *Avian Diseases* **50,** 55–8.

Friedman C.R., Hoekstra R.M., Samuel M. *et al.* (2004) Risk factors for sporadic *Campylobacter* infection in the United States: a case-control study in FoodNet sites. *Clinical Infectious Diseases* **38**(Suppl. 3), S285–96.

Gu W., Siletzky R.M., Wright S., Islam M. & Kathariou S. (2009) Antimicrobial susceptibility profiles and strain type diversity of *Campylobacter jejuni* isolates from turkeys in eastern North Carolina. *Applied and Environmental Microbiology* **75,** 474–82.

Hu L., McDaniel J.P. & Kopecko D.J. (2006) Signal transduction events involved in human epithelial cell invasion by *Campylobacter jejuni* 81-176. *Microbial Pathogenesis* **40,** 91–100.

Huang D.W., Sherman B.T. & Lempicki R.A. (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocols* **4,** 44–57.

Jenner R.G. & Young R.A. (2005) Insights into host responses against pathogens from transcriptional profiling. *Nature Reviews. Microbiology* **3,** 281–94.

Junttila M.R., Li S.P. & Westermarck J. (2008) Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB Journal* **22,** 954–6.

Kogut M.H., Genovese K.J. & He H. (2007) Flagellin and lipopolysaccharide stimulate the MEK-ERK signaling pathway in chicken heterophils through differential activation of the small GTPases, Ras and Rap1. *Molecular Immunology* **44,** 1729–36.

Krause-Gruszczynska M., Rohde M., Hartig R., Genth H., Schmidt G., Keo T., Konig W., Miller W.G., Konkel M.E. & Backert S. (2007) Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*. *Cellular Microbiology* **9,** 2431–44.

Lee S., Park C. & Choi Y. (1996) T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain

in association with tumor necrosis factor receptor-associated factors. *J Exp Med* **183,** 669–74.

Li X., Chiang H.I., Zhu J., Dowd S.E. & Zhou H. (2008a) Characterization of a newly developed chicken 44K Agilent microarray. *BMC Genomics* **9,** 60.

Li X., Swaggerty C.L., Kogut M.H., Chiang H., Wang Y., Genovese K.J., He H., Stern N.J., Pevzner I.Y. & Zhou H. (2008b) The paternal effect of *Campylobacter jejuni* colonization in ceca in broilers. *Poultry Science* **87,** 1742–7.

Li X., Swaggerty C.L., Kogut M.H., Chiang H..I., Wang Y., Genovese K.J., He H. & Zhou H. (2010) Gene expression profiling of the local cecal response of genetic chicken lines that differ in their susceptibility to *Campylobacter jejuni* colonization. *PLoS One* **5,** e11827.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25,** 402–8.

MacCallum A., Haddock G. & Everest P.H. (2005) *Campylobacter jejuni* activates mitogen-activated protein kinases in Caco-2 cell monolayers and *in vitro* infected primary human colonic tissue. *Microbiology* **151,** 2765–72.

Nakayama K.I. & Nakayama K. (2006) Ubiquitin ligases: cell-cycle control and cancer. *Nature Reviews Cancer* **6,** 369–81.

Oswald E., Nougayrède J.-P., Taieb F. & Sugai M. (2005) Bacterial toxins that modulate host cell-cycle progression. *Curr Opin Microbiol* **8,** 83–91.

Pispa J., Mikkola M.L., Mustonen T. & Thesleff I. (2003) Ectodysplasin, Edar and TNFRSF19 are expressed in complementary and overlapping patterns during mouse embryogenesis. *Gene Expression Patterns* **3,** 675–9.

Rottner K., Lommel S., Wehland J. & Stradal T.E. (2004) Pathogen-induced actin filament rearrangement in infectious diseases. *J Pathol* **204,** 396–406.

Silke J. & Brink R. (2010) Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. *Cell Death and Differentiation* **17,** 35–45.

Stern N.J., Meinersmann R.J., Cox N.A., Bailey J.S. & Blankenship L.C. (1990) Influence of host lineage on cecal colonization by *Campylobacter jejuni* in chickens. *Avian Diseases* **34,** 602–6.

Tabbara K., Al Muraikhi A., Bazzi A. & Botta G. (2010) Severity of intestinal disease following *Campylobacter jejuni* infection correlates with level of TNF-{alpha} production and macrophage apoptosis. *Journal of Immunology* **184,** 42.6.

Watson R.O. & Galan J.E. (2005) Signal transduction in *Campylobacter jejuni*-induced cytokine production. *Cellular Microbiology* **7,** 655–65.

Widmann C., Gibson S., Jarpe M.B. & Johnson G.L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiological Reviews* **79,** 143–80.

Wooldridge K.G., Williams P.H. & Ketley J.M. (1996) Host signal transduction and endocytosis of *Campylobacter jejuni*. *Microbial Pathogenesis* **21,** 299–305.

Yang Y.H., Dudoit S., Luu P., Lin D.M., Peng V., Ngai J. & Speed T.P. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Research* **30,** e15.

Zhang Y. & Dong C. (2007) Regulatory mechanisms of mitogen-activated kinase signaling. *Cellular and Molecular Life Sciences* **64,** 2771–89.

Zheng J., Meng J., Zhao S., Singh R. & Song W. (2008) *Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB. *Infection and Immunity* **76,** 4498–508.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Overlapping genes between AC/AN and BC/BN.

Table S2 Functional analysis of immune-related genes in the comparison of AC and AN.

Table S3 Functional analysis of immune-related genes in the comparison of BC and BN.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.