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Antimicrobial activities of isothiocyanates against *Campylobacter jejuni* isolates

Virginie Dufour^{1†}, Bachar Alazzam¹, Gwennola Ermel^{1†}, Marion Thepaut¹, Albert Rossero^{2,3}, Odile Tresse^{2,3} and Christine Baysse^{1†*}

¹ Duals Team, UMR6026-CNRS, University of Rennes 1, Rennes, France

² INRA UMR1014 SECALIM 1014, Nantes, France

³ LUNAM Université, Oniris, University of Nantes, Nantes, France

Edited by:

Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:

Qijing Zhang, Iowa State University, USA

Kelli L. Hiett, Agricultural Research Service, USA

*Correspondence:

Christine Baysse, DUALS Team, UMR6026-CNRS, University of Rennes 1, 262, Avenue du Général Leclerc, F-35042 Rennes, France.
e-mail: christine.baysse@univ-rennes1.fr

† Present address:

Virginie Dufour, Gwennola Ermel and Christine Baysse, Microbiology Team, EA1254, University of Rennes 1, 35042 Rennes, France.

Food-borne human infection with *Campylobacter jejuni* is a medical concern in both industrialized and developing countries. Efficient eradication of *C. jejuni* reservoirs within live animals and processed foods is limited by the development of antimicrobial resistances and by practical problems related to the use of conventional antibiotics in food processes. We have investigated the bacteriostatic and bactericidal activities of two phytochemicals, allyl-isothiocyanate (AITC), and benzyl isothiocyanate (BITC), against 24 *C. jejuni* isolates from chicken feces, human infections, and contaminated foods, as well as two reference strains NCTC11168 and 81-176. AITC and BITC displayed a potent antibacterial activity against *C. jejuni*. BITC showed a higher overall antibacterial effect (MIC of 1.25–5 $\mu\text{g mL}^{-1}$) compared to AITC (MIC of 50–200 $\mu\text{g mL}^{-1}$). Both compounds are bactericidal rather than bacteriostatic. The sensitivity levels of *C. jejuni* isolates against isothiocyanates were neither correlated with the presence of a GGT (γ -Glutamyl Transpeptidase) encoding gene in the genome, with antibiotic resistance nor with the origin of the biological sample. However the *ggt* mutant of *C. jejuni* 81-176 displayed a decreased survival rate compared to wild-type when exposed to ITC. This work determined the MIC of two ITC against a panel of *C. jejuni* isolates, showed that both compounds are bactericidal rather than bacteriostatic, and highlighted the role of GGT enzyme in the survival rate of *C. jejuni* exposed to ITC.

Keywords: *Campylobacter jejuni*, isothiocyanates, gamma glutamyl transpeptidase, antimicrobials, plant extract, glucosinolate

INTRODUCTION

Campylobacter jejuni is a food-borne pathogen responsible of severe gastrointestinal diseases worldwide. In the US, the incidence of *C. jejuni* infections is the second largest after *Salmonella* cases (Gillis et al., 2011), whereas in European Union, *Campylobacter* infections are the most commonly reported bacterial gastrointestinal diseases (European-Food-Safety-Authority, 2011). *C. jejuni* can colonize poultry, cattle, pigs, and sheep asymptotically, and poultry is a particular common source of humans contamination (Friedman et al., 2004): humans are exposed to *C. jejuni* infection through handling and consuming contaminated meat, water, or raw milk. Infections result in severe diarrhea; moreover, serious sequels such as reactive arthritis and Guillain-Barré syndrome, a neurodegenerative complication, can result from *C. jejuni* infections (Nachamkin, 2002).

The research on natural preservatives to reduce meat contamination is therefore a major interest, and volatile substances like isothiocyanates (ITC), that may not influence processed food, are promising candidates for pathogen reduction. ITC are degradation products from glucosinolates, secondary metabolites which constitute a group of more than 140 different compounds, found in all plants belonging to the *Cruciferae* family (Fahey et al., 2001). Glucosinolates are stored in the cell vacuole and come into contact with the enzyme myrosinase (a thioglucosidase) located in cell wall

or cytoplasm during tissue damage (Fenwick et al., 1983; Poulton and Moller, 1993; Magrath et al., 1994). Glucosinolates are then hydrolyzed to a number of products, ITC being the quantitatively dominant compound. It is known that glucosinolates degradation products possess biological activities including beneficial effect on human health, fungicidal, herbicidal, and nematocidal properties (Fahey et al., 1997; Bonnesen et al., 1999; Lazzeri et al., 2004; Keum et al., 2005). Amongst them, ITC exhibit biocidal activities against various bacterial pathogens. There is now ample evidence for the antimicrobial properties of ITC (Aires et al., 2009a,b), but reports of suppression of bacteria by ITC are still limited to some bacteria, and nothing is known about their activity against *C. jejuni*.

Allyl ITC (AITC) is already used as preservative in food industry (Delaquis and Mazza, 1995; Masuda et al., 2001). AITC is generated from its precursor, allyl glucosinolate, namely, sinigrin (1-thio-L-D-glucopyranose 1-N-(sulfoxy)-3-buteneimide; Kawakishi and Namiki, 1969; Masuda et al., 1996) which is particularly abundant in horseradish (*Armoracia lapathifolia*) and wasabi (*Wasabia japonica*). AITC reportedly has antimicrobial activity against a wide range of microorganisms (Kyung and Fleming, 1997; Lin et al., 2000a,b; Masuda et al., 2001).

Jang et al. (2010) recently reported a greater antimicrobial activity of aromatic isothiocyanates, such as Benzyl isothiocyanate (BITC), compared to aliphatic ones, using four Gram-positive

bacteria (*Bacillus cereus* KCCM 11204, *Bacillus subtilis* KCCM 11316, *Listeria monocytogenes* KCCM 40307, and *Staphylococcus aureus* KCCM 12214) and seven Gram-negative bacteria (*Aeromonas hydrophila* KCTC 2358, *Pseudomonas aeruginosa* KCTC 1636, *Salmonella choleraesuis* KCCM 11806, *Salmonella enterica* KCTC12400, *Serratia marcescens* KCTC 2216, *Shigella sonnei* KCTC 2009, and *Vibrio parahaemolyticus* KCCM 11965). Recent data also had shown a bactericidal effect of BITC against Gram-negative periodontal pathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Sofrata et al., 2011). The vegetable source of BITC is the glucotropaeolin glucosinolate, found in Cabbage (Tian et al., 2005), Wasabi (Sultana et al., 2003), Papaya (Kermanshai et al., 2001), and Mustard (Dorsch et al., 1984).

MPITC was first isolated from the seeds of *Iberis sempervirens* (Kjaer et al., 1955) and more recently from the Egyptian plant *Capparis cartilaginea* (Hamed et al., 2007). MPITC and AITC are both constituents in horseradish and are on the GRAS list permitted for such flavors (Waddell et al., 2005). In nature, AITC constitute 37% of horseradish volatiles while MPITC is a minor constituent (about 1%). The antibacterial effect of this ITC is not yet determined.

Sulforaphane is generated from glucoraphanin, an abundant glucosinolate in some varieties of broccoli. It was found to be active against *Helicobacter pylori*, a microaerophilic epsilon proteobacteria such as *C. jejuni*, which dramatically enhances the risk of gastric cancer in infected patients (Fahey et al., 2002).

The antimicrobial activity of ITC is suggested to involve a reaction with thiol groups of glutathione or redox-active proteins, with subsequent inhibition of sulfhydryl enzyme activities and inhibition of redox-based defenses (Tang and Tang, 1976; Kolm et al., 1995; Jacob and Anwar, 2008). The addition of exogenous thiol groups can suppress the antimicrobial effect of ITC (Tajima et al., 1998). The activity of ITC varies with the structure of the molecule, but variations are also noticed amongst identical bacterial species for one ITC. Therefore, we can postulate that the efficacy of the ITC may depend on both the rate of spontaneous degradation of ITC-thiol conjugates and of the detoxification mechanisms of the bacterial isolate. The specific processes for ITC resistance of bacteria are still unknown. In rats and possibly other mammals, benzylITC (BITC) is degraded via conjugation with glutathione by the glutathione-S-transferase (GST), and transformed to cysteinyl glycine and cysteine conjugate by the Gamma Glutamyl Transpeptidase (GGT). The latter is N-acetylated to form mercapturic acid excreted in the urine (Brusewitz et al., 1977). In bacteria, the only report on ITC detoxification concerned cyanobacteria, and pointed out the role of GST and glutathione (Wiktelius and Stenberg, 2007).

Some strains of *C. jejuni*, including the highly virulent strain 81-176, possess the GGT, while all *C. jejuni* strains are unable to synthesize glutathione (Hofreuter et al., 2006). GGT was found to be important for the chick colonization rate and is suspected to contribute to virulence of some *C. jejuni* isolates (Barnes et al., 2007; Hofreuter et al., 2008; Feodoroff et al., 2010). However, its role in the detoxification of electrophilic compound, such as ITC, has not been investigated.

This study aims to analyze the antibacterial activity of two ITC against 24 *C. jejuni* isolates from various origins: chicken feces, human infections (blood or feces) and contaminated processed meats. Additionally, we investigated whether or not the presence of GGT in these isolates affects their sensitivity to ITC.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

The *C. jejuni* isolates used in this study are listed in Table 1. Strains NCTC11168 and 81-176 are widely used reference strains which genome sequences have been published (Parkhill et al., 2000; Hofreuter et al., 2006). Other isolates were selected for having different origins and various antibiotic resistance profiles and were isolated from independent pork or poultry slaughterhouses and processings, or independent human cases. All isolates were streaked on Mueller Hinton agar (MHA) and grown microaerobically at 37°C for 24 h, then the cells were harvested in 2 mL Mueller Hinton broth (MHB) and diluted in the same medium to the appropriate concentration ($OD_{600\text{ nm}} = 0.05$). All cultures were grown under microaerobic atmosphere (CampyGen, Oxoid) at 37°C with 150 rpm shaking.

PULSE FIELD GEL ELECTROPHORESIS

Pulse field gel electrophoresis (PFGE) was performed according to Ribot et al. (2001) and CAMPYNET protocol (<http://Campynet.vetinst.dk/PFGE.html>). Briefly, isolates were subcultured on Karmali at 42°C for 2–3 days under microaerobic atmosphere. Bacterial colonies were harvested and re-suspended in 1 mL of Tris buffer (100 mol L⁻¹ Tris, pH 8, and 100 mmol L⁻¹ EDTA). About 200 µL of suspension was subsequently mixed with an equal volume of 2% agarose (BioRad, Marnes-la-Coquette France) at 56°C. The mixture was molded into plugs and allowed to set at 4°C until totally gelified. The agarose plugs were placed in ETSP buffer (50 mmol L⁻¹ EDTA, 50 mmol L⁻¹ Tris pH 8, 1% Sarcosyl, and 1 mg mL⁻¹ Proteinase K) and incubated at 54°C overnight. Then, plugs were washed in TE buffer (10 mmol L⁻¹ Tris pH 8, 1 mmol L⁻¹ EDTA) four times for 0.5 h. Half of each plug was digested overnight with *Sma*I (New England BioLabs, Saint Quentin en Yvelines, France) at 24°C, and the resulting macrorestriction digests were electrophoresed using CHEF-DRIII system (BioRad) in 1.3% agarose gel in 10 times diluted 5× TBE buffer (0.45 mol L⁻¹ Tris-Borate, 0.01 mol L⁻¹ EDTA, pH 8.3) at 6 V cm⁻¹. Pulsing was ramped from 6 to 30 s over 21 h then 2–5 s over 3 h at 14°C. Gels were stained with ethidium bromide for 2 h, destained in water for 20 min and photographed under UV light with ChemiDoc™ XRS (BioRad). A lambda ladder PFG marker (New England BioLabs) was used for fragment size determination. Bands were analyzed using BioNumerics v 3.5 (Applied Maths Kortrijk, Belgique). Pulsotype grouping was performed with the band position tolerance of the Dice coefficient at 2.0%. When identical profiles were observed between strains with *Sma*I, *Kpn*I macrodigestion was performed in the same conditions as described above with the following modifications: electrophoresis was performed in 1.2% agarose gel and a pulsing ramping from 3 to 30 s over 22 h at 14°C.

Table 1 | *Campylobacter jejuni* isolates used in this study and some of their features.

Name	Provided by	Origin	Isolated from	Antibiotic resistance
NCTC11168	A. Stintzi ¹	Clinical isolate	Diarrheic patient, 1977	ND
81-176	O. Tresse ²	Clinical isolate	Diarrheic patient, 1985	ND
2-77	CNR Bordeaux ³	Clinical isolate	Human stools sample, 2010	Ampicillin, tetracycline, fluoroquinolones
2-78	CNR Bordeaux ³	Clinical isolate	Human stools sample, 2010	None
2-79	CNR Bordeaux ³	Clinical isolate	Human blood sample, 2010	Fluoroquinolones
2-80	CNR Bordeaux ³	Clinical isolate	Human blood sample, 2010	Tetracycline
2-81	CNR Bordeaux ³	Clinical isolate	Human intestinal biopsy, 2010	Fluoroquinolones
3-1	ANSES Ploufragan ⁴	Environmental	Pork slaughter house, 2009	Streptomycin, tetracycline
3-2	ANSES Ploufragan ⁴	Environmental	Pork slaughter house, 2009	Streptomycin, tetracycline
3-3	ANSES Ploufragan ⁴	Environmental	Pork slaughter house, 2009	Tetracycline
3-4	ANSES Ploufragan ⁴	Environmental	Pork slaughter house, 2009	Ciprofloxacin, nalidixic acid
3-5	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	Tetracycline, nalidixic acid
3-6	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	Tetracycline, ciprofloxacin, Nalidixic acid
3-7	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	Tetracycline, ciprofloxacin, Nalidixic acid
3-8	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	Ciprofloxacin, nalidixic acid
3-9	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	None
3-10	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	None
3-11	ANSES Ploufragan ⁴	Environmental	Poultry slaughter house, 2009	Tetracycline
3-12	ANSES Ploufragan ⁴	Food industry	Processed poultry, 2009	Tetracycline, ciprofloxacin, nalidixic acid
3-13	ANSES Ploufragan ⁴	Food industry	Processed poultry, 2009	None
3-14	ANSES Ploufragan ⁴	Food industry	Processed poultry, 2009	Tetracycline
3-15	ANSES Ploufragan ⁴	Food industry	Processed poultry, 2009	None
3-16	ANSES Ploufragan ⁴	Food industry	Processed poultry, 2009	None
3-17	ANSES Ploufragan ⁴	Food industry	Processed poultry, 2009	None

ND, not determined. These isolates were kindly provided by:

¹Alain Stintzi, Ottawa Institute of System Biology, Ottawa, Canada.

²Odile Tresse, SECALIM, Oniris, Nantes, France.

³Francis Mégraud, Centre National de Référence des Campylobacters et Helicobacters, Bordeaux, France.

⁴Katell Rivoal, Hygiene, and Quality of Poultry and Pork Products, ANSES, Ploufragan, France.

GROWTH ANALYSES

A quantity of 500 μ L of each isolates was inoculated at $OD_{600\text{ nm}} = 0.05$ in triplicate on 48-wells plates, and incubated microaerobically at 37°C under shaking. The $OD_{600\text{ nm}}$ was measured every 3 h to monitor the growth with an automatic plate reader (BioTek). The growth of each isolate was monitored at least in triplicate.

ISOTHIOCYANATES SOLUTIONS

Isothiocyanate commercial pure solutions [Allyl-isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (ETIC), and 3-(methylthio)propyl isothiocyanate (MTPITC)] were purchased from Sigma-Aldrich. Pure solutions were diluted in absolute ethanol to 100 mg mL^{-1} (AITC and EITC) or 10 mg mL^{-1} (BITC and MPITC) stock solutions.

MINIMUM INHIBITORY CONCENTRATION DETERMINATION

Minimum inhibitory concentrations (MICs) were determined by two different versions of the agar dilution method.

Briefly, twofold serial dilutions of isothiocyanate stock solutions were added to 50°C molten MHA to get the final desired concentrations (from 6.25 to 200 $\mu\text{g mL}^{-1}$ AITC and EITC or

from 0.625 to 20 $\mu\text{g mL}^{-1}$ for BITC and MPITC) and then the media were poured on 45 or 100 mm Petri dishes.

Cells were inoculated at $OD_{600\text{ nm}} = 0.05$ in triplicate in 48-well plates, and incubated microaerobically for 6 h with shaking.

Each 6 h-culture was diluted to $OD_{600\text{ nm}} = 0.01$ and 40 μ L, corresponding to approximately 5×10^5 CFU mL^{-1} , were spread on 45 mm plates. MIC was defined as the lowest isothiocyanate concentration in solid MHA where no growth was observed after 48 h of 37°C microaerobic incubation.

Alternatively, 10-fold serial dilutions of 6 h-cultures were made in MHB and 5 μ L of each dilution were spotted on 100 mm plates. Hundred microliters of some dilutions were also spread on Columbia agar plates to determine colony-forming units per milliliter concentration of each culture. MIC was defined as the lowest isothiocyanate concentration that inhibited any visible growth of a 10^5 - to 5×10^5 -CFU spot after 48 h of 37°C microaerobic incubation.

In both cases, ethanol was added to MHA as a negative control and inoculated plates without any addition were used as positive growth controls. Each plate was done in triplicates and each experiment was repeated twice.

Minimal inhibitory concentrations and MBCs were also assayed in liquid medium. Strains were inoculated in 5 mL MHB at $OD_{600\text{ nm}} = 0.05$, then 10 μ L of either isothiocyanate dilution

(in absolute ethanol) or absolute ethanol or sterile water (controls) were added. Final ITC concentrations were 10, 5, 2.5, and 1.25 $\mu\text{g mL}^{-1}$ for AITC, and 1.25, 0.625, 0.312, and 0.156 $\mu\text{g mL}^{-1}$ for BITC. $\text{OD}_{600\text{ nm}}$ were measured before and after 18 h of microaerobic incubation at 37°C with shaking. Minimal Inhibitory Concentration was defined as the lowest concentration of ITC that inhibits any visible growth after 18 h of incubation. Cells were also spread before and after incubation on MHA for colony counting. MBC was defined as the lowest concentration of ITC that kills 99.9% of the bacteria (i.e., 3 log reduction) after 18 h of incubation.

SURVIVAL ASSAYS

Campylobacter jejuni strains were grown in MHB in 50-mL sterile culture flasks. After ITC addition, (final concentrations AITC: 50, 100, or 200 $\mu\text{g mL}^{-1}$; BITC: 0, 2.5, 5, or 10 $\mu\text{g mL}^{-1}$), samples were collected at 0, 6, 12, and 24 h of growth, and the viable cells were numbered by plating serial dilutions onto MH agar plates and colony counting after incubation for 24 h at 37°C in microaerobic conditions. The experiment was performed twice with triplicate assays.

CONSTRUCTION OF *C. JEJUNI* 81-176 *ggt* MUTANT

To investigate the roles of GGT, the chromosomal region in *C. jejuni* containing the *ggt* gene was deleted and replaced with the 3'-amino-glycoside phosphotransferase type III gene (*aphA-3*, Km cassette) through a homologous recombination event. The plasmid containing the $\Delta\text{ggt}::\text{aphA-3cat}$ gene was constructed as follows. The plasmid pE1509 containing the upstream sequence of the *ggt* gene was obtained by cloning the PCR product amplified with primers *ggtD21Lxba* (5'-GAAGATAGTATAAAATGCACTCTAGAAAAG-3') and *ggtD22Rnde* (5'-ACTTAGCGTGATTGAAATCGCATATGTAG-3') in the pGEMTeasy (Promega). After digestion by *NdeI*, the insert was introduced into the plasmid pE1520 containing the downstream sequence of the *ggt* gene which was obtained by cloning the PCR product obtained with primers *ggtD21Rxba* (5'-GCGATGATAATAGGACTTGCTCTAGACACTAT-3') and *ggtD22Lsac* (5'-ATCCAAAAATGGAATAATCCGCGGCTCT-3') in the pGEMTeasy (Promega) to give the plasmid pE2057. The Km cassette obtained by PCR amplification using primers LKmSma (5'-TGCCCCGGGACAGTGAATTGGAG-3') and RKmSma (5'-CCCCCGGGCATTGCAATCCTAA-3') and restricted by *SmaI* was inserted at the *PfiMI* site of the plasmid pE2057 between the upstream and downstream regions of the *ggt* gene. The resulting suicide vector pE2088 was electroporated into the *C. jejuni* 81-176 strain. Km resistant clones were selected on Colombia agar (Oxoid) containing kanamycin (50 $\mu\text{g mL}^{-1}$). The double crossing over was checked using PCR amplification with either primers *ggtV1for* (5'-GCTTCCCACCGCAGGATCGC-3') and *KmV1rev* (5'-ACCTGGGAGACAGCAACATC-3'); or primers *KmV1for* (5'-TTCCTTCCGTATCTTTTACGC-3') and *ggtV1rev* (5'-GCTTTTGCTTGTGCTTTTGCGGGA-3'). The construction in *C. jejuni* 81-176 was finally checked by DNA sequencing.

PCR SCREENING OF *ggt* GENE IN *C. JEJUNI* ISOLATES

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) for use as a PCR matrix. Presence

of a GGT encoding gene on the *C. jejuni* isolates genomes was checked by PCR using two primer pairs specific for both 5' and 3' conserved regions of the *ggt* gene in *C. jejuni* and related strains. The *ggt* genes of *C. jejuni* 81-176, *C. jejuni* subsp. *doylei* 269.97, *C. jejuni* subsp. *jejuni* 260.94, *C. jejuni* subsp. *jejuni* HB93-13 and *H. pylori* 26695 were aligned using the MultAlin software (Corpet, 1988; **Figure A1** in Appendix). PCR amplification with *ggt1* (5'-CACGCTAAGTTTTGGTGAG-3') and *ggt4* (5'-GTCCTTCCTTTGCAATA-3') primers produces a PCR product of 622-bp starting at base 33 from ATG of *C. jejuni* *ggt* genes, while utilization of *ggt3* (5'-TACATGGGCGATCCTGATTT-3') and *ggt6* (5'-GCATTAGCTTCTCCGCCTA-3') amplifies a 330-bp-long DNA fragment of *ggt* starting at base 960 from ATG of *C. jejuni* *ggt* genes. Genomic DNA from *C. jejuni* NCTC1168 and 81-176 were used as negative and positive controls respectively.

PCR amplifications with *C. jejuni* 16S RNA gene specific primers, 16SCJA (3'-AGAGTTTGATCCTGGCTCAG-5') and 16SCJB (5'-TGCTCAGTTCAGTGTGACT-3') were performed on each DNA sample as supplementary control.

STATISTICAL ANALYSES

Data from all three technical replicates of the two independent survival assays were analyzed by Student *t*-test. Possible correlations between origin, antibiotic resistance, presence, or absence of the *ggt* gene, and sensitivity to isothiocyanates of all 24 isolates were assessed by Fisher's exact test. For all tests, *p*-values < 0.05 were considered significant.

RESULTS

GROWTH RATE OF *C. JEJUNI* ISOLATES AND SELECTION

Twenty four isolates (including the two reference strains *C. jejuni* NCTC1168 and 81-176, plus the *ggt* mutant of 81-176) were selected for their similar growth rate in 48-well plates on MHB medium at 37°C in microaerobic conditions. As shown in **Table 2**, most *C. jejuni* isolates display a mean growth rate constant between 0.29 and 0.15 h^{-1} in such conditions. Two isolates (3-14 and 3-6) with a reduced growth rate as well as an isolate (3-16) with a higher growth rate were selected to evaluate the impact of the generation time on the sensitivity to ITC. However, all cultures reached a similar biomass, i.e., a final $\log_2(100 \times \text{OD}_{600\text{ nm}})$ from 4.3 to 6.2, after 24 h growth, indicating that the cell viability was not affected in slower growth isolates (**Table 2**). Moreover, the correlation between $\text{OD}_{600\text{ nm}}$ (0.05 and 0.15) and the number of colony-forming units per milliliter was the same for every isolate (data not shown).

PULSE FIELD GEL ELECTROPHORESIS OF *C. JEJUNI* ISOLATES

To assess the genomic diversity of the 24 isolates used in this study, PFGE was performed using *SmaI* and *KpnI*. Each of the 24 isolates has a different *SmaI* or *KpnI* pulsotype (**Figure 1**). Two pairs of isolates (3-8 and 3-17, 3-13 and 2-77) displayed very similar *SmaI* pulsotypes (S9 and S9', S18 and S18') but had different pulsotypes after *KpnI* digestion (**Figure A2** in Appendix).

Table 2 | Growth of *C. jejuni* isolates.

Isolates	Growth rate, mean (h^{-1}) \pm SD	Final $\log_2(100 \times OD)$, mean \pm SD
NCTC1168	0.262 \pm 0.046	5.637 \pm 0.028
81-176	0.195 \pm 0.034	5.786 \pm 0.071
2-77	0.292 \pm 0.003	5.368 \pm 0.048
2-78	0.285 \pm 0.043	5.749 \pm 0.030
2-79	0.275 \pm 0.056	5.809 \pm 0.017
2-80	0.322 \pm 0.054	5.869 \pm 0.072
2-81	0.146 \pm 0.026	5.339 \pm 0.405
3-1	0.046 \pm 0.019	4.345 \pm 0.172
3-2	0.158 \pm 0.008	6.038 \pm 0.043
3-3	0.068 \pm 0.010	4.348 \pm 0.024
3-4	0.261 \pm 0.085	5.357 \pm 0.030
3-5	0.259 \pm 0.054	5.213 \pm 0.090
3-6	0.068 \pm 0.006	4.945 \pm 0.117
3-7	0.171 \pm 0.006	5.492 \pm 0.071
3-8	0.236 \pm 0.023	6.199 \pm 0.037
3-9	0.132 \pm 0.044	5.208 \pm 0.314
3-10	0.194 \pm 0.009	5.368 \pm 0.008
3-11	0.285 \pm 0.020	5.327 \pm 0.035
3-12	0.370 \pm 0.056	5.823 \pm 0.237
3-13	0.291 \pm 0.028	5.056 \pm 0.126
3-14	0.239 \pm 0.012	5.953 \pm 0.150
3-15	0.256 \pm 0.041	5.323 \pm 0.123
3-16	0.450 \pm 0.061	5.190 \pm 0.189
3-17	0.353 \pm 0.045	5.725 \pm 0.039

C. jejuni isolates were grown on 48-well plates in MH medium, in microaerobic conditions (CampyGen, Oxoid) at 37°C. Growth rates were calculated as the slope of the growth curves during logarithmic phase and expressed as h^{-1} . Final OD were measured at 600 nm after 24 h growth. SD (minimum of three replicates).

MINIMAL INHIBITORY CONCENTRATIONS OF ISOTHIOCYANATES AGAINST *C. JEJUNI* ISOLATES

AITC, BITC, and MPITC were preliminary chosen for their chemical properties (Figure 2), their occurrence in natural plant compounds and for their reported antibacterial activity.

EITC was selected as a negative control since it has a high volatile property (Figure 2) and there is no report of an antibacterial effect such as when tested against intestinal bacteria including *E. coli*, *Clostridia*, *Lactobacilli*, and bifidobacteria (Kim and Lee, 2009).

Although sulforaphane was previously reported to display an inhibitory effect against the *C. jejuni* closely related genus *Helicobacter* (Fahey et al., 2002), and despite preliminary experiments carried-out in our laboratory that demonstrated a MIC of 15 $\mu\text{g mL}^{-1}$ against *C. jejuni* NCTC1168 (Ermel, G., unpublished), the high cost of chemically purified sulforaphane dissuaded us from using it in a large scale study.

A first assay was performed with the spread-based method to determine the MIC of the four ITC against the reference strain *C. jejuni* NCTC1168. As expected, the MIC of EITC was higher than 200 $\mu\text{g mL}^{-1}$ (upper limit of the assay) while the MIC of AITC was of 200 $\mu\text{g mL}^{-1}$. MPITC and BITC displayed an identical MIC of 5 $\mu\text{g mL}^{-1}$. Therefore, BITC was selected instead

of MPITC for further study according to its widespread natural occurrence.

When the MIC assay was extended to the 24 *C. jejuni* isolates by the spot-based method, two groups of sensitivity appeared (Figure 1). The nine more resistant isolates are NCTC1168, 2-77, 2-78, 2-80, 3-2, 3-4, 3-6, 3-16, and 3-17, on which the MIC of AITC was of 200 $\mu\text{g mL}^{-1}$ and the MIC of BITC was of 5 $\mu\text{g mL}^{-1}$. For the majority of tested *C. jejuni* isolates ($n = 11$) the MIC were of 100 $\mu\text{g mL}^{-1}$ for AITC and 2.5 $\mu\text{g mL}^{-1}$ for BITC. The growth of the most sensitive isolate (3-13) was inhibited at minimal concentrations of 50 $\mu\text{g mL}^{-1}$ of AITC and 1.25 $\mu\text{g mL}^{-1}$ of BITC. Additionally, four isolates (3-5, 3-9, 3-12, and 3-15) displayed miscellaneous sensitivity to AITC (from 50 to 200 $\mu\text{g mL}^{-1}$) and BITC (from 2.5 to 5 $\mu\text{g mL}^{-1}$). Isothiocyanate sensitivity profiles did not correlate with similarities between isolates as determined by PFGE. Even closely related isolates (3-8 and 3-17, 3-13 and 2-77) have different sensitivities to AITC and BITC (Figure 1).

It is interesting to note that the *ggt* mutation in *C. jejuni* 81-176 did not impact on the MIC of AITC and BITC (Figure 1).

COMPARISON BETWEEN ITC RESISTANCE AND *ggt* DETECTION ON GENOMES

Using two distinct pairs of primers specific for conserved regions of *ggt* in four *C. jejuni* genomes, DNA fragments were amplified by PCR with both primer pairs in *C. jejuni* isolates 81-176 (positive control), 2-77, and 3-16.

This low prevalence of the *ggt* gene in our *C. jejuni* isolate panel is in accordance with a previous study that described the presence of GGT in only 15 out of 166 *C. jejuni* human isolates (Feodoroff et al., 2010) whereas Gonzalez et al. (2009) identified 36.6% of their chicken isolates (out of 205) as *ggt*-positive.

There is no correlation neither between the MIC of AITC and BITC and the presence of GGT nor between the MIC and the origin or pulsotype of the *C. jejuni* isolates (Figure 1; Fisher's exact test, p -value > 0.05).

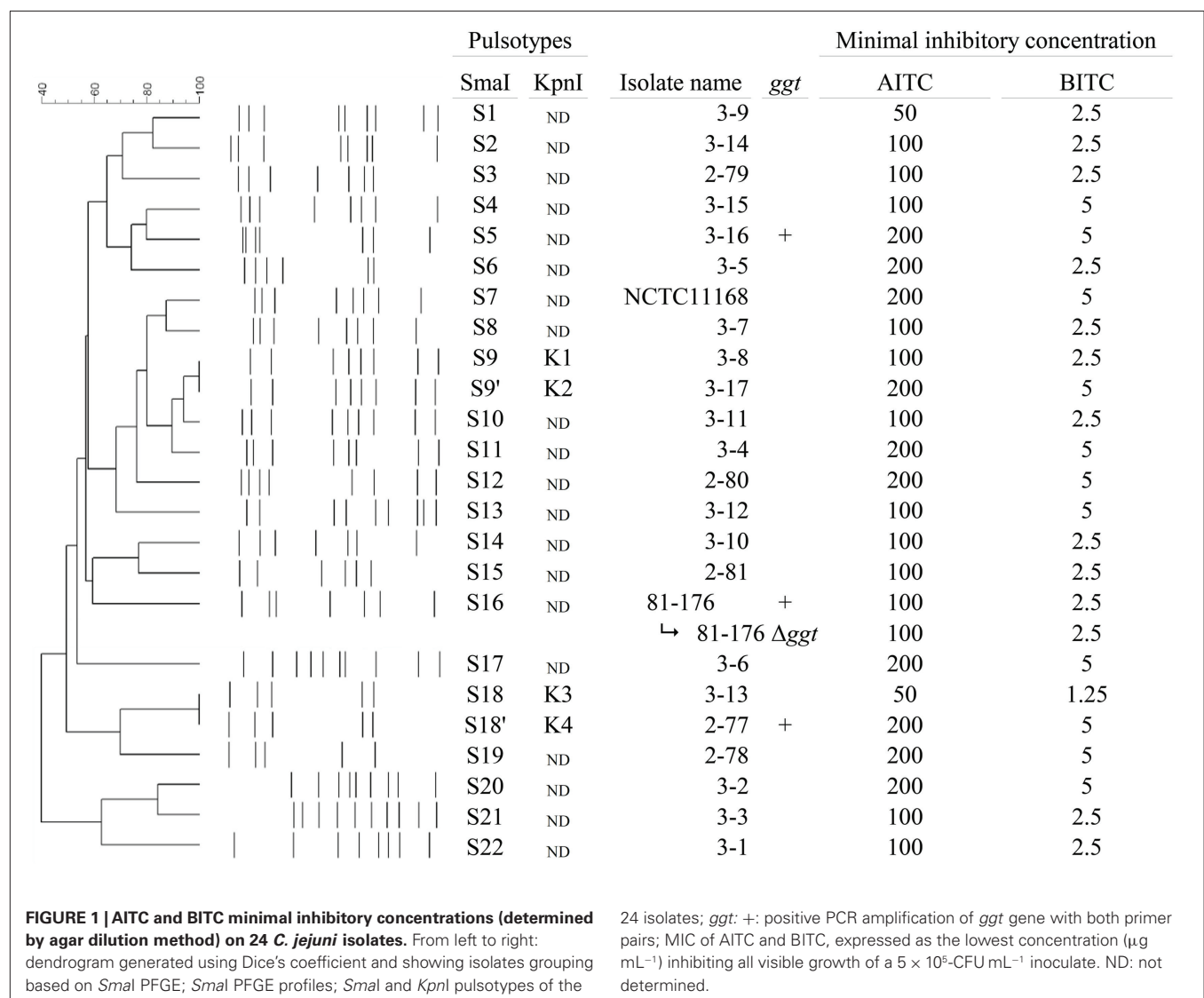
DETERMINATION OF THE MINIMAL BACTERICIDAL CONCENTRATION

For both ITC, MIC, and MBC values were identical (Table 3.). Therefore, we can affirm that AITC and BITC have a bactericidal effect on *C. jejuni*. Moreover the MIC values are higher against *C. jejuni* NCTC1168 than against 81-176, and not affected by the *ggt* mutation, as found with the agar dilution method (Figure 1).

AITC and BITC MIC and MBC values are the same on wild-type 81-176 and *ggt* mutant. However, MBC are discrete values, so the wild-type and mutant strain can have a different killing rate while displaying the same MBC value. While counting colonies for this MBC assay, we noticed that there were 100–1000 times less *ggt* mutant cells than 81-176 wild-type cells after treatment with 5 $\mu\text{g mL}^{-1}$ AITC or 0.625 $\mu\text{g mL}^{-1}$ BITC (corresponding to MBC values). As a consequence, we decided to carry on a more dynamic sensitivity experiment on these two strains.

SURVIVAL ASSAYS OF *C. JEJUNI* 81-176 AND *ggt* MUTANT

The standard assay for testing the antibiotic susceptibility of bacteria is MIC, but this method is of limited value in determining the susceptibility kinetic of bacteria and the ratio of cells surviving to MIC. An alternative approach to measurement of the bactericidal activity of antimicrobial agents is the time-kill method, which



examines the impact of antimicrobial exposure on the bacterial population at multiple time-points rather than the single time point used in the MIC method, and evaluates the rate of survival rather than the inhibition of growth.

To determine whether AITC and BITC were as effective in killing the *C. jejuni* 81-176 and *ggt* mutant, cell viability was measured by bacterial plating at concentrations closer to the MIC and at completely lethal concentrations (Figure 3).

For the strain *C. jejuni* 81-176 on which AITC displayed a MIC of $100 \mu\text{g mL}^{-1}$ in solid medium, an exposure of 24 h to $100 \mu\text{g mL}^{-1}$ AITC is sufficient to kill the whole population. Interestingly, the isogenic *ggt* mutant of 81-176 had a lower survival rate than the wild-type strain when exposed to AITC (Figure 3A, $p = 0.0001$); however both populations are erased by 24 h-exposure to $50 \mu\text{g mL}^{-1}$ AITC. A similar pattern was found when *C. jejuni* 81-176 strain and *ggt* mutant were exposed to BITC in liquid MH although the difference in survival rates between the two strains and mutant were less marked than with AITC but still significant (Figure 3B, $p = 0.001$).

DISCUSSION

The development of antibiotic resistance by *C. jejuni* strains, mainly to fluoroquinolone and macrolides, is a major concern for human health and poultry industry (Luangtongkum et al., 2009; Smith and Fratamico, 2010). Although antibiotics have been banned by the poultry industry, there is a persistence of antibiotic resistant strains of *C. jejuni* in animal reservoirs. Zhang et al. (2003a) demonstrated that these resistant strains survived more successfully in their hosts than the non-resistant strains even without selection pressure.

Cross-contamination from various sources during slaughter occurred, but the majority of *Campylobacter* contamination on carcasses appeared to originate from the slaughtered flock itself (Wirz et al., 2010). Therefore, by reducing the colonization of chick intestine by *C. jejuni*, the human infection through contaminated food consumption may be reduced.

It has been suggested that *C. jejuni* was more sensitive to natural antimicrobials such as olive leaf extract compared to other pathogenic microorganisms (Sudjana et al., 2009). Other examples of

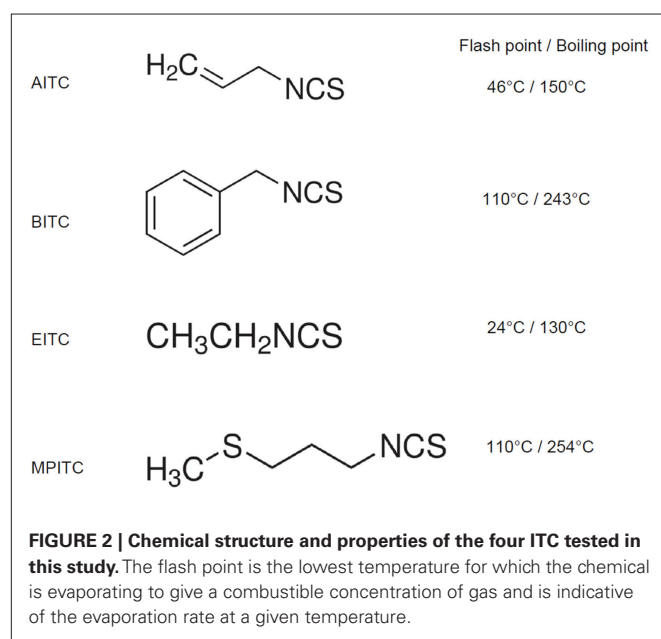


Table 3 | Minimal inhibitory (MIC) or bactericidal (MBC) concentrations in broth cultures.

Strain	MIC		MBC	
	AITC	BITC	AITC	BITC
NCTC11168	10	1.25	10	1.25
81-176	5	0.625	5	0.625
81-176 Δ ggt	5	0.625	5	0.625

Values are given in $\mu\text{g ml}^{-1}$.

efficient natural antimicrobials against *C. jejuni* include linalool vapor of bergamot and linalool oils (Fisher and Phillips, 2006) and essential oil from *Origanum minutiflorum* (Aslim and Yucel, 2008).

It is only recently that plant-derived food ingredients have been explored for their antimicrobial properties. So far, essential oils were the most frequently used form of plant extract tested for antimicrobial activity in foods. Isothiocyanates are other plant extracts with flavoring properties that are arising as promising antimicrobial agents, with activities often rivaling synthetic chemicals. Researches are focusing on the use of active packaging with ITC (Shin et al., 2010), or their combination with existing antibiotic treatments (Palaniappan and Holley, 2010). However there is only one report about efficiency of ITC (sulforaphane) against *C. jejuni* (Woelffel, 2003).

Our work assessed *C. jejuni* sensitivity to isothiocyanates using several methods: minimal inhibitory concentration (MIC) determination in solid and liquid media, MBC determination, and survival assays.

Not surprisingly, the behavior of *C. jejuni* strains in liquid culture plus ITC appears different than onto solid medium. MIC values of AITC and BITC are lower when measured in MH broth than in agar dilution method (Figure 1; Table 3). MIC values are

not a biological constant. The MIC is influenced by many factors such as the interaction between the antimicrobial agent, the bacterial cell and the medium, and the physiological status of the cells. It is known that for most antimicrobial, the concentration required to kill sessile bacteria may be greater than those required to kill planktonic bacteria. MIC values can only be compared if they are measured under well standardized conditions, which are not yet been defined for such volatile compounds as ITC. We have adapted the agar dilution methods for plant extracts previously described by Klancnik et al. (2010). These authors already pointed out a discrepancy between the antibacterial activity levels obtained by the agar dilution method and the broth dilution method for Gram-negative bacteria: a lower concentration of antimicrobial was required for growth inhibition in liquid culture. Moreover, the evaporation rate of ITC may be higher in liquid culture than when ITC are embedded into agar medium. Previous data have shown that AITC vapor was more effective as antimicrobial agent than liquid AITC (Shin et al., 2010); thus it may partially explained the differences we have observed between the two methods.

To investigate the bacteriostatic or bactericidal effect of isothiocyanate on *C. jejuni*, we carried on a MIC determination experiment in liquid media on the two reference strains NCTC11168 and 81-176, and the *ggt* mutant. By counting the viable cells at different ITC concentrations, the MBC can be determined. A given compound can be called bactericidal when it kills bacteria rather than it inhibits the metabolism, i.e., when the MIC and the MBC value are identical. The MBC of AITC and BITC was measured as the minimum concentration needed to kill most (99.9%) of the *C. jejuni* cells (NCTC1168 and 81-176 strains) after incubation for 18 h under a given set of conditions: in MH broth at 37°C in microaerobic conditions. While sulforaphane was reported as a bacteriostatic antimicrobial compound (Woelffel, 2003), our work shows that AITC and BITC clearly display bactericidal activities. MIC and MBC of AITC were higher than those of BITC on each of the 24 *C. jejuni* tested isolates.

In addition, the growing number of reports on antibacterial and anticancer activities of ITC has increased their interest as food supplements. Dietary ITC exert a cancer chemopreventive effect in animals. ITC are able to reduce carcinogen-induced tumorigenesis by inducing carcinogen detoxification (Hecht, 1995), to interfere with angiogenesis (Thejass and Kuttan, 2007a,b), and to induce cell cycle arrest and cell death in cancer cells, while decreasing cancer cell invasion and metastasis (Zhang et al., 2003b). The most intensively studied ITC as chemopreventive agents are AITC, BITC, PEITC, and sulforaphane. For a recent review see Zhang (2012). The effect of ITC as chemopreventive agents is predominantly mediated by the formation of inactive labile thiocarbamate adducts by reaction with the thiol groups of target proteins, while the reaction with amine groups to give thiourea derivatives is less common due to a lesser affinity and a significantly lower reaction rate. The reactivity of specific amines and thiols in target proteins also depends on their own pK_a values (Podhradsky et al., 1979). ITC accumulate as GSH conjugates in the cells and binds to target protein via thiols exchange reactions (Zhang, 2000; Mi et al., 2010).

Concerning thiols exchanges reactions, examples of well studied direct ITC targets are the cytochrome P450 monooxygenase

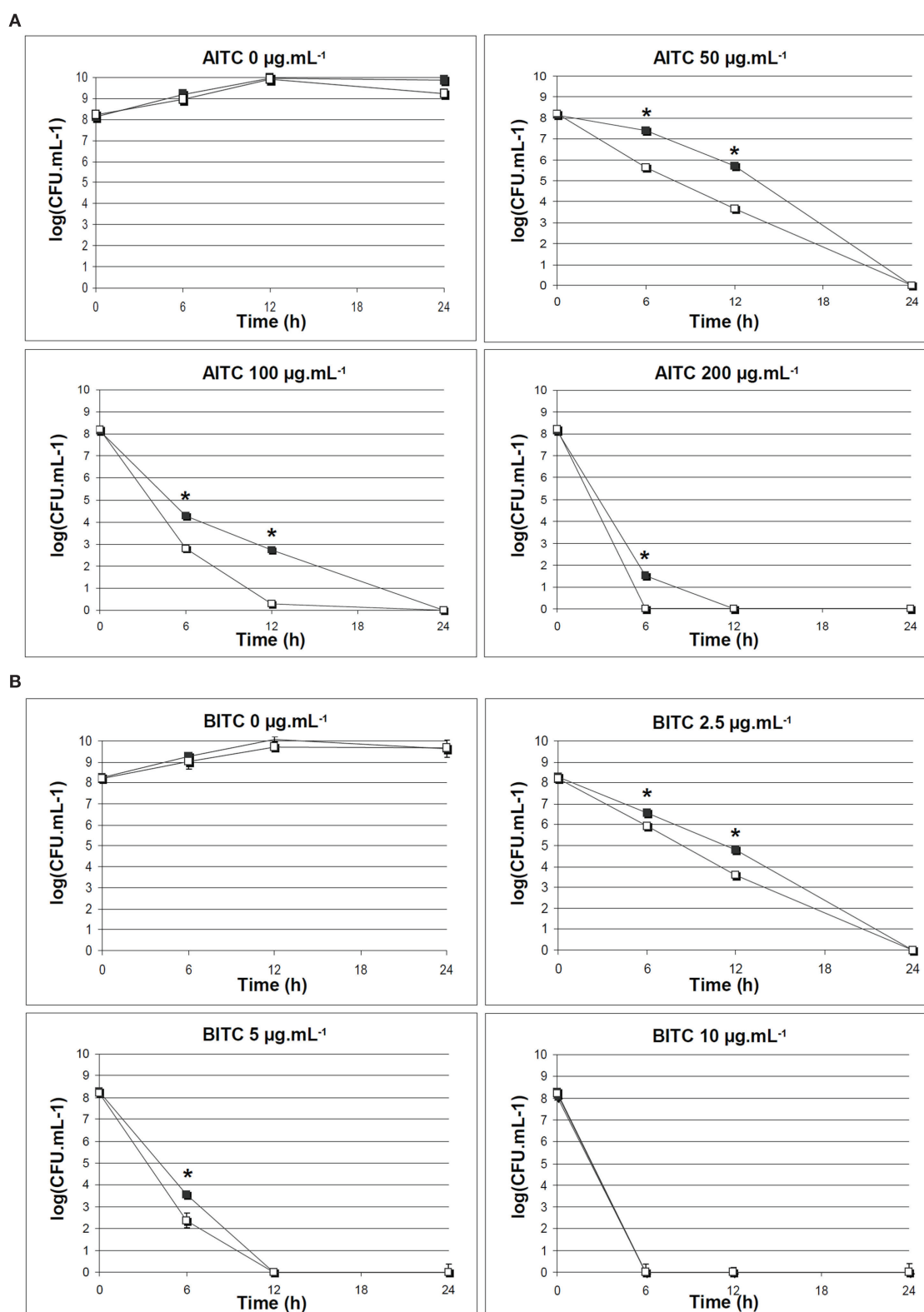


FIGURE 3 | Survival rate of *C. jejuni* 81-176 WT and *ggt* mutant exposed to AITC or BITC. *C. jejuni* strains were grown in MH Broth at 37°C in microaerobic conditions and viable cells were numbered after 6, 12, or 24 h-exposure to ITC. Black squares: *C. jejuni* 81-176 WT; white squares:

C. jejuni 81-176 *ggt* mutant. **(A)** AITC at 0, 50, 100, or 200 µg.mL⁻¹; **(B)** BITC at 0, 2.5, 5, or 10 µg.mL⁻¹. The experiment was performed twice with triplicate assays, each point represents mean of 6 data points with SDs. **p* < 0.05, *t*-test.

(a detoxifying enzyme responsible for the activation of numerous carcinogens; Moreno et al., 1999), the Keap1 protein which inactivation results in the accumulation and activation of the major regulator of the antioxidant response Nrf2 (Zhang and Hannink, 2003), and the α -tubulin thus leading to cell cycle arrest (Smith et al., 2004). ITCs disrupt the redox homeostasis of the cells, both via a long lasting adaptive response depending on the activation of Nrf2 (transcription of antioxidant and repairs enzymes; Jeong et al., 2005) but also by depleting the levels of available intracellular GSH (Zhang, 2000) and by inhibiting glutathione/thioredoxine reductases (Hu et al., 2007). However the biological effect of ITC is dose dependant: at high concentration, the modification of mitochondrial functions leads to proapoptotic cytochrome *c* release, reactive oxygen species (ROS) generation, respiratory alteration, and necrotic cell death. The resulting inflammation may lead to carcinogenic effects (Nakamura et al., 2002).

As we reported in this paper about the MIC of various ITC against *C. jejuni*, variation in the R groups of ITC also accounts for different chemopreventive biological efficiencies of ITC in animals, since the side chain may modify the electrophilicity of –NCS groups, the accessibility to nucleophilic centers, and the lipophilicity of ITC and therefore their location in cell compartments (Zhang, 2012). Our study also showed that the resistance levels of several *C. jejuni* strains for a given ITC are varying, and this variation is probably related to the specific genetic potential of each strain for ITC-conjugate and/or ROS detoxification.

In most living organisms, GGT catalyzes the first step in the degradation of GSH by cleavage and transfer of the γ -glutamyl moiety from GSH to an amino acid acceptor (or hydrolysis to release glutamate). This reaction leads to a release of cysteinyl glycine. GGT enzyme is involved in ITC detoxification in eukaryotes after their conjugation to GSH by GST (glutathione-S-transferase) enzymes (Brusewitz et al., 1977); ITC detoxification in cyanobacteria, also involve GST and glutathione (Wiktelius and Stenberg, 2007).

Our result also suggest that the GGT enzyme may be involved in ITC detoxification in *C. jejuni* since a *ggt* mutant displayed a decreased survival rate to ITC compared to its isogenic wild-type. Despite the absence of glutathione biosynthesis and GST in *C. jejuni*, our results suggest that GGT may be able to detoxify conjugates with other low molecular weight thiols present in *C. jejuni* cells, such as cysteine or *N*-acetylcysteine. The exact mechanism of GGT-mediated ITC resistance remains to be unveiled.

However, only few *C. jejuni* isolates encode a *ggt* gene. Additionally, the presence or absence of the *ggt* does not correlate with isothiocyanate sensitivity, indicating that ITC resistance probably depends on several factors, the presence of the *ggt* being only one of many genetic differences between *C. jejuni* isolates. For example, efflux systems could be involved, as it has been described for *Pseudomonas syringae* pathovars *maculicola*: the *sax* genes encoding resistance–modulation–division efflux systems were found to be required to overwhelm aliphatic isothiocyanate-based defenses of Arabidopsis plants (Fan et al., 2011). Even if homologs of these genes have not been found in *C. jejuni* genomes, Campylobacters possess efflux systems (Lin et al., 2002; Mamelli et al., 2005). Moreover, the resistance levels of *C. jejuni* isolates to ITC do not correlate with their resistance to antibiotics (this study, Tables 1 and 3; Woelffel, 2003) suggesting that these resistance mechanisms are distinct.

Although the antibacterial activity of ITC seems to be directed by their non-specific binding to sulfhydryl groups on the active sites of enzymes and of glutathione (Tang and Tang, 1976; Kolm et al., 1995), the exact mechanisms for ITC antibacterial activity are not completely known. It has been shown that AITC inhibited the catalysis by both thioredoxin reductase and acetate kinase in *E. coli* O157:H7 (Luciano and Holley, 2009). ITC also cause membrane damage and leakage of cellular metabolites (Lin et al., 2000b).

From a transcriptomic analysis, it was recently reported that the isothiocyanate iberin from Brassicaceae, specifically blocks expression of quorum sensing regulated genes in *Pseudomonas aeruginosa* and induced the MEF–OprN efflux pump (Jakobsen et al., 2012).

We are currently investigating the role of several disulfide oxidoreductases of *C. jejuni* in the resistance to AITC and BITC. Moreover, by analyzing the data from a carried-out transcriptomic identification of the *C. jejuni* transcripts induced or repressed by subinhibitory concentrations of BITC, we expect to unravel the whole *C. jejuni* response to ITC.

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APPENDIX

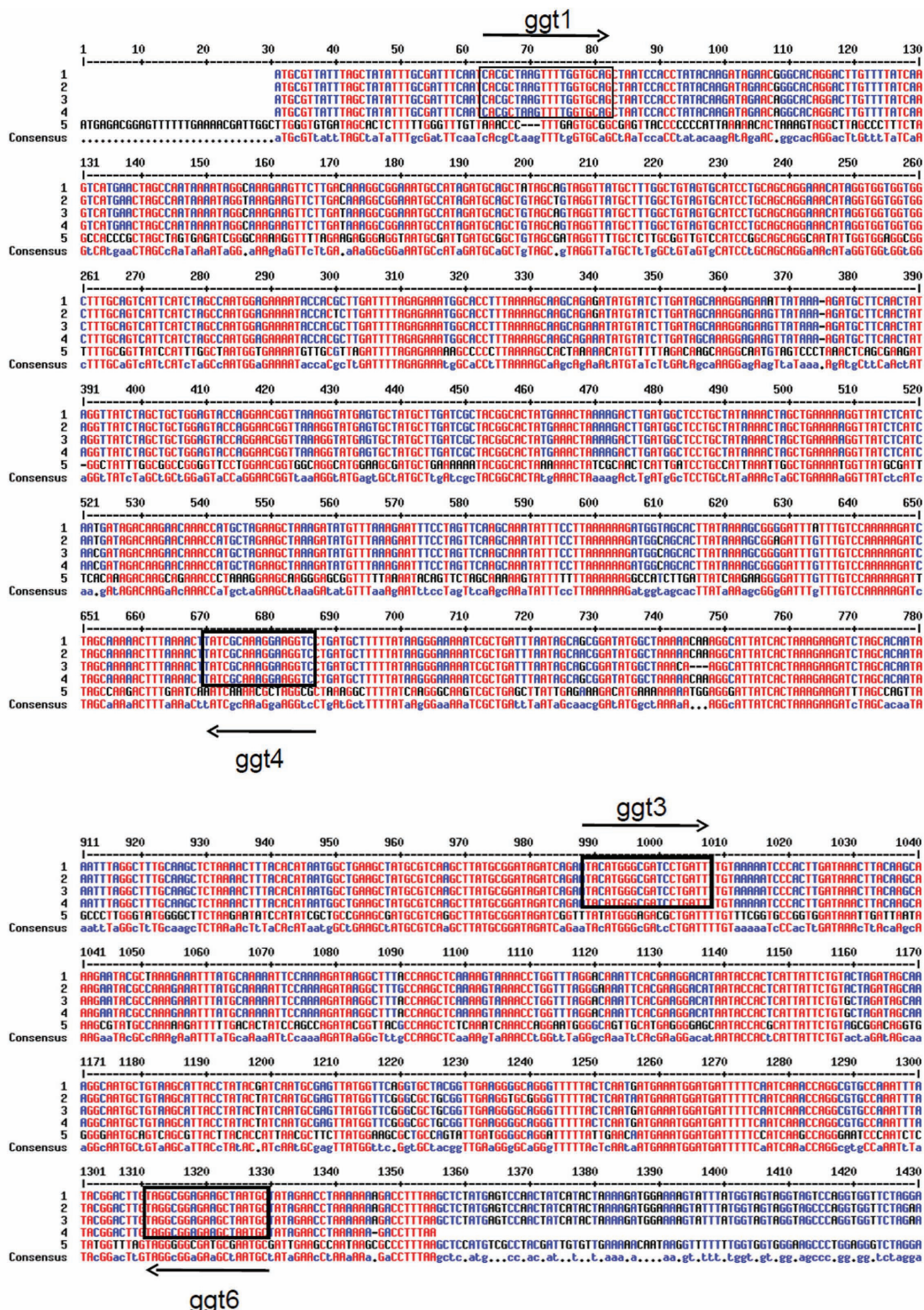


FIGURE A1 | Alignment by MultAlin software (Corpet, 1988) of *ggt* genes from: 1. *C. jejuni* 81-176; 2. *C. jejuni* subsp. doylei 269.97; 3. *C. jejuni* subsp. jejuni 260.94; 4. *C. jejuni* subsp.

jejuni HB93-13; 5. *Helicobacter pylori* 26695 Arrow: position of primers used for PCR amplification of *ggt* gene in *C. jejuni* isolates.

