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Effect of β-lactam antibiotic resistance gene expression on the radio-resistance profile of *E. coli* O157:H7

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Abstract

Some pathogens might develop favorable global adaptation in response to certain stress treatments resulting in enhanced virulence and/or resistance to a different stress. β-lactam resistance, as well as *ampC* and *ampG* genes involved in this resistance, were studied to evaluate their possible role in *Escherichia coli* O157:H7 (*E. coli*) radioresistance. *E. coli* adapted to 25, 15 or 7 μg/mL of kanamycin or carbenicillin, were produced and treated with sensitization (0.4 kGy) or lethal (1.5 kGy) irradiation doses. In *E. coli* O157:H7, irradiation treatment at 0.4 kGy dose increased *ampC* and *ampG* expression respectively by 1.6 and 2-fold in the wild type strain (Wt) but up to by 2.4 and 3.4-fold when the strain was beforehand adapted to 25 μg/mL of carbenicillin (Carb25). Accordingly, Δ*ampC* and Δ*ampG* mutants and *E. coli* adapted to 25 μg/mL of kanamycin were more sensitive to 0.4 kGy treatment than Wt. While, *E. coli* Carb25 or overexpression of *ampC* and *ampG* provided complete resistance to 0.4 kGy and were even able to survive and grow after exposure to a normally lethal 1.5 kGy irradiation dose. We further noticed that these strains can tolerate other stresses like oxidative, cold and heat shocks. This demonstrates that carbenicillin adaptation promotes resistance to γ-irradiation and to other stresses,

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likely at least through increased AmpC and AmpG expression. These results are important for the food industry and particularly when considering the use of irradiation for food preservation of meat obtained directly from animals fed β-lactam antibiotics.

Keywords: Biotechnology, Microbiology, Molecular biology

1. Introduction

The bacterium *E. coli* serotype O157:H7 was identified in 1989 as a human pathogen that can produce one of several Shiga toxins known to cause haemorrhagic colitis and hemolytic uremic syndrome (Karmali et al., 1983). *E. coli* O157:H7, found in the feces of healthy cattle, can be acquired by direct contact with infected animals or people, by consumption of contaminated food (i.e. meat, fruits, vegetables and poultry), or by wastewater of slaughterhouses (Karmali et al., 1983; Phillips, 1999; Loukiadis et al., 2006). The emergence of antibiotic use in animal feed to treat, control and prevent clinical diseases or to enhance animal growth promotes bacterial resistance by natural selection of mutant population unresponsive to the antibiotics (Courvalin, 2008). Furthermore, spontaneous antibiotic-resistant *E. coli* O157:H7 strains are emerging, such as ampicillin and chloramphenicol resistant mutants of strain EDL933, which led to increased motility, altered sensitivity to phages, and altered generation time *in vitro* resulting in a rise in their virulence (Tóth et al., 2003; Carone et al., 2014). As a result, Canadian authorities have recognized that some antimicrobial drugs used in veterinary medicine, e.g. β-lactams, can promote the development of resistance and might have serious consequences on human health (Health Canada, 2002).

Bacterial resistance to β-lactam antibiotics induces SOS response, through RecA and DpiA, and is driven by either β-lactamases modifying penicillin-binding proteins (PBPs), or by increased active efflux or decreased permeability, dependent on permeases (Poole, 2004). In many *Enterobacteriaceae* and other Gram-negative bacteria, the expression of the β-lactamase gene *ampC* is inducible by various β-lactam antibiotics (Sanders Jr and Sanders Jr, 1986; Zeng and Lin, 2013). Some mutations can alter this inducibility, resulting in increased β-lactamases synthesis and consequently raised resistance to new β-lactam antibiotics (Sanders and Sanders Jr, 1983; Juan et al., 2006). AmpC belongs to the group I, class C of β-lactamases and it is expressed at very low levels in wild type strains. AmpC levels increase significantly in *E. coli* in the presence of certain β-lactam antibiotics (Livermore, 1995).

The expression of *ampC* is induced by several genes products leading to peptidoglycan recycling, required for cell growth and division (Normark, 1995; Park and

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Uehara, 2008). The peptidoglycan layer is composed of β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues. Peptidoglycan degradation, caused for instance by the action of β-lactam antibiotics, leads to the generation of a pool of GlcNAc-anhMurNAc-peptides which can be transported to the cytoplasm by the permease AmpG (Park and Uehara, 2008) and act as signals involved in *ampC* induction and metabolism through the transcriptional regulator AmpR and the repressor AmpD. Therefore, mutation in *ampD* or in *ampR* may lead to overproduction of AmpC beta-lactamase (Kaneko et al., 2005; Schmidtke and Hanson, 2008).

In several food industries, γ-irradiation is used to prolong shelf-life of food products and to reduce the growth of pathogens (Koutchma et al., 2009; Ben-Fadhel et al., 2016). At doses of 2−7 kGy, γ-irradiation can eliminate potentially pathogenic bacteria such as *E. coli* O157:H7. The microbial inactivation mechanism of γ-irradiation is explained by oxidative radicals resulting from the radiolysis of water, generating an oxidative stress that will cause damage to nucleic acids, proteins and whole cells (Farkas, 2006). The increase of these free radicals induces an oxidative stress response in the bacteria. However, many pathogens can develop physiological and molecular mechanisms of resistance that can disrupt the efforts of food industries to control food contamination and to insure food safety. Bacterial radioresistance is a complex phenomenon that is still poorly understood.

The aim of this study is to assess the impact of the resistance to antibiotics widely used in animal feed (β-lactams) on the radioresistance of *E. coli* O157:H7, more specifically by evaluating the effect of AmpC β-lactamase and of AmpG permease.

2. Materials and methods

2.1. Material

Isopropyl-β-D-thiogalactopyranoside (IPTG), N,N′-dimethyl-4,4′-bipyridinium dichloride (Paraquat) carbenicillin, ampicillin, piperacillin and kanamycin were obtained from Sigma Aldrich, Ontario, Canada. PureZOL was provided from Life Science Research, Ontario, Canada.

2.2. Bacterial strains, plasmids and growth conditions

E. coli strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated overnight (O/N) in Tryptic Soy Broth (TSB, Difco, Becton Dickinson, Sparks, MD, USA) or on Tryptic Soy Agar plate (TSA, Difco, Becton Dickinson, Sparks, MD, USA). The media was supplemented with: (i) 100 μg/mL carbenicillin and incubated at 30 °C for *E. coli* O157:H7 (pKD46) (Wt and mutants) or at 37 °C for *E. coli* O157:H7 (Ptrc99A) (Wt and carrying *ampC* or *ampG*), (ii) 30 μg/mL

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Table 1. List of *E. coli* strains and plasmids used in this study.

E. coli stains and plasmids	Description	Source or reference
O157:H7 str. EDL933	Pathogenic strain isolated from Michigan ground beef	Provided by Pr. Charles Dozois (INRS-IAF)
K-12		
Δ*ampC*	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔampC777::kan*, *hsdR514*	Genetic stock center (Baba et al., 2006)
Δ*ampG*	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *ΔampG790::kan*, *λ*⁻, *rph-1*, *Δ(rhaD-rhaB) 568*, *hsdR514*	Genetic stock center (Baba et al., 2006)
Plasmid	Sensitive plasmid	Genetic stock center
pKD46	F-, *λ*⁻, *rph-1*, pKD46	(Datsenko and Wanner, 2000)
pTrc99A	*trcP* vector, *lacI* promoter, pUCl8 EcoRI-SmaI,	Provided by Pr. Charles Dozois (INRS-IAF)

kanamycin and incubated at 37 °C for *E. coli* O157:H7 without pKD46, or (iii) without antibiotics for strain K-12 (Wt and mutants). Transformed cells were recovered in 1 mL SOC medium (Thermo Fisher Scientific, Burlington, Ontario, Canada) (pKD46) or 1 mL TSB and incubated at 30 °C for 1 h. IPTG was added to TSB medium to obtain a final concentration of 1 mM for *E. coli* O157:H7 carrying pTRC99A plasmid. To reach the bacterial exponential phase, the O/N culture was diluted at 1/100th and incubated at 37 °C for 3−4 h until an $OD_{600} \approx 1$ was obtained. OD_{600} for all cell cultures used in this work was measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Burlington, Ontario, Canada) and a path length correction was applied by multiplying the given value by a factor of 10.

2.3. Construction of mutants and complementation/ overexpression vectors

Deletion mutants were derived using one-step PCR fragment-mediated lambda red recombination mutagenesis (Datsenko and Wanner, 2000). The pKD13 strain has been used to create KEIO mutants (Baba et al., 2006). Using primers listed in Table 2, the *ampC::kan* and *ampG::kan* alleles were amplified directly from K-12 mutant strains JW4111-2 (Δ*ampC*::*kan*) and JW0423-1(Δ*ampG*::*kan*) respectively, obtained from the Keio collection to generate *ΔampC*::*kan* and Δ*ampG*::*kan* in O157:H7, a derivate of *E. coli* K-12 carrying pKD46 (Sabri et al., 2009). The purified PCR fragment was transformed into DH5α competent cells carrying pKD46 by heat shock. The extracted plasmid-Δ*gene* (*ampC* or *ampG*) was transformed by electroporation (1800 volts, 200 ohms, 25 μF) into *E. coli* O157:H7 competent cells. The homologous recombination between pKD46 and chromosomic genes was then evaluated. *E. coli* O157:H7 mutants were verified by amplification and

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Table 2. List of primers used in this study.

Genes	Sequence (5′ to 3′)
Homologous primers	
ampC. Fwd	GAGCTTCATTGGTCGCGTAT
ampC. Rev	TTCACTCTGCTGCCACATTC
ampG. Fwd	ATATCGCCATCATCGCTACC
ampG. Rev	CAAAAGGGGAGTGCAGAAGA
Primers for overexpression	
ampC.over-EcoRI-Fwd	**CTCTGAATTC**TTTTCTACG GTCTGGCTGCT
ampC.over-SmaI-Rev	**CTCTCCCGGG**CAGGCGC ATAAATGTTTCCT
ampG.over-EcoRI-Fwd	**CTCTGAATTC**TGCTGACCGA TACCATCGCT
ampG.over-SmaI-Rev	**CTCTCCCGGG**GCTAAGCCGCACAAAAGAAC
Primers for verification	
ampC.over-Fwd	TTTTCTACGGTCTGGCTGCT
ampC.over-Rev	CAGGCGCATAAATGTTTCCT
ampG.over-Fwd	TGCTGACCGATACCATCGCT
ampG.over-Rev	GCTAAGCCGCACAAAAGAAC
Primers for qRT-PCR	
ampC. Fwd	CGCTAAGGAGTGCAACATGA
ampC. Rev	CGCAGCATGTAAAAGCGATA
ampG. Fwd	GCCGCCCTTAATTTTTCTTC
ampG. Rev	GCAATGGAAACGGTAAAAGC
rpoH Fwd	TCGTCAAAGTTGCGACCACCAAAG
rpoH rev	ATCGTCGTCGGAAGACAGGTCAAA

sequencing of *ampC::kan* or *ampG::kan* using primers cited in Table 2. Overexpression of *ampC* and *ampG* was achieved using the high-copy number inducible plasmid pTRC99A (Amann et al., 1988). These two genes were amplified using *E. coli* O157:H7 DNA as the template and the primers cited in Table 2 designed to have EcoRI and SmaI cohesive ends. The resulting PCR amplicon was digested by EcoRI and SmaI (New England Biolabs, Massachusetts, USA) and used for ligation to EcoRI-/SmaI-treated pTrc99A. The recombinant plasmid was transformed into DH5α competent cells by heat shock. After miniprep, it was transformed by electroporation (1800 volts, 200 ohms, 25 μF) into *E. coli* O157:H7 competent cells. Transformants carrying pTRC99A-*gene* or pTRC99A-without gene were selected on TSA plates supplemented with 100 μg/mL of carbenicillin. Plasmids were digested by EcoRI and migrated on 1% agarose gel to confirm gene insertion.

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2.4. Antibiotic susceptibility assays

The minimum inhibitory concentration (MIC) of the antibiotics was determined as recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2014). The evaluated antibiotics were carbenicillin, ampicillin, piperacillin and kanamycin. All antibiotics were prepared in distilled water to a final concentration of 50 μg/mL for kanamycin and 100 μg/mL for the others. A Blank Antimicrobial Susceptibility Disks (Thermo Ficher Scientific, Burlington, Ontario, Canada) imbibed with either 5 μL or 10 μL of one of the antibiotics, corresponding to 250 μg and 500 μg for kanamycin and 500 μg and 1000 μg for the other antibiotics, were used for the antibiotic susceptibility assays on *E. coli* O157:H7 mutants.

2.5. Development of antibiotic-adapted O157:H7 strains

E. coli O157:H7 was adapted to 25, 15 or 7 μg/mL of carbenicillin or kanamycin by successive passages ($n = 4$) in TSB supplemented with the respective antibiotic at the specified concentration. The selected concentrations of each antibiotic used in this study were enough to slow down the bacterial growth. After the adaptation step and for all following experiments, adapted bacteria were cultivated in TSB without antibiotic.

2.6. γ-Irradiation treatments

Cell cultures (all strains) from the exponential phase of growth ($OD_{600} \approx 1$) were transferred into microfuge tubes and subjected to one of two different irradiation treatments: the sensitive dose of 0.4 kGy to only damage the cells or the lethal dose of 1.5 kGy (Caillet et al., 2005). The experiments were performed at room temperature (20 ± 1 °C) at the Canadian Irradiation Center, where samples were irradiated in a UC-15A irradiator (Nordion Inc., Kanata, ON, Canada) equipped with a ^{60}Co source with a dose rate of 16.8 kGy/h.

2.7. Viability and growth study

All strains were evaluated immediately after irradiation treatment for their viability and growth. The obtained working cultures were serially diluted and plated on TSA plates and then incubated overnight at 37 °C. For the growth assays, the obtained working cultures were diluted in TSB to an $OD_{600} = 0.05$ and 200 μL of cell culture were distributed wells of sterile microtiter plates. This experiment was performed in a Bioscreen C apparatus (Growth Curves USA) where the cultures were incubated at 37 °C for 20 h and the OD_{600} growth measurements were performed every 30 minutes after a homogenization period of 30 seconds.

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2.8. RNA preparation and quantitative real-time PCR (qRT-PCR)

E. coli O157: H7 Wt and Carb25 adapted bacteria were incubated in TSB at 37 °C and irradiated at the sensitive dose at the exponential phase of growth ($OD_{600} \approx 1$). Control and irradiated cultures were incubated at 37 °C for 60 min and then RNA extraction was performed. Bacterial culture was centrifuged at 12,000 x *g* for 5 min and the cells were suspended in 1 mL PureZOL. RNA extraction was carried out as recommended by the supplier. The iScript reverse transcription kit (Life Science Research, Ontario, Canada) was used to synthesize cDNA from 50 ng of RNA and quantitative real-time PCR (qRT-PCR) was then performed using the SsoAdvanced™ Universal SYBR® Green Supermix reagent (Life Science Research, Bio-Rad) on a Rotor-Gene 6000 thermocycler (Corbett). Modulation of gene expression from qRT-PCR was normalized to the expression of the internal *rpoH* gene. The primers used in this study are listed in Table 2. The variation of gene expression was calculated using the $-2^{\Delta\Delta Ct}$ method of Livak and Schmittgen (2001). The relative expression of Wt was used as a reference to calculate the *ampC* and *ampG* expression levels.

2.9. Statistical analysis

Each experiment was done in triplicate (n = 3) and experimental values are presented as means +/- standard deviation. Statistical analysis was done using Prism5 (GraphPad Software, Inc.) and differences between means were considered significant when the confidence interval is lower than 5 % (***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; student t test).

3. Results

Two populations of *E. coli* O157:H7 were subcultured four times on various concentrations of carbenicillin (Carb25, Carb15 and Carb7) or kanamycin (Kan25, Kan15 and Kan7) until adaptation. Once these adapted bacteria were obtained, all remaining experiments were performed without antibiotic treatment. The bacterial growth rate increased according the number of passages and to the antibiotic concentration. O157:H7 was more easily adapted to kanamycin than to carbenicillin (Figs. 1 and 2). Adapted subpopulations were cultivated in TSB medium O/N at 37 °C, diluted at $1/100^{th}$ and incubated at 37 °C until $OD_{600} \approx 1$. The viability was then tested vs the Wt strain immediately following γ-irradiation treatment with 0.4 kGy (Fig. 3). As expected, irradiation significantly ($P \leq 0.001$) reduced the counts of viable Wt bacteria (1.6 log reduction as compared to the control). The growth rate was also affected. In contrast, the same treatment had no significant effect on the survival ($P > 0.05$) of the carbenicillin-adapted subpopulations (Fig. 3). However,

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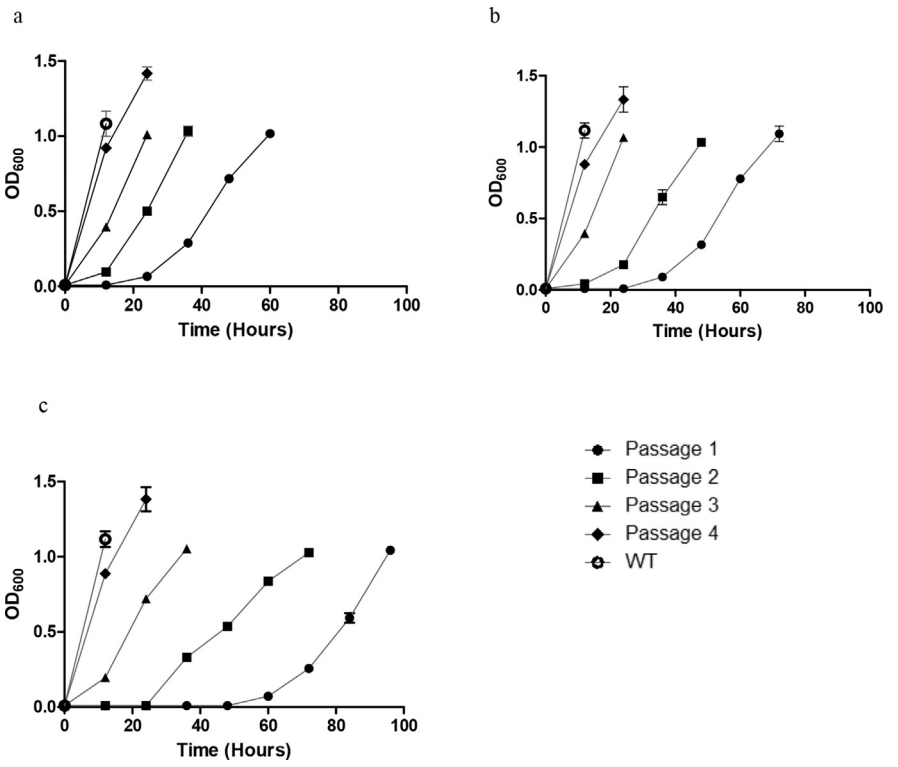


Fig. 1. Growth of carbenicillin-adapted *E. coli* O157:H7 strains to 7 μg/mL (a), 15 μg/mL (b) or 25 μg/mL (c) of carbenicillin by successive passages in TSB supplemented with antibiotic at the specified concentration (n = 4). Bacteria were incubated at 37 °C and 1/100th dilution was carried out from the culture at $OD_{600} \approx 1$ and then sub-cultured for the next passage. 200 μL of each cell culture were used for growth monitoring.

irradiation seems to have little effect on their growth (Fig. 4); and after 400 min and 600 min post-irradiation, respectively Carb25 and Carb15 were completely resistant (Fig. 4a and b).

These observations show that bacterial adaptation to high concentrations of carbenicillin (i.e. ≥15 μg/mL) confers resistance to γ-irradiation. In contrast to carbenicillin, the adaptation of *E. coli* O157:H7 to different concentrations of kanamycin did not increase its radioresistance and a significant difference was observed between irradiated and non-irradiated cells adapted to the three concentrations of kanamycin (Figs. 5 and 6). The reduction level was 1.4, 1.8, 2 and 1.6 $\log_{10}$ CFU/mL for Wt and adapted *E. coli* to 7, 15 and 25 μg/mL, respectively (Fig. 5). These results were confirmed by evaluating *E. coli* growth after treatment (Fig. 6). A significant difference was observed throughout the whole experimental period between irradiated and non-irradiated *E. coli* cells adapted to the three concentrations of kanamycin. These results indicate that kanamycin has no effect on the development of resistance to 0.4 kGy irradiation treatment in *E. coli* O157:H7. The transcription of *ampC* and *ampG* was assessed by qRT-PCR 60 min after irradiation at 0.4 kGy. The results (Fig. 7a)

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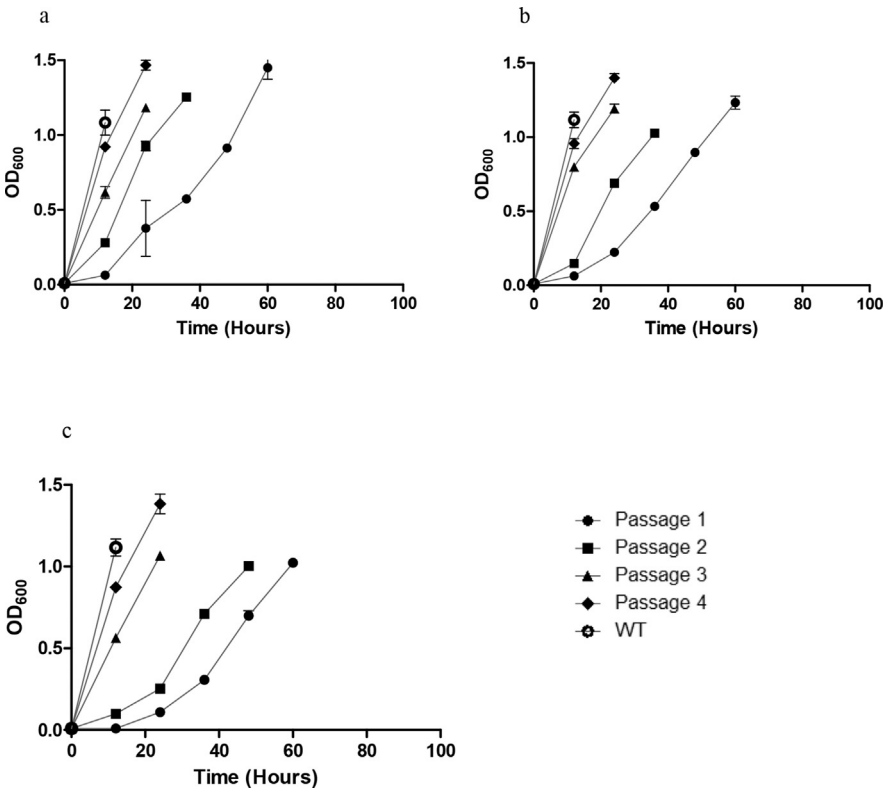


Fig. 2. Growth of kanamycin-adapted *E. coli* O157:H7 strains to 7 μg/mL (a), 15 μg/mL (b) or 25 μg/mL (c) of kanamycin by successive passages in TSB supplemented with antibiotic at the specified concentration (n = 4). Bacteria were incubated at 37 °C and 1/100th dilution was carried out from the culture at $OD_{600} \approx 1$ and then sub-cultured for the next passage. 200 μL of each cell culture were used for growth monitoring.

demonstrate that irradiation treatment of *E. coli* led to a significant increase ($P \leq 0.01$) in the expression of *ampC* and *ampG,* showing respectively an increase of 1.6 and 2-fold at 60 min post-treatment compared to the non-irradiated control. Interestingly, even without irradiation treatment, the expression of *ampC* was 2.4-fold higher in Carb25 than in the Wt. No significant difference ($P > 0.01$) was observed on the *ampG* expression. Moreover, irradiation treatment at 0.4 kGy further increased the expression of both *ampC* and *ampG* genes by 1 $\log_2$ in Carb25 bacteria (Fig. 7b). Sequencing of the Carb25 *ampC* and *ampG* genes revealed no mutation, compatible with an adaptation to carbenicillin promoted by modifications in gene expression.

To further investigate the effect of these genes on the radioresistance of *E. coli* O157:H7, mutants in these genes were generated. As expected, the antibiogram results showed that Δ*ampC* and Δ*ampG* mutants are very sensitive to β-lactam antibiotics (ampicillin, piperacillin and carbenicillin) (Fig. 8 a,b,c). This sensitivity is dose-dependent and was higher with carbenicillin compared to the other antibiotics. In contrast, no significant effect of kanamycin on the Δ*ampG* mutant and only a little

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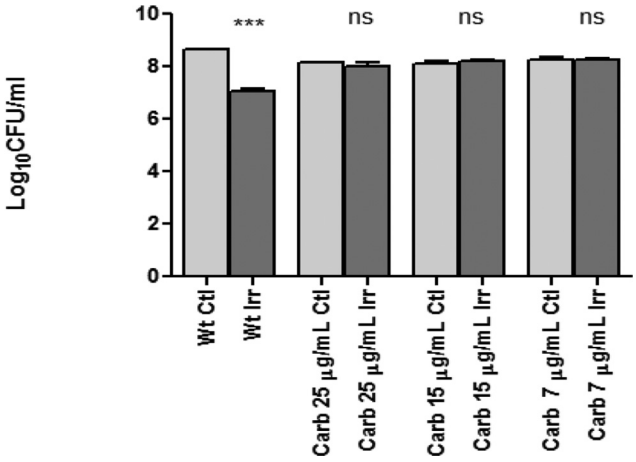


Fig. 3. Viability of *E. coli* O157:H7 (Wt or carbenicillin-adapted subpopulations) treated or not with irradiation at 0.4 kGy. Immediately after irradiation treatment, five µL of each cell culture were used. Results are represented by $\log_{10}$ of CFU/mL. Carb: carbenicillin adapted bacteria; Ctl: control non-irradiated; Irr: irradiated.

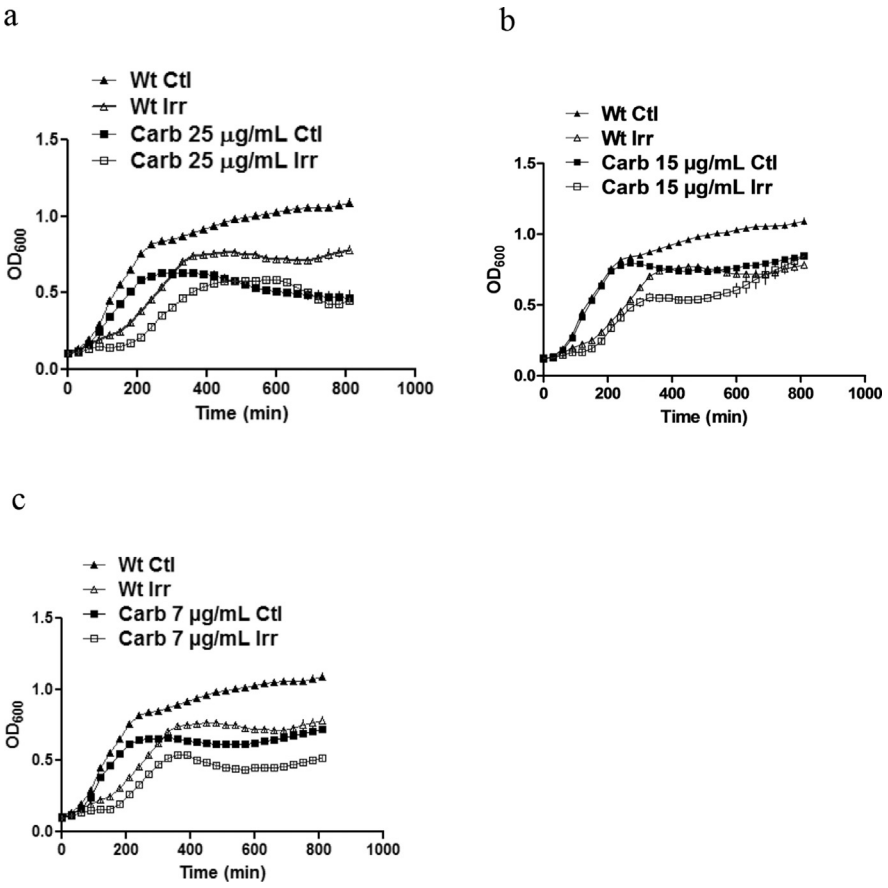


Fig. 4. Growth of *E. coli* O157:H7, wild type and subpopulations -adapted to (a) 25 µg/mL, (b) 15 µg/mL or (c) 7 µg/mL carbenicillin, then treated or not with irradiation at 0.4 kGy. 200 µL of each cell culture were used. Carb: carbenicillin adapted bacteria; Ctl: control non-irradiated; Irr: irradiated.

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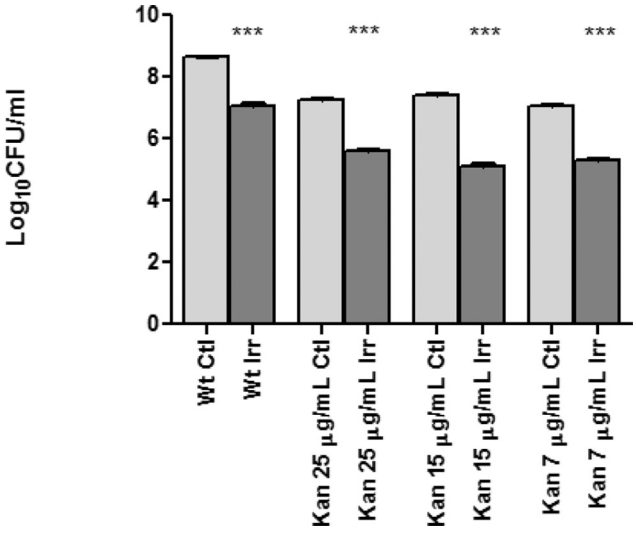


Fig. 5. Viability of *E. coli* O157:H7 (wild type and kanamycin adapted type) treated or not with irradiation at 0.4 kGy. 5 µL of each cell culture were used. Results are represented by $\log_{10}$ of CFU in 1 mL. Kan: kanamycin adapted bacteria; Ctl: control non-irradiated; Irr: irradiated.

effect on the Δ*ampC* mutant were observed (Fig. 8d). Since the carbenicillin-adapted subpopulations were more resistant to γ-irradiation treatments, we tested whether the mutants with more sensitivity to β-lactam antibiotics would behave the opposite way. The effects of γ-irradiation at sensitization dose of 0.4 kGy and lethal dose of 1.5 kGy on the viability and the growth of *E. coli* Δ*ampC* and Δ*ampG* mutants are shown in Figs. 9a and 10a. The data demonstrate that inactivation of *ampC* and *ampG* increased the sensitivity of the cells to irradiation treatment at a dose of 0.4 kGy. Viability was reduced by 2 and 2.8 $\log_{10}$ CFU/mL in Δ*ampC* and Δ*ampG* mutants, respectively, as compared to only 1.1 $\log_{10}$ CFU/mL reduction in the Wt. To verify the effect of *ampC* and *ampG* deletion in radiosensitivity, *E. coli* Δ*ampC* and Δ*ampG* mutants were complemented respectively, by *ampC* and *ampG* genes on pTRC99A plasmid which is inducible by IPTG. Irradiation response of Δ*ampC* overexpressing *ampC* (Δ*ampC*[*ampC*+]) and Δ*ampG* overexpressing *ampG* (Δ*ampG*[*ampG*+]) was compared to Wt[pTRC99A] as a control. Very interestingly, the complemented mutants overexpressing the corresponding gene (Δ*ampC* [*ampC*+] and Δ*ampG*[*ampG*+]) became completely resistant to irradiation at the sensitization dose (Fig. 9) with only a limited growth difference with the Wt (Fig. 10). Even more, these overexpressing mutants were able to survive and grow after irradiation treatment at a dose of 1.5 kGy which is completely lethal for Δ*ampC* and Δ*ampG* mutants and for Wt O157:H7 (Figs. 9 and 10). Our findings suggest that AmpC and AmpG may intervene in the radioresistance of *E. coli* O157:H7.

Since response mechanisms to stresses are often redundant, we also evaluated the effect of these two genes in the response to other stress like oxidative (paraquat),

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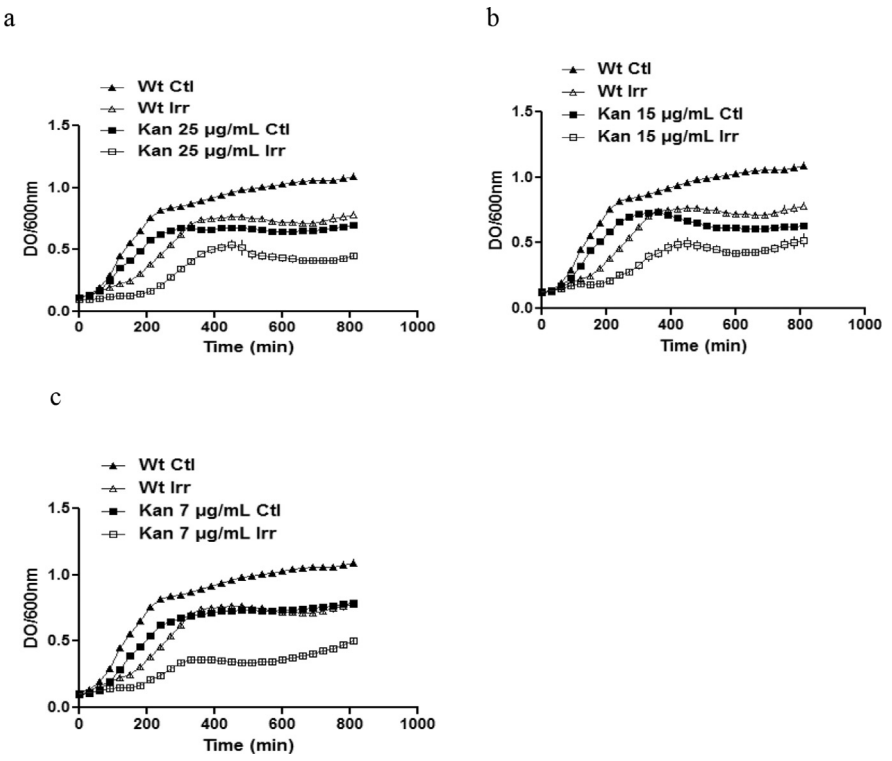


Fig. 6. Growth of *E. coli* O157:H7, wild type and kanamycin adapted type to (a) 25 µg/mL (b) 15 µg/mL and (c) 7 µg/mL treated or not with irradiation at 0.4 kGy. 200 µL of each cell culture were used. Kan: kanamycin adapted bacteria; Ctl: control non-irradiated; Irr: irradiated.

heat and cold stress. Interestingly, in contrast with the Wt and the ampC and ampG mutants, Carb25, Δ*ampC*[*ampC*+], and Δ*ampG*[*ampG*+] strains were all able to survive and grow after incubation at 50 °C for 2 h or 1 week at 4 °C and in presence of 0.5 mM or 1 mM paraquat, metabolic generator of superoxide (Figs.11 and 12).

4. Discussion

In the present study, we have demonstrated that the resistance of *E. coli* O157:H7 to some antibiotic types could reduce its sensitivity to irradiation treatment at a dose that is normally lethal. In fact, our results show that an *E. coli* O157:H7 subpopulation adapted to carbenicillin recovered growth faster than wild type bacteria following an irradiation treatment at the sensitization dose (Figs. 3 and 4). This was not the case for a subpopulation of *E. coli* O157:H7 adapted to kanamycin (Figs. 5 and 6). We also demonstrated that the viability and the growth of the adapted bacteria were affected as compared to the Wt. This could be the result of a trade-off phenomenon, which is a negative correlation between stress resistance/adaptation and growth bacteria like pathogenic *E. coli* (Basra et al., 2018). These findings suggest that the acquisition of radioresistance after a bacterial adaptation to antibiotics depends on the class of antibiotic.

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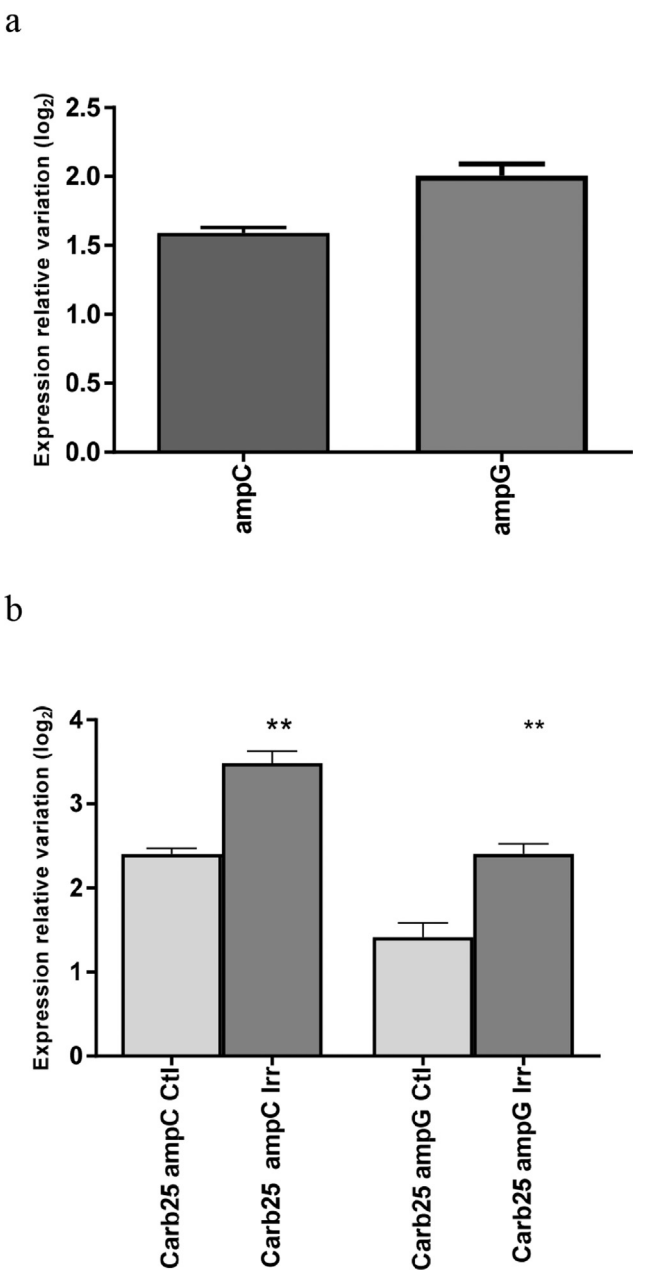


Fig. 7. Effect of γ-irradiation on *ampC* and *ampG* expression of *E. coli* O157:H7; (a): in wild type bacteria. (b): in 25 μg/mL carbenicillin adapted bacteria (Carb25). Relative gene expression represents the change in transcription in bacteria 60 minutes after irradiation treatment at a dose of 0.4 kGy. Results are represented by $\log_2$ RNA expression using $-2^{\Delta\Delta Ct}$. The difference was considered as significant when the expression relative variation ≥ 1.5 $\log_2$. Carb25: 25 μg/mL carbenicillin adapted bacteria, NI: not irradiated and Irr: irradiated.

Wesche et al. (2009) reported that microorganisms that survive a given stress could gain resistance to that stress or another one. This phenomenon is attributable to the cross-protection mechanisms found in the bacteria and has an important impact on the industrial process conditions and food safety. While there is an increasing

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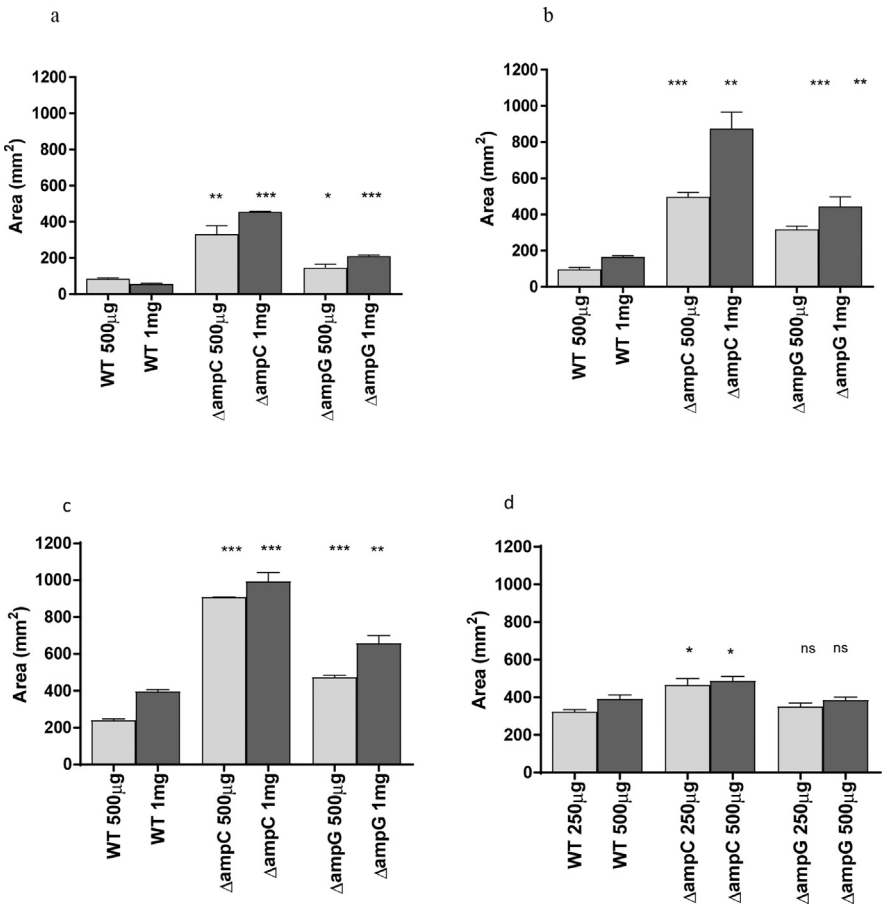


Fig. 8. Antibiotics susceptibility assays on *E. coli* O157:H7 *ampC*, *ampG* or *ampR* mutants. The disk containing 500 µg or 1 mg of (a) ampicillin, (b) piperacillin and (c) carbenicillin or 250 or 500 µg of (D) kanamycin was placed onto the surface of the agar plates. Inhibition area = ($R^2 \times \pi$).

evidence of a relationship between different stress resistances, few studies have focused on the evaluation of the effect of bacterial resistance to a given stress on radioresistance. Collectively, it is possible that resistance to β-lactam antibiotics is responsible for some mechanism of resistance to an irradiation treatment at a dose of 0.4 kGy.

Peptidoglycan turnover and recycling are among the most important processes in bacterial life cycle. These mechanisms are involved in the regulation of virulence and the resistance response (Folkesson et al., 2005; Boudreau et al., 2012). The β-lactamase AmpC, produced by some Gram-negative bacteria, can interact with many β-lactam antibiotics including ampicillin, carbenicillin, penicillin and piperacillin (Sanders Jr and Sanders Jr, 1986). One of the regulators of this protein is the permease AmpG which imports peptidoglycan-derived product, named muropeptide, to the cytoplasm where it activates the transcription of *ampC* (Korfmann and Sanders, 1989; Yang et al., 2013).

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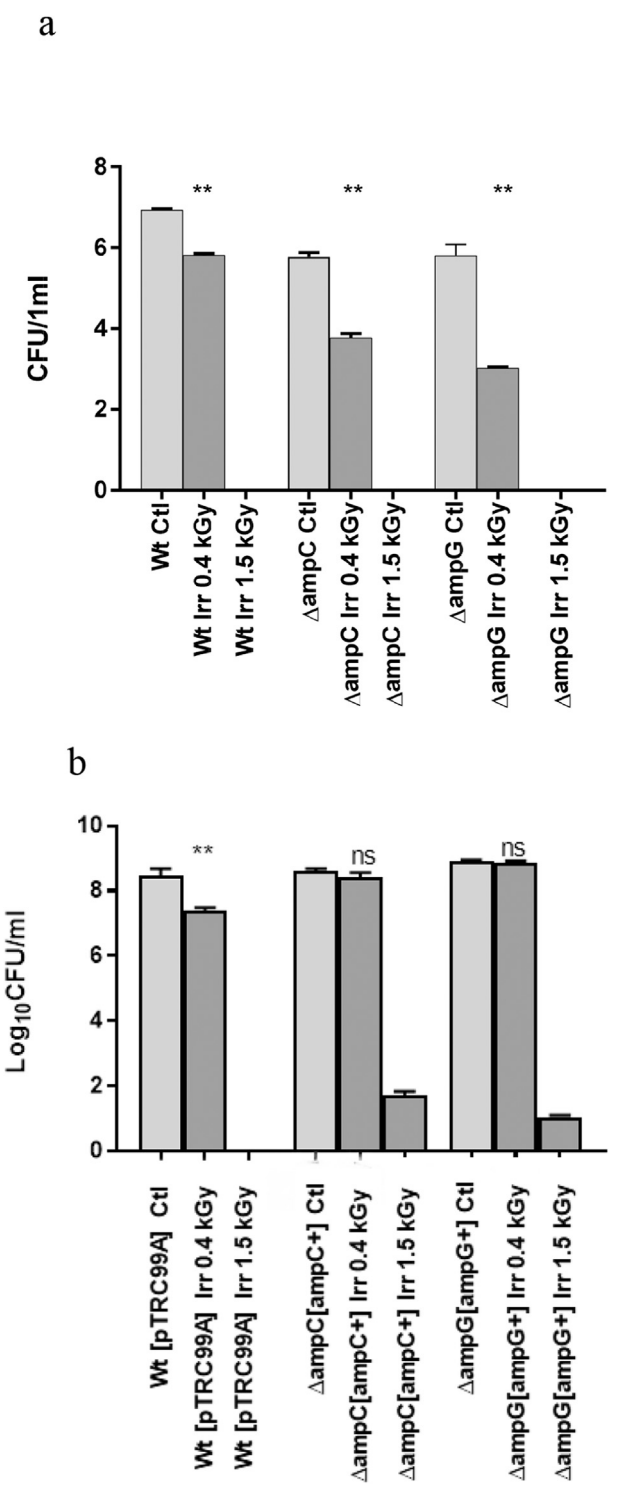


Fig. 9. Viability of *E. coli* strains: a: viability of Δ*ampC* and Δ*ampG. E. coli*. b: viability of Δ*ampC* [*ampC*+] and Δ*ampG*[*ampG*+]. *E. coli.* 5 μL of each cell culture were used. Results are represented by $\log_{10}$ of CFU in 1 mL. Ctl: control non-irradiated; Irr: irradiated; 0.4 kGy: sensitive dose; 1.5 kGy: lethal dose.

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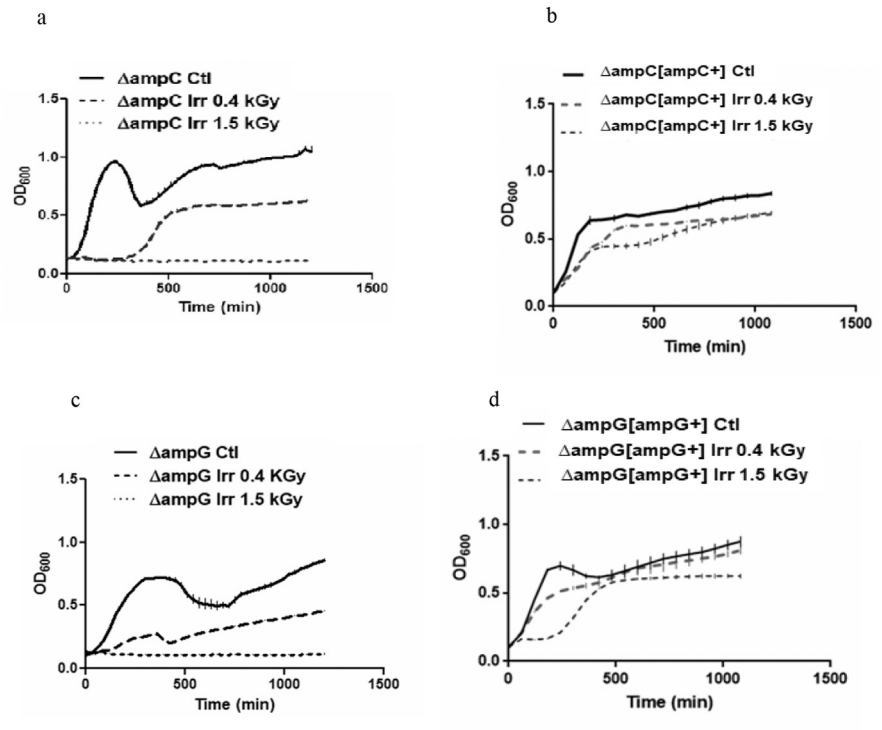


Fig. 10. Growth after γ-irradiation of *E. coli* O157:H7 Δ*ampC* (a), Δ*ampC-ampC* (b), Δ*ampG* (c) or Δ*ampG-ampG* (d). 200 μL of each cell culture were used. Ctl: control non-irradiated; Irr: irradiated; 0.4 kGy: sensitive dose; 1.5 kGy: lethal dose.

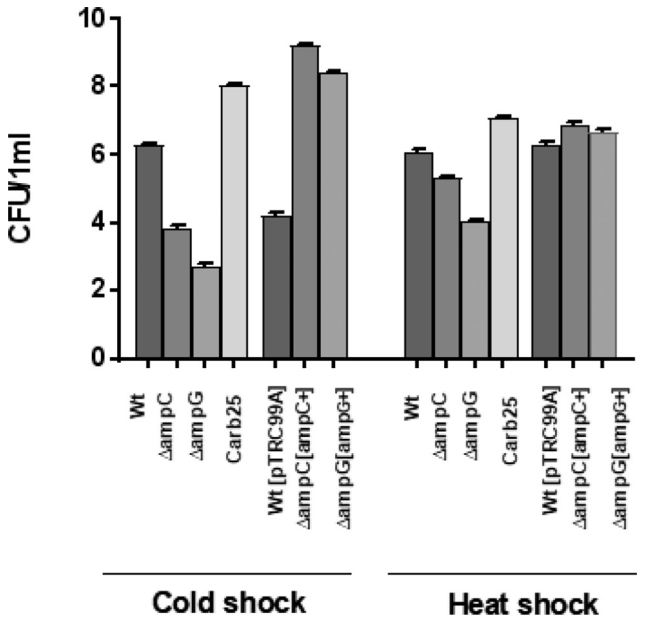


Fig. 11. Viability of *E. coli* O157:H7 strains under thermal stress: The bacteria at the exponential phase of growth ($OD_{600} \approx 1$) are cultured in TSB and incubated at 50 °C for 2h (heat shock) or at 4 °C for 1 week (cold shock). 5 μL of each cell culture were used. Results are represented by $\log_{10}$ of CFU.

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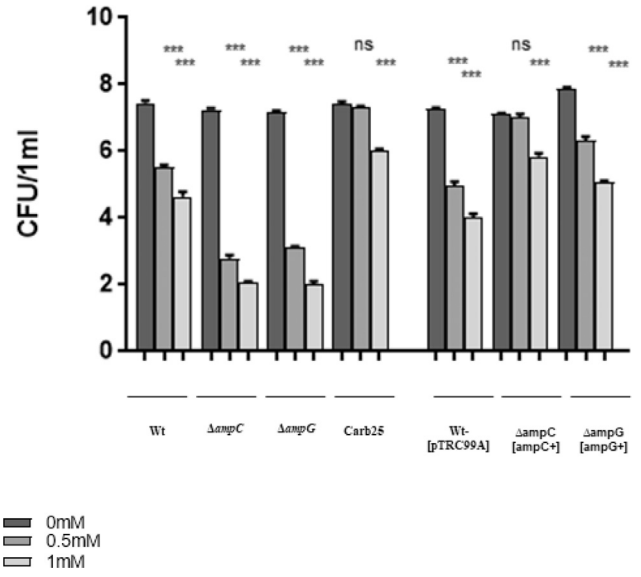


Fig. 12. Viability of *E. coli* O157:H7 strains under paraquat stress: The bacteria at the exponential phase of growth ($OD_{600} \approx 1$) are cultured in TSB supplemented with 0.5 or 1mM of paraquat and incubated at 37 °C for 40 min. 5 μL of each cell culture were used. Results are represented by $\log_{10}$ of CFU.

To evaluate the impact of the β-lactam resistance on the *E. coli* O157:H7 radioresistance, we assessed the expression of two genes required for β-lactamase activity in *E. Coli* (i.e. *ampC* and *ampG*) under γ-irradiation treatment. These marker genes were selected based on RNA-seq results demonstrating that their expression increases in *E. Coli* O157:H7 following 0.4 kGy irradiation treatment (unpublished data). We have showed that the expression of both genes was significantly increased (Fig. 7) confirming our RNA-seq results. Collectively, these findings indicate that AmpC and AmpG could be related to *E. coli* O157:H7 radioresistance. In addition, ΔampC and ΔampG mutants in strain O157:H7 showed an important sensitivity towards different β-lactam antibiotics (Figs. 9a and 10a), consistent with previous studies that have reported a role of these genes in the resistance of other *E. Coli* strains to β-lactams (Sanders, 1987; Lindquist et al., 1993).

In the current study, we have also demonstrated that γ-irradiation up-regulates *ampC* and *ampG* expression of both Wt and Carb25 O157:H7 (Fig. 7). This could be explained by the response of *E. coli* to muropeptides distribution by increasing *ampC* and *ampG* expression, which then promotes resistance. In fact, Caillet et al. (2005) reported that irradiation treatment affects the peptidoglycan recycling by modification of the distribution of muropeptides on *E. coli*, probably as a result of modification to the glycan-binding peptides. Peptidoglycan recycling is important for cell growth and division (Park and Uehara, 2008). The AmpG permease can transport GlcNAc-anhMurNAc-peptides, a peptidoglycan degradation product, to the cytoplasm where it is lysed by the β-glucosaminidase NagZ to produce anhMurNAc-peptides, which are then metabolized to UDPMurNAc-pentapeptides or by *ampD*

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to generate GlcNAc, anhMurNAc, murein tripeptide, and D-Ala. Murein tripeptide is ligated to UDP-MurNAc, therefore returning it to the murein synthesis pathway. In absence of β-lactam, UDPMurNAc-pentapeptide binds AmpR and converts it into a repressor of *ampC* transcription. In presence of a β-lactam, anhMurNAc-peptides accumulate and bind AmpR which then leads to the activation of *ampC* transcription (Dietz et al., 1997; Jacobs et al., 1997). The inactivation of *ampG* results in non-inducible and micro-constitutive β-lactamase phenotypes to the bacterial cell, correlating peptidoglycan recycling and β-lactamase induction (Korfmann and Sanders, 1989; Lindquist et al., 1993).

On the other hand, Δ*ampC*[*ampC*+] and Δ*ampG*[*ampG*+] strains overproducing AmpC and AmpG respectively and of Carb25 were paraquat resistant (Fig. 11). In fact, sub-lethal concentrations of ampicillin or norfloxacin were associated with ROS-induced mutagenesis in *E. coli* and *Staphylococcus aureus (S. Aureus)* (Cuirolo et al., 2009; Kohanski et al., 2010). In addition, β-lactam-induced clinical hetero-resistant *S. aureus* is associated with oxidative stress via increasing ROS production and DNA damage, both factors responsible of SOS response induction (Rosato et al., 2014). This suggests that carbenicillin, similarly to ampicillin, leads to this mutagenesis induced by ROS and activates genes involved in the SOS response. Consequently, when *E. coli* O157:H7 was irradiated, it had all the resistance machinery already activated, which may have contributed to its radioresistance when adapted to carbenicillin, not to kanamycin (Figs. 3 and 6).

Also, in *E. coli* O157:H7, we see that overexpression of *ampC* or *ampG* promotes heat and cold stress resistance (Fig. 12), maybe because AmpR effect on thermic tolerance, is similar to what is observed in *Pseudomonas aeruginosa* and this by increasing the expression of DnaJ-DnaK-GrpE Hsp70 system, involved in heat stress response (Balasubramanian et al., 2014) and by controlling cold stress response. On the basis of these observations, it appears that the type of stress applied on *E. coli* O157:H7 affects the response to other types of stress.

Gene *ampR* is involved in the oxidative stress response of *Pseudomonas aeruginosa* (Balasubramanian et al., 2014). In the present study, the Δ*ampC* and Δ*ampG* mutants were more sensitive to irradiation treatment compared to the Wt. In the Δ*ampG* mutant, anhMurNAc-peptides probably accumulate in the periplasm, resulting in no production of UDPMurNAc-pentapeptide. It is thus possible that UDPMurNAc-pentapeptide plays a role in the activation of the oxidative stress response by its interaction with *ampR*. In this context, *E. coli* becomes less resistant to an irradiation treatment when there is no interaction. These observations suggest that AmpC may be involved in *E. coli* O157:H7 radioresistance by a direct ROS genes synthesis control or interaction with *ampR* (Figs. 9-a-10b). Moreover, different growth profiles of Δ*ampC* and Δ*ampG* mutants were observed (Fig. 10). This could be related to the perturbation in peptidoglycan

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recycling caused by the absence of *ampC* or *ampG*, thus affecting cell growth and division (Park and Uehara, 2008).

In *E. coli*, various mutations in the *ampC* promoter/attenuator region, in *ampG*, in *ampD* or in *ampR* genes may lead to constitutive overproduction of AmpC beta-lactamases (Kopp et al., 1993; Caroff et al., 1999; Kuga et al., 2000). We have amplified and sequenced the *ampC* and *ampG* genes of Carb25 adapted bacteria and no mutation was detected in *ampC* promoter region, in *ampC* gene or in *ampG* genes, suggesting that carbenicillin adaptation leads to AmpC overproduction (Fig. 7b) by mutation in other regulatory elements, such as *ampR* or *ampD* but not in *ampG*. In this case, we can suppose that in Carb25 adapted bacteria, irradiation stress is important to induce the positive regulatory mechanism involving AmpG. In absence of this stress, AmpC overexpression maybe is favored especially by AmpR and/or AmpD.

In conclusion, the results of this study clearly demonstrate that in addition to their contribution in antibiotic resistance, *ampC* and *ampG* genes modulate the resistance of *E. coli* O157:H7 to γ-irradiation and may be to other stress like oxidative, cold and heat stress. Transcriptomic studies of Δ*ampC* and sequencing of Carb25 complete genome could be assessed to confirm respectively the hypothesis of the role of *ampC* in inducing radioresistance by the control of the peptidogycan recycling and to determine the genes involved in carbenicillin adaptation in *E. coli* O157:H7. These results can be of great interest to the food industry and especially for irradiation treatment of meat from β-lactam treated animals, which could affect the efficiency of irradiation treatment. Since kanamycin did not show any effect on the radioresistance, it will be important to investigate the effects of additional antibiotic classes currently used in animal production to verify their effects on the radioresistance or resistance to other stress of *E. coli* O157:H7.

Declarations

Author contribution statement

Ghizlane Gaougaou, Yosra Ben-Fadhel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Eric Deziel, Monique Lacroix: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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