NOTE

Contribution of surface polysaccharides to the resistance of *Campylobacter jejuni* to antimicrobial phenolic compounds

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Campylobacter spp., a particular *Campylobacter jejuni*, is a leading bacterial cause of food-borne gastroenteritis worldwide.¹ In addition, the increasing resistance of *C. jejuni* to clinically important antibiotics for the treatment of human campylobacteriosis, such as fluoroquinolones and macrolides, has become a serious public health concern worldwide.² Several molecular mechanisms for antibiotic resistance that have been reported in *C. jejuni* include the alteration of cellular targets for antibiotics,³ removal of antibiotics by efflux pumps,⁴ enzymes that hydrolyze or modify antibiotics⁵ and limitation of entry of antibiotics into the cell.⁶

Phenolic compounds are a group of secondary metabolites naturally present in a wide range of plants. They have been extensively investigated owing to their antioxidant properties and beneficial health effects, such as anticancer and antimicrobial activities.⁷ Antibacterial effects of phenolic compounds have been reported in several different food-borne pathogens, including *Campylobacter*, and the antimicrobial activities of phenolic compounds vary depending on the structure of phenolic compound and the bacterial strain.^{8,9} Although a recent study showed that the multidrug efflux pump CmeABC has an important role in the resistance of *C. jejuni* to phenolic antimicrobials,¹⁰ the molecular mechanisms for resistance to phenolic antimicrobials have not yet been defined well in *Campylobacter*. In this study, we compared the anti-*Campylobacter* activities of two major classes of phenolic compounds, such as flavonoids and phenolic acids, and demonstrated the role of surface polysaccharides in the resistance of *C. jejuni* to phenolic antimicrobials.

Five *C. jejuni* strains were used in this study, including two genome-sequenced *C. jejuni* strains (NCTC 11168 and 81–176),^{11,12} two human clinical isolates (HCJ3599 and HCJ2082), and a poultry isolate (PCJ497). *C. jejuni* strains were routinely grown with Mueller–Hinton (MH) media at 42 °C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂). Occasionally, culture media were supplemented with kanamycin (50 µg ml⁻¹) and tetracycline (5 µg ml⁻¹). As surface polysaccharides are an important permeability barrier against antibiotics, we investigated their role in resistance to phenolic antimicrobials. In this study, we used previously reported *kpsS* and *waaF*

mutants. The *kpsS* gene encodes a β-Kdo-transferase (Kdo: 3-deoxy-D-manno-octulosonic acid) that has a key role in the CPS synthesis.¹³ The *waaF* gene encodes a heptosyltransferase, the inactivation of which results in the truncation of the inner core moiety of LOS in *C. jejuni*;¹⁴ therefore, the *kpsS* mutant (*kpsS::aphA3*) and the *waaF* mutant (*waaF::tetO*) produce truncated CPS and LOS, respectively.⁶ The *waaF* mutants of 81–176, PCJ497, HCJ3599 and HCJ2082 were constructed by natural transformation with the genomic DNA of the *waaF* mutant of NCTC 11168 as described previously.⁶ Briefly, the recipient *C. jejuni* strains were grown overnight on MH agar and resuspended in MH broth to an OD at 600 nm of 0.1. After shaking incubation at 200 r.p.m. for 3 h, the genomic DNA of the *waaF* mutant was added to the culture. The *C. jejuni* strains were further grown for 6 h and plated on MH agar containing 5 µg ml⁻¹ of tetracycline to select the *waaF* mutants. The minimum inhibitory concentrations (MICs) of phenolic antimicrobials were determined with a microtiter broth dilution method.⁴ All phenolic compounds were purchased from Sigma-Aldrich (St Louis, MI, USA).

Flavonoids, such as morin, quercetin, hesperidin and taxifolin, exhibited antimicrobial effects on *C. jejuni* NCTC 11168 (Table 1). The anti-*Campylobacter* activities of synthetic phenolic compounds, including butylated hydroxytoluene, lauryl gallate and propyl gallate, were also examined. The synthetic phenolic compounds are considered to be generally recognized as safe and often used as antioxidant food additives to prevent lipid oxidation.¹⁵ Interestingly, these synthetic phenolic compounds demonstrated a high anti-*Campylobacter* activity in comparison with natural phenolic compounds (Table 1). We also investigated the role of surface polysaccharides in *C. jejuni*'s resistance against antimicrobial phenolic compounds by using CPS and LOS mutants. *C. jejuni* possesses CPS and LOS on the bacterial surface. Unlike other Gram-negative bacteria that usually harbor lipopolysaccharides (LPS) consisting of lipid A, core and O-antigen structures, the LOS of *C. jejuni* does not contain an O-antigen polysaccharide chain.¹⁶ Mutations of CPS and LOS increased the susceptibility of *C. jejuni* NCTC 11168 to phenolic compounds (Table 1). An LOS mutation (that is, the *waaF* mutant) reduced

Table 1 Susceptibility of surface polysaccharide mutants of *C. jejuni* NCTC 11168 to phenolic antimicrobials

Class	Subclass	Compound	<i>C. jejuni</i> NCTC 11168 ^a			
			WT	$\Delta kpsS$	$\Delta waaF$	<i>waaF</i> comp ^b
Phenolic acid	Hydroxycinnamic acid	<i>p</i> -Coumaric acid	1024	512 (2)	128 (8)	1024
		Caffeic acid	1024	512 (2)	256 (4)	1024
		Sinapic acid	512	256 (2)	64 (8)	512
	Hydroxybenzoic acid	Vanillic acid	1024	512 (2)	512 (2)	1024
		Salicylic acid	512	512	128 (4)	512
		Gallic acid	512	512	128 (4)	512
		Lauryl gallate	256	128 (2)	16 (16)	256
		Propyl gallate	128	64 (2)	16 (8)	256 (1/2)
		Epigallocatechin gallate	512	256 (2)	64 (8)	512
		Morin	256	256	32 (8)	512 (1/2)
Flavonoid	Flavan-3-ol	Quercetin	256	128 (2)	64 (4)	512 (1/2)
		Chrysin	1024	512 (2)	512 (2)	1024
	Flavanone	Naringenin	512	512	256 (2)	512
		Hesperidin	256	128 (2)	32 (8)	256
	Flavanolol	Taxifolin	256	128 (2)	64 (4)	256
		Butylated hydroxytoluene	256	128 (2)	16 (16)	256
	Other phenolic					

Abbreviation: WT, wild type.

The table shows MIC ($\mu\text{g ml}^{-1}$) and fold changes in parenthesis.^aThe results are representative of three independent experiments that produced similar results.^bA *waaF* complementation strain.**Table 2** Increased susceptibility to phenolic antimicrobials by an LOS mutation in multiple *C. jejuni* strains

Class	Subclass	Compound	<i>C. jejuni</i> strains ^a							
			81-176		PCJ497		HCJ3599		HCJ2082	
			WT	$\Delta waaF$	WT	$\Delta waaF$	WT	$\Delta waaF$	WT	$\Delta waaF$
Phenolic acid	Hydroxycinnamic acid	<i>p</i> -Coumaric acid	512	256 (2)	512	256 (2)	512	256 (2)	512	256 (2)
		Caffeic acid	1024	128 (8)	512	256 (2)	512	256 (2)	512	256 (2)
		Sinapic acid	512	128 (4)	512	256 (2)	256	128 (2)	256	128 (2)
	Hydroxybenzoic acid	Vanillic acid	512	256 (2)	512	128 (4)	512	256 (2)	512	256 (2)
		Salicylic acid	512	256 (2)	512	256 (2)	1024	256 (4)	1024	256 (4)
		Gallic acid	1024	256 (4)	1024	256 (4)	512	128 (4)	512	256 (2)
		Lauryl gallate	256	128 (2)	512	128 (4)	512	64 (8)	512	64 (8)
		Propyl gallate	256	64 (4)	256	64 (4)	512	64 (8)	512	128 (4)
		Epigallocatechin gallate	512	128 (4)	512	256 (2)	256	128 (2)	512	128 (4)
		Morin	512	128 (4)	256	256	512	128 (4)	512	256 (2)
		Quercetin	256	128 (2)	256	128 (2)	128	32 (4)	128	32 (4)
Flavonoid	Flavone	Chrysin	256	128 (2)	512	256 (2)	512	256 (2)	256	128 (2)
		Naringenin	256	64 (4)	256	64 (4)	128	32 (4)	256	64 (4)
	Flavanone	Hesperidin	512	64 (8)	512	128 (4)	256	128 (2)	256	256
		Taxifolin	512	128 (4)	256	256	256	128 (2)	256	64 (4)
	Flavanolol									
	Other phenolic									

Abbreviation: WT, wild type.

The table shows MIC ($\mu\text{g ml}^{-1}$) and fold changes in parenthesis.^aThe results are representative of three independent experiments that produced similar results.

MIC levels in all the tested phenolic antimicrobials, whereas a CPS mutation (that is, the *kpsS* mutant) presented only minor MIC changes in some phenolic compounds (Table 1). In particular, the LOS mutation resulted in 16-fold MIC reductions in butylated hydroxytoluene and lauryl gallate and 8-fold MIC reductions in *p*-coumaric acid, sinapic acid, propyl gallate, epigallocatechin gallate, morin and hesperidin (Table 1). Also, the MIC changes in the LOS mutant were successfully restored to the wild-type level by

complementation (Table 1), suggesting that the increased susceptibility to phenolic antimicrobials by the LOS mutation is specifically related to the function of LOS. The results show that LOS is the major permeability barrier conferring resistance to phenolic antimicrobials. As the hypervariability in surface polysaccharides has been reported in *C. jejuni*,¹² we also determined the effect of an LOS mutation on the susceptibility to phenolic antimicrobials in different *C. jejuni* strains. Anti-*Campylobacter* activities of phenolic compounds were confirmed

in several *C. jejuni* strains (Table 2). Particularly, quercetin, naringenin and butylated hydroxytoluene consistently exhibited active anti-*Campylobacter* activities in the strains (Table 2), and the LOS mutation in all the strains reduced the MIC levels of phenolic compounds (Table 2).

The outer membrane and the surface polysaccharides are an important permeability barrier to toxic compounds in Gram-negative bacteria.¹⁷ The LPS structures that are embedded in the outer leaflet of the outer membrane often contribute to bacterial resistance to hydrophobic antibiotics.¹⁸ In *Campylobacter*, the LOS confers resistance to not only hydrophobic drugs (for example, erythromycin)⁶ but also bile salts, affecting *C. jejuni* colonization of chicken intestines.¹⁹ In this study, we successfully demonstrated that LOS also significantly contributed to the resistance of *C. jejuni* to phenolic antimicrobials. Presumably, the effect of LOS in the resistance would result from direct inference with the attachment of hydrophobic molecules to hydrophilic LOS structures on the bacterial surface. In addition, direct or indirect interaction with membrane proteins, such as drug efflux pumps, would affect the resistance role of LOS in *C. jejuni*. It has been reported that the multidrug efflux pump AcrB and LPS synergistically contribute to the intrinsic resistance of *Salmonella*.²⁰ As the multidrug efflux pump CmeABC has an important role in *C. jejuni* resistance to phenolic compounds,¹⁰ we cannot exclude the possibility that LOS would interplay with CmeABC in *C. jejuni* resistance to phenolic compounds. Given the increasing number of newly reported phenolic antimicrobials from various sources, the findings in this study improve our understanding of the molecular mechanisms of *C. jejuni* resistance to phenolic antimicrobials in association with surface polysaccharides.

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