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SHORT COMMUNICATION

Sub-lethal Concentrations of Phytochemicals (Carvacrol and Oregano) Select for Reduced Susceptibility Mutants of *Escherichia coli* O23:H52

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Abstract

In vitro studies aimed at studying the mechanism of action of carvacrol and oregano as natural anti-bacterial agents to control multiple antibiotic-resistant avian pathogenic *Escherichia coli* (APEC) strain O23:H52 isolated from chicken were performed. Derivatives with increased minimum inhibitory concentrations (MIC) to the phytochemicals were selected after growing *Escherichia coli* (*E. coli*) strain O23:H52 at sub-lethal concentrations of carvacrol and oregano for a period of 60 days. Whole-genome sequencing (WGS) of two derivatives revealed a missense mutation in *cadC* and *marR*: the genes responsible for survival mechanisms and antibiotic resistance by efflux, respectively.

Key words: *E. coli*, APEC, phytochemicals, antibiotic resistance, WGS

Introduction

Phytochemicals are natural plant products produced as secondary metabolites of which some possess anti-microbial effects (Wink 2004), and therefore, may present a promising alternative strategy to antibiotics especially against antibiotic-resistant bacteria (Wong et al. 2006; Marcus et al. 2019) as well as pathogenic bacteria (Jayalakshmi et al. 2011; Rajamanickam et al. 2019). Several studies have investigated the anti-bacterial properties of a number of phytochemicals, but their mechanisms of action remain ill-defined. The interaction between phytochemicals and bacteria at the cellular level is due to the hydrophobic nature of phytochemicals which enables their entry into the lipid bilayer of the cytoplasmic membrane, to act as a membrane destabilizing agent that induces structural changes modifying the functionality of the lipid membrane and associated proteins (Sikkema et al. 1995; Luz et al. 2014; Yuan et al. 2019). Thus, the effects are pleiotropic and include: altering surface charge of the bacterial membrane (Cristani et al. 2007), ion (H^+ and K^+) transport (Ultee et al. 2002), stress responses (Richter et al. 2010; Di Pasqua et al. 2013), conjugation (Skalicka-Woźniak et al. 2018), motility and quorum sensing (Monte et al. 2014), amongst other effects.

Carvacrol and oregano exhibit anti-microbial activities against pathogenic microorganisms whether bacteria or fungi, irrespective of origin from the plant, animal or human sources (Baricevic and Bartol 2002; Mathlouthi et al. 2012). Given the focus of this study is on *E. coli*, previous studies have shown that oregano oil containing carvacrol and thymol is effective against *E. coli* in a dose-dependent manner (Friedman et al. 2002; Al-Mnaser 2019; Alvarez et al. 2019). Another study has shown that exposing *E. coli* to sub-lethal concentrations of carvacrol leads to changes in the ratio of unsaturated and saturated fatty acid components of the cell membrane (Di Pasqua et al. 2006) suggesting that *E. coli* develops an adaptive response upon exposure. To interrogate the many target sites of the *E. coli* cell, which could be affected by carvacrol and oregano, an approach used here was to grow and continuously expose *E. coli* cells for 60 days at sub-MIC level of phytochemicals (carvacrol and oregano) in growth medium, to generate derivatives that have reduced sensitivity (an increased resistance). This approach will select both temporary adaptations as well as mutational events. Focusing on the latter should identify the genes encoding cellular functions involved in response to the stress of carvacrol and oregano. Therefore, this work aimed at similar investigations into the anti-bacterial role of carvacrol and oregano at the genetic level.

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Experimental

Materials and Methods

***E. coli* strain and growth conditions.** One *E. coli* strain, designated C1, of poultry origin and previously characterized as harboring five virulence determinants (*fim1*, *csgA*, *crl*, *astA,* and *hlyA*) (Al-Mnaser 2019) and, therefore, potentially an APEC strain (Johnson et al. 2008), and being resistant to five antibiotics (cefotaxime, nalidixic acid, cefotaxime, ampicillin, and tetracycline) (Al-Mnaser 2019) was selected as a suitable poultry isolate for this study. Strain C1 was shown to have a MIC of 0.3 µg/ml against aqueous carvacrol and oregano. The *E. coli* strain was grown overnight in Luria-Bertani (LB) broth (inoculation was from a pure stock culture preserved in a cryotube at −80°C) at 37°C to yield approximately 10^9 CFU/ml (OD_{600} = 1.00) of which 100 µl was used to inoculate each of three sets of tubes (total volume of 10 ml) supplemented with 0.2 µg/ml aqueous carvacrol, with 0.2 µg/ml aqueous oregano, and without any supplement as a control. Re-inoculation by transfer of 100 µl to freshly prepared media was done every 48 h over a period of 60 days after which the bacteria were diluted and spread on non-selective LB agar to generate single well-defined colonies. Two derivative *E. coli* strains were chosen randomly and were designated as 22M and 26M; carvacrol-derivative strain and oregano derivative strain, respectively.

Determination of MIC values of the derivative *E. coli* strains against aqueous phytochemicals. The two derivative *E. coli* strains (22M and 26M) were used to determine their MIC values against aqueous carvacrol and oregano using a quasi-microdilution method. 96-well plates with LB supplemented with a dilution series of oregano and carvacrol were inoculated with 22M and 26M and the OD_{600} was measured spectrophotometrically every 1 h for 24 h under aerobic conditions and at a temperature of 37°C (Fluostar Omega). The OD_{600} readings were used to plot the relationship between time and OD. Plots were used to calculate bacterial growth to determine the MIC value of carvacrol or oregano against the *E. coli* strains. The same procedure was done after two weeks of storage in cryotubes containing non-selective medium at −80°C, to ensure that the increase in MIC values was stable and not a result of an adaptative change.

WGS of the derivative *E. coli* strains. Strains 22M and 26M with their original wild-type strain C1 were sent to MicrobesNG at the University of Birmingham for WGS. *In silico* serotyping analysis using Serotype Finder 1.1 website (https://cge.cbs.dtu.dk/services/SerotypeFinder/) (Joensen et al. 2015) and MLST analysis using MLST 2.0 software (https://pubmlst.org/) (Sepehri et al. 2009) were performed prior to full genome analysis.

Results and Discussion

In this study, we have investigated the antibacterial properties of two phytochemicals; carvacrol (the active ingredient of oregano) and oregano using wild-type *E. coli* strain of poultry origin as a starter strain, which to our knowledge, this has not been done before. This initial *in vitro* study aimed at increasing our understanding of the mechanism of action of these phytochemicals to control APEC strain (the causative agent of colibacillosis disease in poultry) with multiple antibiotic-resistance, which will enable us to evaluate their anti-bacterial properties as possible feed additives in the poultry industry instead of antibiotics.

The continuous exposure of *E. coli* cells to sub-lethal concentrations of carvacrol and oregano resulted in an increased resistance (reduced sensitivity) to these phytochemicals, and this was demonstrated by increased MIC values from 0.3 µg/ml to 0.6 µg/ml to both carvacrol and oregano. This step was repeated twice in order to confirm that the elevated MIC was stable. After that, the identity of the derivative strains was confirmed by extracting data from the WGS to ensure that the derivative *E. coli* strains 22M and 26M were true derivatives of the *E. coli* strain C1. WGS data analysis revealed that the three strains shared the same *in silico* serotype and multi-locus sequence typing (MLST) profiles, O23:H52 and ST-373, respectively.

The next objective was to search for the genomic variations in the derivatives compared with the progenitor strain, as this might give us information on the evolution of these derivatives (Tenaillon et al. 2001; Bryant et al. 2012). WGS data analysis showed that there were missense mutations detected in two chromosomal genes; *cadC* which encodes for a transcriptional activator of the *cad* operon (Küper and Jung 2005) and *marR* which encodes for a repressor of *mar* operon (Cohen et al. 1993). These two mutations were found in the carvacrol-derivative strain (22M). However, the oregano-derivative strain (26M) contained only one missense mutation, which was in *cadC*.

The *cad* operon is one of the survival mechanism systems in *E. coli* that is triggered in response to unfavorable acidic conditions (Tetsch et al. 2011). This system is composed of three genes; *cadA* (encodes a cytoplasmic CadA protein responsible for decarboxylation of lysine), *cadB* (encodes a transmembrane CadB protein responsible for excretion of the end products of lysine decarboxylation), and *cadC* (located upstream of the *cadBA* operon and encodes a transmembrane protein CadC) (Watson et al. 1992; Küper and Jung 2005). CadC has a dual function as a transcriptional activator of the *cad* operon in *E. coli* (Küper and Jung 2005) and as a sensor to external changes in pH in the environment (Tetsch et al. 2011). The missense muta-

tion detected in *cadC* gene resulted in an amino acid substitution from tyrosine to histidine at position 504 of the CadC protein, caused by a transition substitution from T to C at the genome position 280821. The visualization of the mutation location in the carvacrol-derivative strain (22M), when compared with the wild-type strain (APEC O23:H52), is shown in Fig. 1. The increased phenotypic resistance to phytochemicals in the derivative strains 22M and 26M can be explained by two possible scenarios, assuming these mutations are not silent: 1) the substitution in *cadC* might effect on the expression of the Cad system, leading its over-expression of the CadC activator/sensor (Tetsch et al. 2008) to compensate for the constant presence of phytochemicals, 2) the substitution in *cadC* might affect the Cad system leading to over-expression of the *speF-potE* operon (another survival mechanism in *E. coli*) to replace the function of *cadBA* operon (Soksawatmaekhin et al. 2004). These options could be further investigated by Real-Time-PCR mRNA expression and/or complementation studies in order to get a clearer picture. These results suggest that carvacrol/oregano can trigger stress responses in multiple antibiotic-resistant APEC strain when used at sub-lethal concentration. Similar findings were documented when using sub-lethal concentrations of carvacrol which led to a missense mutation in *soxR*, which is another oxidative stress defence in *E. coli* (Chueca et al. 2018).

The *mar* operon is responsible for chromosome-mediated multiple antibiotic resistance as a protective mechanism in response to environmental stresses such as the presence of antibiotics and oxidative stress (Ariza et al. 1994). This operon, which is short for multiple antibiotic resistance, consists of four genes; *marA* (encoding an activator protein of *mar* operon), *marR* (encoding a repressor protein of *mar* operon) (Cohen et al. 1993), *marB* and *marC* (with unknown function) (Alekshun and Levy 2004). The *mar* operon is responsible for the increased low level resistance in *E. coli* strains to different classes of antibiotics including tetracycline, chloramphenicol, β-lactams, and fluoroquinolones by efflux mechanisms (George and Levy 1983). Interestingly, the MarR repressor in *E. coli* found in the gut of animal hosts has another function which is detecting phenolic compounds of plant products (Sulavik et al. 1995), further supporting the role of MarR in carvacrol/oregano resistance. The missense mutation detected in *marR* gene was an amino acid substitution from arginine to histidine at position 94 of the protein MarR, caused by a transition substitution from C to T at the genome position 13346. The visualization of the mutation location in the carvacrol-derivative strain (22M), when compared with the wild-type strain (APEC O23:H52), is shown in Fig. 2. Given the increased resistance phenotype of the *E. coli* strains, this substitution is probably a non-silent mutation resulting in an increased activity of the *mar* efflux system due to the repressor failing to repress the *mar* operon, and therefore increased its resistance as was recently discovered (Chueca et al. 2018). Similar findings were demonstrated by previous work from our laboratory (AlKhandari 2017), which showed that thymol-derivative strain showed non-sense mutations in *marR* and *acrR*, genes encoding repressors involving in efflux pump systems were responsible for the reduced susceptibility to phytochemicals. These findings suggest that carvacrol can act as an efflux pump inhibitor when used at high concentrations as proposed in this study (Miladi et al. 2016).

This study might indicate the importance of giving carvacrol, oregano or thymol as a feed additive instead of antibiotics as a feed additive to chicken and what might happen after a long period of time use. However,

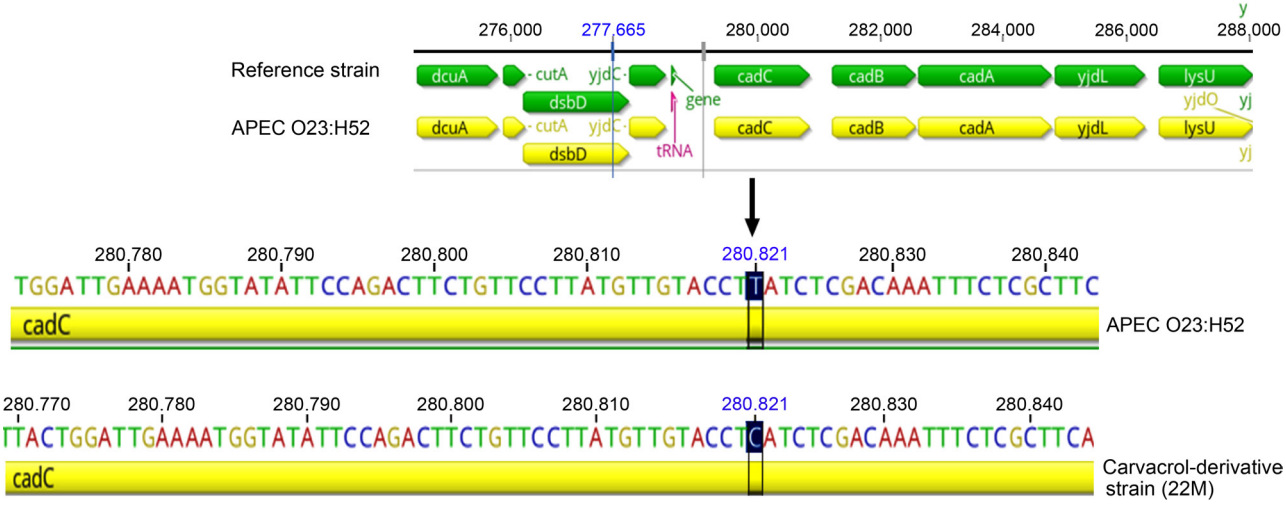

Fig. 1. A diagram showing the presence of T at 280821 in the genome of the C1 strain (APEC O23:H52) that is substituted by C at position 280821 in the genome of the carvacrol-derivative strain (22M). This graph was generated using the Geneious Prime 2019.1.1 (https://www.geneious.com).

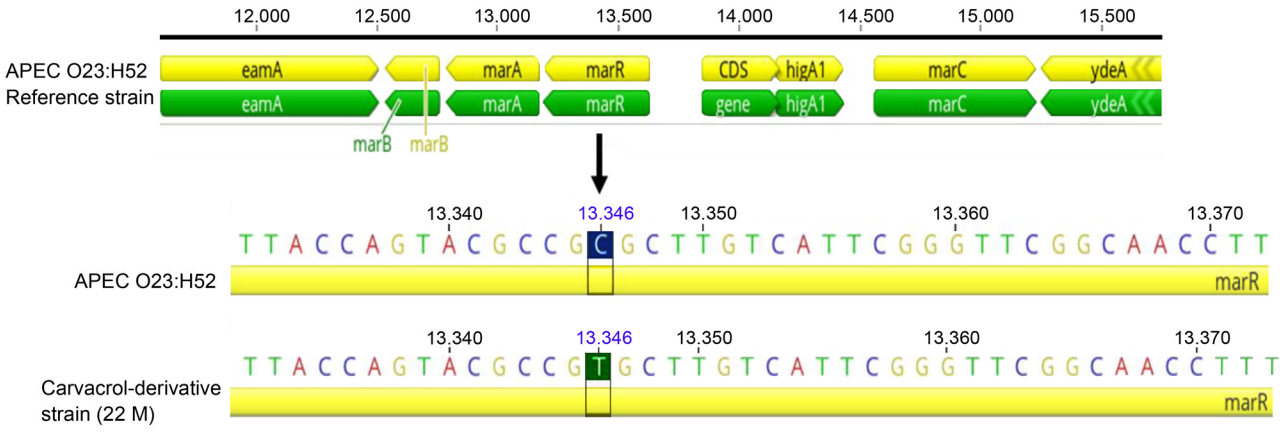

Fig. 2. A diagram showing the presence of C at 13346 in the genome of the C1 strain (APEC O23:H52) that is substituted by T at position 13346 in the genome of the carvacrol-derivative strain (22M). This graph was generated using the Geneious Prime 2019.1.1 (https://www.geneious.com).

this work was performed only once but the body of data from this current study supports the findings of previously mentioned studies which have suggested that exposure to phytochemicals (carvacrol, oregano, and thymol) select for mutants in different genes but each responsible for increased resistance phenotype. To confirm the effect of gene mutations on resistance, further studies could include: 1) gene complementation to show phenotype reversion and 2) use of mutant *E. coli* strains from the Keio library to study *cadC* and *marR* mutants in specific, and to study all the possible mechanisms of actions. In conclusion, the possible mechanisms of action of carvacrol/oregano against *E. coli* seem to be associated with missense mutations in the genes responsible for survival mechanisms under unfavorable conditions (*cadC*) and multiple antibiotic resistance (*marR*).

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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