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Development of transient phage resistance in *Campylobacter coli* against the group II phage CP84

Temporäre Resistenz von Campylobacter coli gegenüber dem Gruppe-II-Phagen CP84

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Summary

Recently, there is a growing interest in the use of bacteriophages for pre- and post-harvest applications to reduce foodborne pathogens (including *Campylobacter*) along the food chain. Quantitative *Campylobacter* reductions of up to three $\log_{10}$ units have been achieved by phage application. However, possible phage resistance might limit this approach. In *Campylobacter* (*C.) jejuni*, phage resistance mechanisms have been described in detail but data on these mechanisms in *C. coli* are still missing.
To study phage resistance in *C. coli*, strain NCTC 12668 was infected with the lytic phage CP84, belonging to group II of *Campylobacter* phages. Resistant and sensitive clones were analysed using phenotypic and genotypic assays. *C. coli* clones acquired only transient resistance against CP84. The resistance led to cross-protection to one out of five other group II phages tested. Phage resistance was apparently neither caused by large genomic rearrangements nor by a CRISPR system. Binding assays demonstrated that CP84 could not adsorb to resistant *C. coli* clones suggesting a bacterial phage receptor to be involved in resistance. However, phage resistant *C. coli* clones did not reveal an altered motility or modified *flaA* sequence.
Considering the loss of binding capacity and the reversion to a phage sensitive phenotype we hypothesize that acquired resistance depends on temporal phase variable switch-off modifications of the phage receptor genes, even though the resistance mechanism could not be elucidated in detail. We further speculate that even closely related phages of the same group use different bacterial receptors for binding on *C. coli*.

Keywords: *Campylobacter*, phage treatment, resistance mechanism, phage receptor

Zusammenfassung

Die Möglichkeit, die Zellzahl wichtiger Lebensmittelinfektionserreger (einschließlich *Campylobacter*) entlang der Lebensmittelkette durch den Einsatz von Bakteriophagen zu reduzieren, hat zunehmend wieder an Interesse gewonnen. Verschiedene Studien zeigten, dass durch die Applikation von Bakteriophagen Reduktionen der *Campylobacter*-Zellzahlen bis zu drei Logstufen erzielt werden können. Allerdings könnte dieser Ansatz durch die Entstehung von Resistenzen gegenüber den Phagen begrenzt werden. Für *Campylobacter* (*C.) jejuni* wurden bereits detailliert einige Resistenzmechanismen gegenüber Phagen beschrieben, jedoch fehlen Daten zu Phagen-Resistenzmechanismen bei *C. coli.*
Zur Erhebung von Phagen-Resistenzdaten wurde *C. coli* NCTC 12668 mit dem virulenten Phagen CP84, einem Gruppe-II-*Campylobacter*-Phagen, infiziert. Die phagenresistenten und -sensitiven Klone wurden phänotypisch und genotypisch weiterführend untersucht. Die resistenten *C. coli*-Klone zeigten hierbei nur eine vorübergehende Resistenz gegen den Phagen CP84. Diese Resistenz führte zu einer Kreuzresistenz gegenüber einem weiteren von fünf getesteten Gruppe-II-

Bakteriophagen. Die Phagenresistenz wurde offenbar weder über große genomiche Reorganisationen noch über das CRISPR-System hervorgerufen. Bindungsstudien zeigten, dass CP84 nicht mehr an resistente *C. coli*-Klone binden konnte, was auf eine Beteiligung des bakteriellen Phagenrezeptors an dem Resistenzmechanismus hinweist. Resistente *C. coli*-Klone zeigten keine veränderte Beweglichkeit und keine veränderte *flaA*-Sequenz.
Auch wenn der Resistenzmechanismus von *C. coli* NCTC 12668 gegenüber dem Phagen CP84 bisher nicht nachgewiesen werden konnte, lässt sich die Hypothese aufstellen, dass die erzielte Resistenz durch vorübergehende phasenvariable switch-off-Modifikationen der Phagenrezeptor-Gene erfolgte. Die gezeigten Daten deuten darauf hin, dass auch verwandte Bakteriophagen derselben Gruppe verschiedene Rezeptoren zur Bindung an *C. coli* verwenden.

Schlüsselwörter: *Campylobacter*, Phageneinsatz, Resistenzmechanismus, Phagenrezeptor

Introduction

Campylobacteriosis is one of the most frequently reported foodborne illnesses in humans and is most commonly associated with the species *Campylobacter (C.) jejuni* and *C. coli*. So far no compliant elimination strategy of *Campylobacter* in food producing animals has been ascertained. Considering the growing antimicrobial resistance of many foodborne pathogens, a pre-harvest application of phages to reduce the bacterial count on food products is getting more attention. In addition, post-harvest application of phages could be a useful strategy to reduce the bacterial count on food products.

Almost all *Campylobacter* phages described so far belong to the family *Myoviridae* (Javed et al., 2013). According to their genome size and head diameter, they are classified into the groups I, II and III (Connerton et al., 2011). Javed et al. (2013) recently showed high (at least 76%) homology of the core genes of phages within each group II and III, respectively, while homology between these two groups were less than 40%. While group III phages mainly infect *C. jejuni* strains, many group II members as phage CP84 lyse *C. jejuni* and *C. coli* (Frost et al., 1999). Phages of both groups were similarly efficient in reducing the *C. jejuni* count in chickens (1.5 to 5 $\log_{10}$ CFU/g) (Connerton et al., 2011). However, in chicken studies higher MOIs of group II phage CP220 were needed to reduce the bacterial count of *C. coli* to the same extent as of *C. jejuni* (El-Shibiny et al., 2009). Like in other bacteria, acquired phage resistance is widespread in *Campylobacter* with incidences of 2% in vivo and 91% in vitro (Scott et al., 2007b; Carvalho et al., 2010; El-Shibiny et al., 2009). In several studies phage resistance mechanisms of *C. jejuni* have been analysed. Besides the complete loss of the capsule or alterations of capsular structures caused by phase variations in the capsular locus genes *cj1421* and *cj1422*, the expression of the ganglioside-like lipooligosaccharide structures GM1 and GD1, an altered motility and large genomic rearrangements within the host chromosome have been identified as resistance mechanisms (Coward et al., 2006; Scott et al., 2007b; Sorensen et al., 2011; Sorensen et al., 2012; Louwen et al., 2013). In contrast, data on acquired phage resistance of *C. coli* are still lacking.

In our study, we focused on the acquired resistance of *C. coli* NCTC 12668 to group II phage CP84 (NCTC 12684). We investigated phenotypically (i) the acquisition and stability of resistance, (ii) cross-resistance to other group II phages, (iii) motility of resistant clones and (iv) binding of phages to resistant clones. Genetically, phage resistance was investigated by fluorescence Amplified Fragment Length Polymorphism (fAFLP) analysis to detect potential genomic rearrangements in the bacterial host, and by sequence determination of putative phage receptors.

Material and Methods

Bacterial strain and bacteriophages

C. coli strain NCTC 12668 was obtained from the National Collection of Type Cultures (NCTC), Health Protection Agency, United Kingdom. It was grown on selective modified Charcoal-Cefoperazon-Desoxycholat (mCCDA) agar (Oxoid, Wesel, Germany), non-selective Mueller-Hinton-blood (MHB) agar (Oxoid) as well as in non-selective Sodium-NZamines-Casaminoacids-Yeast-Magnesiumsulfate (NZCYM) medium (Roth, Karlsruhe, Germany) at 37°C under microaerobic conditions. The phages CP84, CP75, CP83 and CP21 (Tab. 1) have been previously described (Sails et al., 1998; Hammerl et al., 2012). Other group II bacteriophages used in this study (CP7 and CP68) were isolated from food and environment in Germany in 2008. Propagation of all phages was performed on *C. coli* NCTC 12668.

Lytic activity of phage CP84 on *C. coli* NCTC 12668

Overnight cultures of *C. coli* NCTC 12668 were diluted to 1 x 10^5 CFU/ml in NZCYM medium and 1 ml was added to 1 ml CP84 phage lysate with a phage titer of 10^7 plaque forming units (PFU)/ml or NZCYM medium and incubated under microaerobic conditions at 37°C. Bacterial counts and phage titers were determined at several time points. Numbers of viable bacteria were determined

TABLE 1: *Phages used in this study*

Phage	NCTC number	Reference
CP84	NCTC 12684	Sails et al. (1998)
CP75	NCTC 12675	Sails et al. (1998)
CP83	NCTC 12683	Sails et al. (1998)
CP21	–	Hammerl et al. (2012)
CP7	–	–
CP68	–	–

Pby plating serial dilutions in NZCYM medium on MHB agar and counting colony forming units (CFU) after 48 h of microaerobic incubation. Phage titers were determined by using serial dilutions of the cultures for plaque assays.

Bacteriophage plaque assay

Plaque assays were performed by a modified soft agar method (Sambrook and Russel, 2001). Briefly, bacteriophages were serially diluted in NZCYM medium and mixed 1:1 with 100 µl of overnight culture of *C. coli* NCTC 12668. Co-cultures were incubated for 10 min at room temperature before the addition of 5.5 ml of 0.6% NZCYM agar (Roth). The mixture was poured into an empty petri dish, set to dry and incubated at 37°C for 24 h before plaque counting.

Isolation of phage resistant clones from sensitive *C. coli* NCTC 12668 strain

Overnight cultures of *C. coli* NCTC 12668 were diluted to 1 x 10^5 CFU/ml and 1 ml was added to CP84 phage lysate with a phage titer of 10^7 PFU/ml or NZCYM medium, incubated at 37°C for 48 h, streaked out on MHB plates and further incubated for 48 h. Single colonies of the primary culture (P1–P3), after single culture (S1–S5) and after co-culture with CP84 (Ph1–Ph5) were picked and plaque assays were performed (Tab. 2). Colonies were considered to be phage resistant if CP84 could not form plaques on bacterial lawns. Experiments were done in triplicate. All clones described in our studies originated from one of these replicates. These clones were stored at –80°C in Cryobank vials (Mast Diagnostics, Reinfeld, Germany).

TABLE 2: *Clones isolated in this study*

C. coli NCTC 12668 clone	Isolate time	Source	Phage resistant/ sensitive (R/S)
P1	0 h	Primary culture	S
P2	0 h	Primary culture	R
P3	0 h	Primary culture	S
S1	48 h	Single culture	S
S2	48 h	Single culture	R
S3	48 h	Single culture	S
S4	48 h	Single culture	S
S5	48 h	Single culture	S
Ph1	48 h	Co-culture	R
Ph2	48 h	Co-culture	R
Ph3	48 h	Co-culture	R
Ph4	48 h	Co-culture	R
Ph5	48 h	Co-culture	R

TABLE 3: *Primers and adapters used in this study*

Analysis	Primer	Sequence	Reference
fAFLP	Hhaad1	GACGATGAGTCCTGATCG	Duim et al. (2001)
	Hhaad2	ATCAGGACTCATCG	Duim et al. (2001)
	Hindad1	CTCGTAGACTGCGTACC	Duim et al. (2001)
	Hindad2	AGCTGGTACGCAGTC	Duim et al. (2001)
	Hhapre	GATGAGTCCTGATCGC	Duim et al. (2001)
	Hhasel	GATGAGTCCTGATCGCA	Duim et al. (2001)
	Hindpre	GACTGCGTACCAGCTT	Duim et al. (2001)
	Hindsel	FAM-GACTGCGTACCAGCTTA	Duim et al. (2001)
CRISPR	CAMPDR F	AGCTGCCCTTATGGTGGTG	Schouls et al. (2003)
	CAMPDR R	AAGCGGTTTTAGGGGATTGT	Schouls et al. (2003)
flaA	FLA4 F	GGATTTCGTATTAACACAAATGGTGC	Meinersmann et al. (1997)
	FLA178 F	ACTTTAGGTCAAGCTAT	Meinersmann et al. (1997)
	FLA630 F	GGAACAGGACTTGGAGC	Meinersmann et al. (1997)
	FLA1133 F	CCGATGCTATGGGATTT	Meinersmann et al. (1997)
	FLA1728 R	CTGTAGTAATCTTAAAACATTTTG	Meinersmann et al. (1997)
	FLA1553 R	TGATTTTGTACAGAACC	Meinersmann et al. (1997)
	FLA1133 R	AAATCCCATAGCATCGG	Meinersmann et al. (1997)
	FLA442 R	GAACTTGCGCCGATTTG	Meinersmann et al. (1997)

Cross-resistance assays

Plaque assays were carried out with the group II phages CP7, CP21, CP68, CP75 and CP83 on CP84-resistant *C. coli* NCTC 12668 clones.

Stability of phage resistance

Five resistant (Ph1–Ph5) and one sensitive (P1) *C. coli* NCTC 12668 clone were reactivated from the stock culture and alternatingly cultured in liquid medium (NZCYM broth) for 24 h followed by plating on selective agar (mCCDA) to avoid contamination. Plates were incubated for 48 h. Multiple colonies of these plates were then re-inoculated in liquid media. This procedure was conducted until a phage sensitive phenotype was observed or over a period of six weeks resulting in 16 liquid sub-cultivations. Once per week phage resistance was tested by plaque assays. Reversion to phage sensitivity of the bacterial cultures was defined if phage titers differed only by 2 log levels compared to the positive control.

Fluorescence Amplified Fragment Length Polymorphism (fAFLP)

After DNA extraction, fAFLP was carried out with phage-resistant and phage-sensitive *C. coli* clones as previously described (Duim et al. 2001). Briefly, DNA was digested with HindIII and HhaI and after ligation of adapters (Tab. 3) a pre-selective followed by a selective PCR was performed according to Duim et al (2001). After capillary electrophoresis (LGC Genomics, Berlin, Germany), band patterns were analysed using Bionumerics v6.01 (Applied Maths, Sint-Martens-Latem, Belgium). Lane similarity was calculated by Pearson coefficient correlation. Arrangement into dendrogram was accomplished by unweighted pair-group method using arithmetic averages (UPGMA) according to Alter et al. (2005). Only AFLP profiles in the molecular size range of 35-500 bp were analysed. Correlation levels were expressed as percentage of similarity.

***flaA* sequencing**

The *flaA* gene of *C. coli* NCTC 12668 clones (P1-P3, S1-S5, Ph1-Ph5) was amplified using primers Fla4F and Fla1728R according to Meinersmann et al. (1997). PCR products were sequenced with the corresponding primers and sequences analysed with Bionumerics v6.01 (Applied Maths). Sequencing was performed by GATC (Constance, Germany).

Binding assay
The phage resistant *C. coli* clone Ph1 and the phage sensitive *C. coli* clone (S1) were grown overnight in NZCYM broth, and approximately 1 x 10^4 CFU/ml were mixed with phage CP84 at a MOI of approximately 10. Co-cultures were incubated at 37°C. At several time points, co-cultures were gently inverted, 1 ml centrifuged (16 000 x g) at 4°C for 10 min and the phage titers in the supernatants determined. As negative controls bacteriophages were incubated in NZCYM medium and with overnight cultures of *Yersinia enterocolitica* 83/88/2 (without virulence plasmid).

Motility assay
1 µl of overnight cultures of phage sensitive and resistant *C. coli* clones was spotted onto a *Brucella* broth (Roth) swarming agar (0.4%) plate. Motility was assessed by the evaluation of the diameter of growth zones after 48 h of incubation at 37°C.

Statistical analysis
P-values were calculated by nonparametric Mann-Whitney test (GraphPad Prism v5, La Jolla, CA, USA). Results were considered significant at $p < 0.05$. Data are shown as median ± interquartile range (IQR).

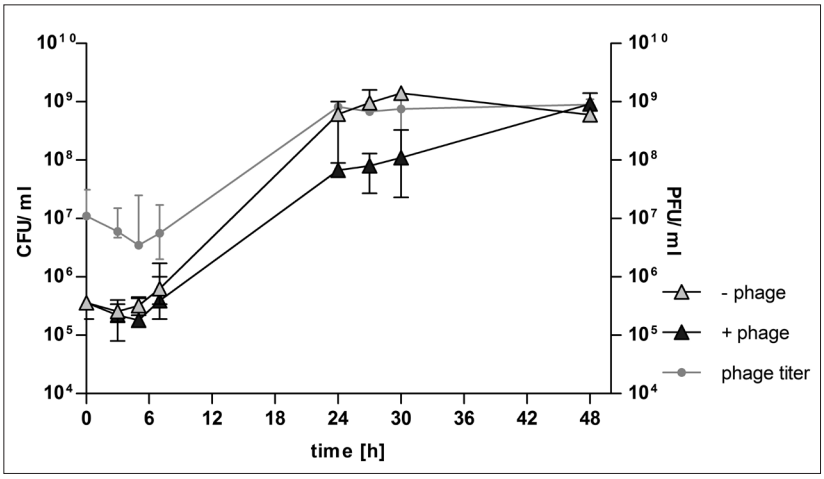

FIGURE 1: *Growth of C. coli NCTC 12668 with and without phage CP84. Cell counts (CFU/ml) and phage titers were determined over a period of 48 h. Shown are medians ±IQR (n = 3). Triangle, CFU/ml; grey, C. coli; black, C. coli with CP84; circles, CP84 titer (PFU/ml).*

Results and Discussion

Acquisition and stability of phage resistance
To study the lytic activity of CP84 on *C. coli* strain NCTC 12668, bacteria were infected with phage CP84 at a MOI of approximately 10 over a period of 48 h at 37°C and bacterial counts as well as phage titers were determined at several time points. After 24 h of incubation, the cell number of an infected culture was 1 $\log_{10}$ unit lower compared to the control without phage (Fig. 1). However, at the end of the experiment, similar cell numbers were determined. This suggests a developing phage resistance of *C. coli* NCTC 12668 during co-cultivation with CP84. Nonetheless, it cannot be excluded that the phage resistant phenotype was already present before the phage lysate was added. The CP84 titer decreased during the first three hours of the experiment as phages bound onto host cells, and increased again after six hours when progeny phages were released. In total, phage titer increased from 5 x 10^7 PFU/ml to 1 x 10^9 PFU/ml within 24 h of infection indicating that the phage replicated in the host. Thereafter, the phage titer remained more or less stable (Fig. 1).

To analyse if the growth of the bacteria was associated with phage resistance, colonies isolated at several stages of the experiment were further investigated by plaque assays. In the absence of phage, only one out of five and one out of seven colonies were resistant at the start and at the end of the experiment, respectively. In contrast, all eight colonies isolated after 48 h incubation with CP84 showed a resistant phenotype. To our knowledge, this is the first report on *C. coli* phage resistance acquired after co-cultivation with a group II phage in vitro. In *C. jejuni* similar phage resistance rates, ranging from 11% to 91%, have been observed in vitro (Loc Carrillo et al., 2005; Scott et al., 2007b). However, resistance rates of *Campylobacter* spp., determined after treatment of chickens with different group II phages, ranged only from 2% to 13% (El-Shibiny et al., 2009; Carvalho et al., 2010). It has been speculated that phage resistance might be associated with a reduced colonization fitness which could explain the lower phage resistance rates in the gut than under in vitro conditions (Scott et al., 2007a).

Stability of phage resistance can be diverse in different bacterial species (O'Flynn et al., 2004; Coward et al., 2006; Kim and Ryu, 2011). Coward et al. (2006) described that *C. jejuni* strains maintained the acquired phage resistance after storage at –80°C as well as after repeated sub-cultivation. In contrast, in our study the isolated *C. coli* colonies reverted to a phage sensitive phenotype after one to three weeks of sub-cultivation (Tab. 4). This variation seems not to be due to a phage-carrier system, as no phages were detected in the supernatant of overnight cultures of phage-resistant *C. coli* clones Ph1–Ph5. The phenomenon of resistant clones reverting back to phage sensitivity was also described for *E. coli* where phage resistant mutants became sensitive after approximately 50 generations (O'Flynn et al., 2004). On the other hand, Kim and Ryu (2011) reported different stability data for SPC35 phage resistance in *E. coli* and *Salmonella*. Whilst *E. coli* clones kept their resistance, *Salmonella* clones reverted to susceptibility in the course of three sub-cultivations. They showed that resistance in *E. coli* was due to an insertion of an IS2 element in the phage receptor protein ButB, but no mutation of this gene could be detected in resistant *Salmonella* clones. The loss of resistance of these *Salmonella* clones led the authors to the hypothesis that a phase-variable mechanism was responsible for resistance in *Salmonella* (Kim and Ryu, 2011).

Even though, we do not know how often Coward et al. (2006) sub-cultivated the resistant *C. jejuni* clones, it could be speculated that varying resistance stabilities against phage CP84 could depend on the different *Campylobacter* species.

Cross-resistance against other group II phages
Coward et al. (2006) showed that resistance of *C. jejuni* isolates to a phage conferred cross resistance against

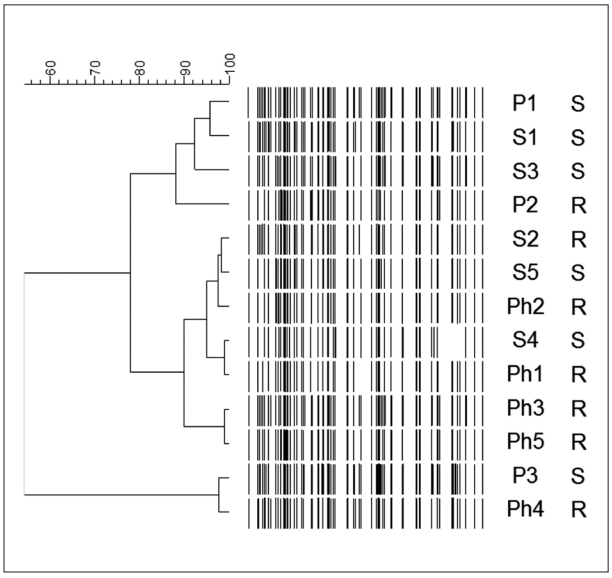

FIGURE 2: *fAFLP-based comparison of phage resistant and sensitive C. coli clones, fAFLP based band patterns of clones isolated from the primary culture (P1–P3), single culture (S1–S5) and co-culture with CP84 (Ph1–Ph5) were compared by the unweighted pair-group method using arithmetic averages (UPGMA). Phage sensitivity (S) or phage resistance (R) of the clones is also indicated. Correlation levels were expressed as percentage of similarity.*

several phages of the same group. We therefore analysed cross-resistance of *C. coli* clones Ph1–Ph5 against other group II phages. CP84 resistant *C. coli* NCTC 12668 colonies showed only cross-resistance against group II phage CP68 but not to four other group II phages (CP7, CP21, CP75 and CP83; data not shown). In contrast, CP84 resistant *C. jejuni* isolates were also resistant against CP75 and CP83 (Coward et al., 2006). This suggests that phage resistance mechanism of *C. jejuni* might lead to higher cross-protection rates than the resistance mechanism of *C. coli*.

Characterization of phage resistance mechanisms

The presence of CRISPR-Cas systems has been described in *C. jejuni* strains but whether they are a functional phage defence mechanism in *C. jejuni* has not been elucidated yet (Louwen et al., 2013; Louwen and van Baarlen, 2013). In our study, primers described by Schouls et al. (2003) were used for CRISPR analysis in *Campylobacter* (Tab. 3). However, no DNA fragment in *C. coli* NCTC 12668 could be amplified. Using the CRISPRs web server no CRISPR system was detected in the genome of the sequenced *C. coli* strain RM2228 (Grissa et al., 2007). This is in accordance with the results of Price et al. (2007), who were unable to detect a CRISPR system in seven *C. coli* isolates.

Phage resistance of *C. jejuni* clones isolated in the course of in vivo experiments was caused by intragenomic inversion or inter-genomic recombination e. g. in Mu-phage like regions in the genome (Scott et al., 2007a; Scott et al., 2007b). Also some *C. coli* strains carry Mu-phage like sequences (data not shown). Using the fAFLP method, we detected three clusters with similarity levels higher than 85%. However, clusters did not coincide with the resistant phenotype Ph1–Ph5, as phage resistant and phage sensitive clones were both present in each of the three clusters (Fig. 2). These data suggest that the observed resistance was not caused by large genomic rearrangements.

As neither a global defence mechanism like the CRISPR-Cas system nor genomic rearrangements were responsible for the resistant phenotypes, we investigated the ability of the phages to adsorb to *C. coli* NCTC 12668 mutant Ph1. Adsorption of CP84 to sensitive bacteria was visible by a phage titer drop of approximately 1.5 $\log_{10}$ units after 10 min of incubation (Fig. 3). Immediate phage binding to host cells was detected at the beginning of the experiment, where the titer of free phages was reduced within seconds. This goes along with the data of Figure 1, where the phage titer decreased at first and increased again after 6 hours of co-culturing. On the other hand, phage titers in the supernatants of phage resistant clones remained constant during the assay, demonstrating that phages were not able to bind to the cells. In accordance with our observations Scott et al. (2007a) described a binding assay with phage CP34, where the phage bound to the sensitive *C. jejuni* strain HPC5 but not to the phage resistant variants R14 and R20. These results indicate that resistance might have been caused by changes in the bacterial receptor protein, a competitive inhibitor or by the production of an extracellular matrix which blocks the phage receptor (Labrie et al., 2010).

Coward et al. (2006) reported resistance of *Campylobacter* to group I and II phages as being linked to an altered motility. Therefore, we performed a motility assay with phage sensitive and resistant *C. coli* NCTC 12668 clones P1–P3, S1–S5 and Ph1–Ph5 on swarming agar. Resistant *C. coli* NCTC 12268 isolates did not show an altered motility compared to the phage sensitive bacteria (data not shown). Even though we investigated only resistance mechanisms against one phage our data suggest that *C. coli* resistance to group II phages is not necessarily linked to motility as proposed by Coward et al. (2006). As no changes in motility of the resistant *C. coli* clones have been observed, the sequence of *flaA* was analysed. FlaA is regarded as a suitable phage receptor since it is extracellularly located (Wassenaar et al., 1995; Nuijten et al., 2000). However, the *flaA* sequences of phage sensitive and resistant *C. coli* clones P1-P3, S1-S5 and Ph1-Ph5 were identical revealing that resistance of *C. coli* NCTC 12668 to CP84 is not caused by an altered FlaA (data not shown).

TABLE 4: *Stability of acquired CP84 phage resistance in C. coli (in weeks). CP84 resistant C. coli clones were sub-cultured for six weeks without phage. Each week, resistance of C. coli to CP84 was tested by a plaque assay. Shown are the number of weeks after which susceptibility was observed (Ph1–Ph5: phage resistant C. coli clones).*

C. coli clone	Replicates		
	I	II	III
Ph1	3	2	2
Ph2	3	2	1
Ph3	4	2	4
Ph4	6	3	3
Ph5	1	2	2

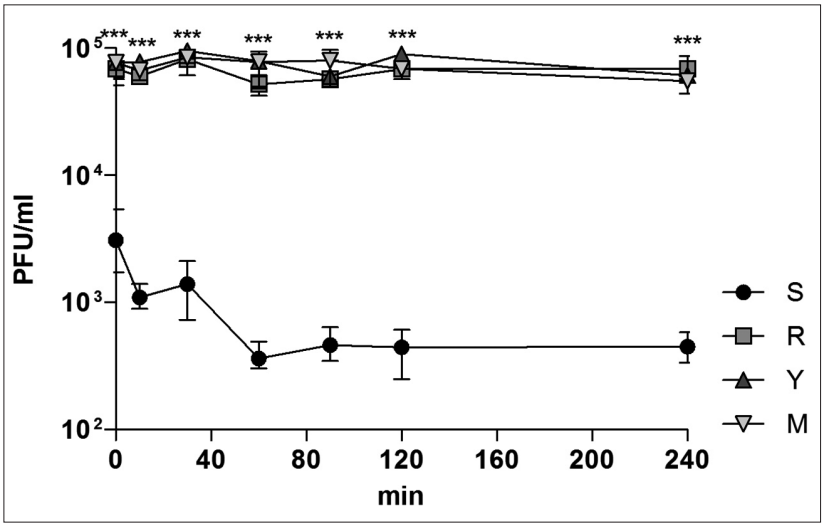

FIGURE 3: *Binding of CP84 to sensitive and resistant C. coli clones. Phage titers in the supernatants were determined after incubation of CP84 with C. coli sensitive (S) and resistant (R) clones at several time points. As negative controls, pure media (M) or Yersinia enterocolitica (Y) were incubated with CP84. Shown are medians ±IQR (n = 3) with statistical significance of *** p ≤ 0.0005.*

No phage receptors in the capsular polysaccharides (CPS) of *C. coli* have been described so far, but changes within the poly-G tract of the CPS locus genes cj1421 and cj1422 of *C. jejuni* NCTC 11168 conferred resistance to group III phage F336 (Sorensen et al., 2011). The CPS of *C. jejuni* strains show high structural diversity, as both, CPS synthesis and modifications, are subjected to phase variation by slip strand mismatch repair (Guerry et al., 2012). In contrast to *C. jejuni* NCTC 11168, the CPS locus of *C. coli* RM2228 contains only two genes (cco15250 and cco1546) with a poly-G tract. We were not able to amplify these genes of the *C. coli* strain NCTC 12668 using primers deduced from the sequence of *C. coli* RM2228. Therefore, it remains open, whether phase variation of capsule locus genes was responsible for the phage resistance of our *C. coli* clones.

In conclusion, we demonstrate that the acquired phage resistance of *C. coli* NCTC 12668 against group II phage CP84 is a transient phenomenon. Even though the resistance mechanism could not be elucidated, we speculate that it depends on temporal phase variable switch-off modifications of the phage receptor gene. However, as phage resistance stabilities, cross-resistance patterns against other group II phages and motility phenotypes of our CP84 resistant *C. coli* NCTC 12268 clones differ obviously from the phenotypes of the CP84 resistant *C. jejuni* clones described by Coward et al. (2006), we speculate that *C. coli* acquired phage resistance against CP84 by another mechanism than *C. jejuni*.

For pre- and post-harvest applications of phages we recommend the use of cocktails of phages provoking different kinds of resistance mechanisms in *Campylobacter* spp., so that resistance against one phage can be compensated by other phages. If the reversion of resistance of *C. coli* to group II phages is common, intermittent phage application can be an effective method to reduce *C. coli* counts. Whether resistance development and its reversion can also be seen in vivo has to be investigated in further studies.

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Conflict of interest: The authors declare that they have no conflict of interest.

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