TITLE: Global transcriptional response of *Escherichia coli* MG1655 cells exposed to the oxygenated monoterpenes citral and carvacrol

Authors: Beatriz Chueca, Elisa Pérez-Sáez, Rafael Pagán, Diego García-Gonzalo

Affiliation: Tecnología de los Alimentos. Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón (IA2), Universidad de Zaragoza-CITA, c/ Miguel Servet 177, 50013. Zaragoza, Spain

Corresponding author: Diego García-Gonzalo

E-mail: Diego.Garcia@unizar.es

Address: Dpto. PACA. Facultad de Veterinaria. Universidad de Zaragoza.

C/ Miguel Servet, 177, 50013, Zaragoza, Spain.

Phone number: 34-976-761581

Fax. number: 34-976-761590

Abstract

DNA microarrays were used to study the mechanism of bacterial inactivation by carvacrol and citral. After 10-min. treatments of *Escherichia coli* MG1655 cells with 100 and 50 ppm of carvacrol and citral, 76 and 156 genes demonstrated significant transcriptional differences ($p \leq 0.05$), respectively. Among the up-regulated genes after carvacrol treatment, we found gene coding for multidrug efflux pumps (*acrA*, *mdtM*), genes related to phage shock response (*pspA*, *pspB*, *pspC*, *pspD*, *pspF* and *pspG*), biosynthesis of arginine (*argC*, *argG*, *artJ*), and purine nucleotides (*purC, purM*). In citral-treated cells, transcription of *purH* and *pyrB* and *pyrI* was 2 times higher. Deletion of several differentially expressed genes confirmed the role of *ygaV, yjbO, pspC, sdhA, yejG* and *ygaV* in the mechanisms of *E. coli* inactivation by carvacrol and citral.

These results would indicate that citral and carvacrol treatments cause membrane damage and activate metabolism through the production of nucleotides required for DNA and RNA synthesis and metabolic processes. Comparative transcriptomics of the response of *E. coli* to a heat treatment, which caused a significant change of the transcription of 1,422 genes, revealed a much weaker response to both individual constituents of essential oils (ICs).Thus, inactivation by citral or carvacrol was not multitarget in nature.

Highlights:

- Transcriptome responses to mildly lethal IC treatments were determined.
- ICs treatments activate DNA and RNA synthesis.
- ICs lack multitarget inactivation.

Keywords: essential oils, *Escherichia coli* MG1655, transcriptome, stress response, heat treatment.

Abbreviations: IC, individual constituent of essential oils; **EO,** essential oil; **PEF**, pulsed electric fields; **TSBYE**, tryptic soy broth with yeast extract; **CFU**, colony-forming units; **MIC**, minimum inhibitory concentration; **TSAYE**, tryptic soy agar with yeast extract; **SC**, sodium chloride; **BS**, bile salts; **GO**, gene ontology; **HSP,** heat shock protein.

1 Introduction

Bacterial inactivation with the purpose of guaranteeing commercial food safety and stability is generally achieved by heat treatments. Despite being the benchmark technology for microbial inactivation, heat treatments have a negative effect on food properties and quality; thus, alternatives are being studied. Individual constituents of essential oils (ICs) have been proposed for use in the food industry due to their natural origin: consumers increasingly prefer foods that resemble fresh products as much as possible, since the latter are appreciated for their enhanced natural appeal and perceived nutritional quality (Gorris and Smid, 2007). Because of the high concentration of ICs required to achieve significant microbial inactivation and the resulting undesirable changes in flavor, essential oils (EOs) and their ICs are normally utilized to inhibit microbial growth rather than to kill microbial cells. In addition, their combination, at small concentrations, with heat has been proposed to confer a synergistic lethal effect, thereby avoiding the nutritional and organoleptic changes that heat otherwise tends to produce (Ait-Ouazzou et al., 2011; Ait-Ouazzou et al., 2013; Espina et al., 2014; Espina et al., 2012).

Citral and carvacrol belong to the group of oxygenated monoterpenes. They are common constituents of many EOs, and they have been extensively studied as antimicrobial agents (Ait-Ouazzou et al., 2011; Ben Arfa et al., 2006; Burt, 2004). However, their mechanisms of microbial inactivation have not been elucidated in full. In general, the main action of these lipophilic compounds seems to be related to a direct interaction with the hydrophobic regions of membrane proteins and protein complexes (Sikkema et al., 1994). In this regard, Ait- Ouazzou et al. (2011) and Somolinos et al. (2010) detected sublethal injuries in bacterial envelopes as a consequence of a treatment with carvacrol or citral. Likewise, Ultee et al. (1998) and Somolinos et al. (2010) showed that carvacrol and citral increased cell membrane permeability. Previous research has not only described an ROS-dependent mechanism in the face of bactericidal antibiotics (Kohanski et al., 2007) and of the IC (+)-limonene (Chueca et al., 2014a), but also an ROS-dependent mechanism leading to *Escherichia coli* death after citral and carvacrol

75 treatments (Chueca et al., 2014b). However, unlike the mechanisms of (+)-limonene and
76 bactericidal antibiotics, citral and carvacrol-mediated bacterial death was independent of the
77 Fenton reaction and the tricarboxylic acid (TCA) cycle. Nevertheless, in-depth studies would be
78 needed to better describe cell response to these ICs. It would be helpful to elucidate the
79 molecular details of inactivation by antimicrobials not only to further understand bacterial
80 resistance mechanisms, but also to improve the efficacy of preservation processes designed to
81 provide safer food with an extended shelf life.
82 Recent techniques enable scientists to gain knowledge about global gene expression. Among
83 them, transcriptomics based on DNA microarrays have brought about profound changes in the
84 study of microbial physiology (Wecke and Mascher, 2011). Comparison of gene RNA
85 transcripts obtained from bacterial cells before and after citral or carvacrol exposure would
86 show sets of genes either up-regulated or down-regulated by the treatments, thereby possibly
87 revealing more about cell resistance mechanisms and/or the regulatory networks that coordinate
88 bacterial stress response (Jordan et al., 2008). This approach has been successfully used to
89 describe the mode of action of many antibiotics (Wecke and Mascher, 2011) as well as the
90 mechanisms of bacterial adaptation and inactivation in connection with food processing
91 technologies such as heat (Guernec et al., 2013; Gunasekera et al., 2008), high hydrostatic
92 pressure (Bowman et al., 2008), chlorine dioxide (Pleitner et al., 2014), pulsed electric fields
93 (PEF) (Chueca et al., 2015) and the IC cinnamaldehyde (Visvalingam et al., 2013).
94 A recent study (Brauner et al., 2016) proposed a classification of bacterial survival strategies by
95 applying three concepts: resistance, which allows a microorganism to grow in the constant
96 presence of an antibiotic at lower concentrations; tolerance, which permits a microorganism to
97 survive high antibiotic concentrations but only during a limited treatment duration; and
98 persistence, similar to tolerance but effective over a longer duration of treatment. That threefold
99 perspective can be further explored by separately evaluating the role of over-expressed genes
100 detected in the microarray analysis and verifying if their contribution to the cell's defense and
101 survival follows a resistance, a tolerance or a persistence strategy when ICs are applied.

The objective of this study was to investigate – via microarray hybridization – the global transcriptional pattern of *E. coli* MG1655 upon exposure to citral or carvacrol treatments. Furthermore, to improve our understanding of the mechanism of bacterial inactivation by citral and carvacrol, we also compared our results with *E. coli* transcriptomic response to lethal heat treatments. As a last step, we evaluated the role of over-expressed genes in microbial resistance and tolerance to citral and carvacrol.

2 Materials and Methods

2.1 Micro-organisms and growth conditions

The strain used for the transcriptomic assays was *Escherichia coli* MG1655. The Keio collection (Baba et al., 2006) strains used in this study were parental strain BW25113 and single-gene deletion mutants Δ*arcA*, Δ*argC*, Δ*argG*, Δ*artJ*, Δ*inaA*, Δ*mdr*, Δ*ndh*, Δ*pspA*, Δ*pspB*, Δ*pspC*, Δ*pspD*, Δ*purC*, Δ*purM*, Δ*ygaV* and Δ*yjbO* for carvacrol resistance experiments; and Δ*purH*, Δ*pyrB*, Δ*pyrI*, Δ*sad/nusB*, Δ*sdhA*, Δ*yaaX*, Δ*ybiJ*, Δ*ybiM*, Δ*yejF*, Δ*yejG*, Δ*ygaV* and Δ*yneI* for citral treatments. Keio collection strains for post-transcriptional assays were supplied by the Japanese National Institute of Genetics. The cultures were maintained in a cryovial at -80°C.

Broth subcultures were prepared by inoculating, with one single colony from a plate, a test tube containing 5 mL of sterile Tryptic Soy Broth (Oxoid, Basingstoke, Hampshire, England) with 0.6% Yeast Extract added (Oxoid) (TSBYE). After inoculation, the tubes were incubated overnight at 37°C. With those subcultures, 250-mL Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final concentration of 10^4 colony-forming units (CFU)/mL. The flasks were incubated under agitation (130 rpm) (Selecta, mod. Rotabit, Barcelona, Spain) at 37°C until the stationary growth phase was reached (24 h / 2×10^9 CFU/mL).

2.2 Bacterial treatments with citral and carvacrol

Citral (95%) and carvacrol (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Following the procedure described by Friedman et al. (2002), a vigorous shaking method was used to prepare antimicrobial compound suspensions. Prior to treatment, microorganisms were likewise centrifuged at 6000 × *g* for 5 min and resuspended to a final concentration of approximately 1 × 10^7 CFU/mL in McIlvaine citrate-phosphate buffer of pH 4.0 with the corresponding concentrations of citral and carvacrol added. Experiments began at room temperature (22±2ºC). Control condition was stablished as bacterial cells held in the same pH 4.0 buffer for 10 min. These reference cells were not inactivated during the treatment. After treatment, 0.1 mL samples were used to count survivors.

For transcriptome evaluation, samples were treated with 50 and 100 ppm of citral and carvacrol, respectively. Immediately after treatment, 10 mL samples were used to extract RNA.

2.3 Minimum Inhibitory Concentration (MIC)

MIC against *E. coli* MG1655 was determined for carvacrol and citral by the tube dilution method with an initial concentration of 10^5 CFU/mL (Rota et al., 2004). The highest and lowest concentrations tested were 2,500 and 50 ppm of ICs respectively. For ICs, we also prepared negative controls containing TSBYE plus 2,500 ppm of ICs, and positive controls containing TSBYE with microorganisms at a final concentration of 10^5 CFU/mL. After 24 h incubation at 37°C, the MIC was determined as the lowest concentration of each IC in the presence of which bacteria failed to grow, i.e. at which no visible changes could be detected in the broth medium (Clinical and Laboratory Standards Institute, 2012).

2.4 Heat treatments

For the transcriptomic assay, a heat treatment was carried out in an incubator (FX Incubator, mod. ZE/FX, Zeulab, Zaragoza, Spain) at 48°C, with a thermocouple (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany) to monitor temperature during treatment. Once temperature had

154 stabilized, 50 μL of a diluted cell suspension was added to a sterile tube containing 450 μL of
155 McIlvaine citrate-phosphate buffer of pH 4.0. The initial bacterial concentration was
156 approximately 2×10^8 CFU/mL. Control condition was stablished as bacterial cells held in the
157 same pH 4.0 buffer for 10 min. These reference cells were not inactivated during the treatment.
158 After 10 min, 0.5 mL samples were immediately used to extract RNA, or 0.1 mL samples were
159 used for purposes of survivor enumeration.
160
161 2.5 Counts of viable and sublethally injured cells
162 The bacterial cells' physiological response was investigated by counting viable cells. After
163 respective treatments, samples were diluted in Phosphate Buffered Saline, pH 7.3 (PBS; Oxoid).
164 Then 0.1 mL samples were pour-plated onto Tryptic Soy Agar (Oxoid) with 0.6% Yeast Extract
165 added (Oxoid) (TSAYE). Treated samples were also plated on TSAYE with 3% (MG1655) or
166 4% (BW25113) of sodium chloride (Panreac, Barcelona, Spain) added (TSAYE-SC) and 0.1%
167 (MG1655) or 0.2% (BW25113) of bile salts (Oxoid) added (TSAYE-BS), to evaluate
168 cytoplasmic membrane damage and outer membrane damage, respectively (Mackey, 2000).
169 These concentrations corresponded to the pre-determined maximum non-inhibitory sodium
170 chloride and bile salt concentrations for native cells (data not shown).
171 Plates were incubated at 37°C for 24 h (TSBYE) or 48 h (TSBYE-SC and TSBYE-BS).
172 Previous experiments showed that longer incubation times had no influence on survival counts.
173 After plate incubation, the colonies were counted with an improved image analyzer automatic
174 counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom), as described in
175 previous research (Condón et al., 1996).
176 Inactivation was expressed as the difference between $\log_{10}$ counts before and after each
177 treatment. The extent of sublethal injury was expressed as the difference between $\log_{10}$ counts
178 on a non-selective medium (TSAYE) and $\log_{10}$ counts on selective media (TSAYE-SC and
179 TSAYE-BS). The error bars in the figures indicate the mean ± standard deviations from the data

obtained from at least 3 independent experiments carried out with different microbial cultures. ANOVA and *t*-tests were performed with GraphPad PRISM® (GraphPad Software, Inc., San Diego, USA) and differences were considered significant if $p \leq 0.05$.

The treatment conditions we selected for both heat and ICs were designed in view of a specific criterion. The goal was to have treatment conditions that would ensure that a level of bacterial cell inactivation of less than 50% could be attained in 10 min. Thus we could obtain a transcriptomic response under conditions that can be considered truly lethal, but within a limited amount of time to ensure that most cells were still alive.

2.6 RNA isolation, labeling and hybridization

RNA samples were obtained from untreated and treated cultures that were prepared as described above. Cultures were immediately pelleted by centrifugation at 6000 × g for 5 min. Three biological replicates were performed under identical conditions. RNA was isolated using the RNeasy Kit (Qiagen). The quality of the isolated RNA was examined by Tape Station using the R6K ScreenTape Kit (Agilent). All samples delivered RNA Integrity Number (RIN) values of >9.

The labeling and hybridization was performed as per the Agilent Two-Color Microarray-Based Prokaryote Analysis Fair Play III Labeling Protocol v. 1.3 by Bioarray SL (www.bioarray.es). Labeled cDNAs were hybridized on Agilent's *E. coli* Microarray Kit 8x15K, ID 020097. Hybridization and subsequent washing of the slides was performed according to the manufacturer's instructions.

The microarray slides were scanned using an Agilent microarray scanner (G2505C), and data were processed with Agilent's Feature Extraction software (v. 10.7).

2.7 Analysis of gene expression

Data were normalized using the *Tquantile* method to allow comparison among all arrays. Multiple testing correction was performed using Benjamini and Hochberg correction

(Benjamini and Hochberg, 1995). Spots with an adjusted *p*-value ≤ 0.05 were considered statistically significant, and the false positive rate remained at less than 5%. Expression levels obtained from 3 independent biological replicates of the treated samples and 4 independent biological replicates of the untreated samples were compared using the Limma package (Smyth, 2004) included in the Bioconductor software (Gentleman et al., 2004). A functional grouping of genes was carried out according to the data from the NCBI (http://www.ncbi.nlm.nih.gov/COG/) using the GOStats package included in the Bioconductor software. Genes are associated to their biological functions through Gene Ontology (GO), which also provides the information about other genes cooperating in such functions. Thus, GO is a useful tool for exploiting the existence of sets of genes involved in a certain cellular process (Coronnello et al., 2016). The expression of the 15 most up-regulated genes for each IC was verified via qPCR (Bioarray SL).

220 **3 Results and Discussion**

221 3.1 Bacterial inactivation with carvacrol, citral and heat treatments

222 To select adequate lethal treatment conditions to be applied before the RNA extraction,
223 inactivation of *E. coli* MG1655 under different concentrations of carvacrol and citral and under
224 different temperatures was evaluated. Since ICs have been proposed to pasteurize low pH food
225 such as fruit juices, the pH of the treatment media was adjusted to a 4.0 value to elucidate the *E.*
226 *coli* response to these ICs under acid conditions (Ait-Ouazzou et al., 2013).

227 The *E. coli* cells were treated for 10 min, a duration selected to obtain transcriptomic data
228 reflecting the immediate response to a lethal treatment with either ICs or heat and allowing, at
229 the same time, for the activation of bacterial stress response systems. Under these treatment
230 conditions, cell inactivation determined in nonselective medium was ca. 0.2 $\log_{10}$ cycles with
231 respect to the initial population after carvacrol treatments (Fig.1A) and ranged from 0.16 to 0.66
232 $\log_{10}$ cycles with respect to the initial population after citral and heat treatments (Figs. 1B and
233 1C). A differential plating technique with selective and nonselective media revealed a
234 population of damaged cells developing a global transcriptional response to the treatments
235 applied. Recovery of cells in a selective medium with sodium chloride (Mackey, 2000) showed
236 a population with sublethal injuries in their cytoplasmic membranes only after heat treatments
237 ($p \leq 0.05$) (Fig. 1C). However, repairable damage in the outer membranes, revealed by recovery
238 in the presence of bile salts, was observed in the wake of the application of all three food
239 preservation technologies herein evaluated (Fig. 1). As previously demonstrated for carvacrol
240 (Ait-Ouazzou et al., 2013), the degree of sublethal injuries to the outer membrane of *E. coli* was
241 greater than to the cytoplasmic membrane, suggesting that damage occurred firstly on the outer
242 membrane. Heat treatment caused outer membrane damage to more than 99 % of survivors (Fig.
243 1C); however, only less than 90 % of survivors showed damages on their outer membranes after
244 carvacrol and citral treatments, at least under the conditions tested in this study (Figs. 1A and
245 1B).

One must bear in mind that the most adequate treatment conditions for this transcriptomic analysis would be those targeting a high fraction (≥90%) of the initial cell population but allowing cells to develop a stress response, i.e. high cell survival in conjunction with a large proportion of sublethally injured cells. Thus, treatments with over 50% of inactivated cells were discarded. Finally, we chose to apply 10 min-treatments of 100 ppm carvacrol (45% of inactivated cells and 60% of the surviving population with damages to their outer membranes), 50 ppm citral (30% of inactivation and 15% of survivors with damages to their outer membranes) and heat at 48°C (leading to 50% of inactivation and 98% of survivors injured in their outer membranes).

3.2 Global transcriptional response in carvacrol and citral-treated cells

To determine the effect of carvacrol and citral on the transcriptome of *E. coli* MG1655, for which the complete genome sequence is available (Blattner et al., 1997), DNA microarrays were used to compare transcript levels within stationary-phase bacterial cells subjected to a lethal IC condition at pH 4.0 for 10 min with regard to bacterial cells held in the same media for 10 min without any additional treatment (control cells). Control cells were not inactivated during the treatment.

Statistical analysis of DNA microarrays indicated that, following carvacrol treatment (100 ppm for 10 min), 76 genes displayed transcriptional differences with an adjusted p-value ≤ 0.05, as compared with control cells. The significance and differences in transcript levels for all genes are depicted as a volcano plot (Fig. 2A). The upper corners of the graph show genes with both large fold changes and statistical significance. It was considered that genes with a negative value of $\log_2$ fold change were repressed with regard to control conditions, while the transcription of those genes with a positive value was activated by carvacrol. Of the 76 genes differentially expressed, 61 genes were up-regulated, while 15 were down-regulated after carvacrol treatment as compared to control cells (Fig. 2A).

Regarding the treatment with citral, 156 genes were transcribed differently than in the control

273 treatment ($p \leq 0.05$), only 27 of which were up-regulated (Fig. 2B). As for the greater number of
274 genes showing transcriptional differences, the citral treatment seems to activate more metabolic
275 pathways in bacterial cells than carvacrol. However, if we take into account the *p*-value and the
276 magnitude of up-regulation, two very different response patterns are shown: the treatment with
277 carvacrol caused a more significant response and higher levels of over-expression of several
278 genes (Fig. 2). Thus, those up-regulated genes might play an important role in bacterial cell
279 survival to carvacrol, and should be carefully examined.

280 Living organisms respond to stressful environmental conditions by redirecting protein synthesis
281 to alleviate cell damage (Harcum and Haddadin, 2006). As a consequence, transcription of
282 genes involved in cell response and repair is stimulated, while those not involved in those
283 functions, such as genes with roles in cell division machinery, are usually down-regulated. Up-
284 regulated genes after carvacrol treatment with available information about their function include
285 multidrug efflux pumps (*acrA, mdtM*), genes related with phage shock response (*pspA, pspB,*
286 *pspC, pspD, pspF* and *pspG*), biosynthesis of arginine (*argC, argG, artJ*) and purine nucleotides
287 (*purC, purM*), and transport of galactonate (*dgoR*) and tryptophan (*mtr*) (Table 1). Gene classes
288 are usually based on GO categories. Arginine biosynthetic process, purine nucleotide
289 biosynthetic process and dicarboxylic acid biosynthetic process are the final GO category for
290 the ontology trees constructed with the up-regulated genes after carvacrol treatment.
291 *acrA* is one of a network of genes that are believed to play a role in promoting the stress-
292 induced mutagenesis response in *E. coli* K-12 (Al Mamun et al., 2012). It has also been linked
293 with antibiotic resistance, since experiments carried out with a mutant lacking the *acrA* gene
294 showed a hypersensitivity to a high number of antibiotics (Liu et al., 2010). Visvalingam et al.
295 (2013) also described an activation of *acrA* expression after cinnamaldehyde challenge. The
296 other up-regulated gene corresponding to a multidrug efflux pump, *mdtM*, acts in synergy with
297 the AcrAB/TolC multidrug efflux system to provide enhanced efflux of unconjugated bile salts
298 in *E. coli* (Paul et al., 2014). Therefore data show a correlation between genes associated with

299 multidrug efflux pumps in aiding *E. coli* cells to cope with carvacrol, as is the case for
300 antibiotics and cinnamaldehyde.
301 The majority of up-regulated genes after a carvacrol treatment belonged to the group of phage
302 shock proteins (*psp*), a system that responds to extracytoplasmic stress (Darwin, 2005) and is
303 induced by dissipation of the proton motive force caused by secretin production (Darwin, 2005;
304 Darwin and Miller, 2001), or after exposure to hydrophobic organic solvents (Kobayashi et al.,
305 1998). In relation with the previously described up-regulated gene *acrA*, Jovanovic et al. (2006)
306 have proposed that ArcB, the sensor of the ArcAB system, is required for *psp* expression.
307 Regarding the action of *psp* response, it has been demonstrated that PspA binds to membrane
308 phospholipids and repairs proton leakage of damaged membranes in *E. coli* (Kobayashi et al.,
309 2007).
310 On the other hand, the up-regulated genes related with the biosynthesis of arginine are classified
311 inside a GO category which statistical study of our transcriptomic assay revealed to be an up-
312 regulated GO functional group. Arginine, along with lysine, constitutes one third of the residues
313 composing a segment of ion channels. Monoterpenes, such as carvacrol, affect several
314 physicochemical properties of lipid bilayer membranes and they act, similarly to other lipophilic
315 compounds, as allosteric modulators of several ion channels (Oz et al., 2015). Cell envelope
316 damage can be observed after carvacrol treatment of *E. coli*: therefore *psp* response could be
317 induced after detection of injuries on ion channels (that would affect the proton motive force
318 and activate the arginine biosynthetic process), and/or after detection of membrane damage by
319 ArcB (which would cause the activation of both *psp* response and *mdtM* efflux pump). These
320 results demonstrate that carvacrol-treated cells activate a response involving components and
321 functions directly associated with the membranes, which are considered one of the main targets
322 of carvacrol treatments (Ait-Ouazzou et al., 2011; Sikkema et al., 1994; Ultee et al., 1998).
323 In the citral-treated samples, transcription of *purH* (purine biosynthesis) and *pyrB* and *pyrI*
324 (pyrimidine biosynthesis) increased more than 2-fold (Table 2). These results would indicate
325 that cell response involves activation of metabolism, since nucleotides are needed to synthesize

326 DNA and RNA for catabolic processes. 11 of the 25 most up-regulated genes were hypothetical
327 proteins with unknown function. Among them, *ybiJ* is the gene showing the highest expression:
328 nearly 10-fold higher after citral treatment. In DNA repair-deficient cells, *ybiJ* was over-
329 expressed, as well as genes corresponding to SOS response, cellular efflux and oxidative stress;
330 the whole can be regarded as a gene regulation strategy to avoid mutations and cell death
331 induced by chlorambucil. Also, Wurpel et al. (2014) identified the protein YbiJ in artificially
332 induced outer membrane vesicles from uropathogenic *E. coli*. Thus, *ybiJ* over-expression could
333 indicate damage to cell envelopes, as well as to DNA, caused by citral.
334 Therefore, our results would support the idea that bacterial envelopes are a main target of
335 carvacrol, along with the existence of an active metabolism triggered in the cell to cope with
336 citral treatment. Comparison of our results with the transcriptome of carvacrol- and citral-
337 treated bacterial cells under different pH treatment media conditions and/or grown in the
338 treatment medium might bring further insights into the mechanism of bacterial inactivation by
339 these ICs.
340 As shown in Table 3, four up-regulated genes were observed after both treatments, among
341 which *ygaV* showed the highest expression. Interestingly, *ygaVP* codes for a protein that is
342 apparently membrane-associated.
343 Unlike the transcriptomic response observed by Chueca et al. (2015) in PEF, no transcriptional
344 differences in genes of acid shock response were observed between the control and treated
345 samples with ICs carvacrol and citral. Thus, the IC treatments used in this study would not
346 impair normal cell response to acidic environment. Interestingly enough, there were up-
347 regulated genes by IC treatments related to the response of *E. coli* to low pH, such as the case of
348 *sad/yneI* after citral treatment (Kannan et al., 2008) and *inaA* after carvacrol treatment, a gene
349 dually regulated by the multiple antibiotic resistance (Mar) and superoxide (SoxRS) stress
350 response systems (Rosner and Slonczewski, 1994). This would be consistent with the oxidative
351 damage by carvacrol described by Chueca et al. (2014b). Although these compounds caused
352 extensive oxidative damages in growing *E. coli* cells when treated in a growth medium (Chueca

et al., 2014b), activation of oxidative stress response by these compounds was not detected in the present study. It might be possible that absence of oxidizable substrates in treatment buffer and/or cell physiological state could affect presence of oxidative damages. Further transcriptome analysis at pH 7.0 would help us compare citral and carvacrol with cinnamaldehyde, since the latter compound caused a repression of acid resistance genes associated with an increase in antibiotic resistance (Visvalingam et al., 2013).

3.3 Comparison of transcriptomics in carvacrol- and citral-treated vs. heat-treated cells

Given the industrial relevance of heat as a food preservation technology, its mechanism of inactivation has been well studied (Gould, 1989), as well as bacterial transcriptome after sublethal (Carruthers and Minion, 2009; Gunasekera et al., 2008; Harcum and Haddadin, 2006) and lethal stresses (Guernec et al., 2013). To compare cell responses after IC and heat treatments under our selected conditions (10 min, pH 4.0), transcriptomics after the carvacrol and citral treatments were compared with those following a heat treatment that inactivated half of the bacterial population and caused damage in the outer membrane of the majority of survivors (≥98%). As described above, the treatments with carvacrol and citral had caused 45% and 30% of inactivation and damage to the outer membrane of 60% and 15% of the surviving population, respectively. In contrast with IC treatments, heat treatment caused the differential expression of 1,422 genes, 508 of which were up-regulated, compared with only 61 and 27 up-regulated genes in carvacrol and citral-treated cells (Fig. 3). This was similar to what was observed by Chueca et al. (2015) in the comparison with PEF, where only 20 genes were up-regulated. Thus, the extensive cell response caused by heat treatments (and, as a result, the structures targeted by heat) remained greater, not only when compared with PEF treatments, but also with carvacrol and citral treatments.

IC inactivation, especially by carvacrol, seemed to be location-specific and related exclusively to cell envelopes, as likewise observed for PEF (Chueca et al., 2015). This contrasts with the multitarget damage caused by heat, which affects ribosomes, membranes, enzymes and DNA

(Gould, 1989).

As previously discussed, the extense of damage to the outer membrane was greater (Fig. 1) in the case of heat treatment than in carvacrol and citral treatments. This would confirm the relevance of bacterial envelopes in the process of cell recovery after stress from damage caused by food preservation technologies, since injured cells activate pathways to overcome the stress and repair the damages. Cells that have already been subjected to a heat treatment could thereby develop cross-resistance to subsequent stresses (Hengge, 2011).

Table 4 shows the genes most up-regulated after a lethal heat treatment. There were 5 up-regulated GO categories in the heat-treated cells: protein folding, response to temperature stimulus, glucose metabolic process, response to drug and pyridine-containing compound metabolic process. “Protein folding” was expected as a consequence of heat treatment, which lead to an increase in unfavorable protein interactions such as misfolding and aggregation. Genes belonging to this category included those encoding major chaperone subunits (*groL, groS, dnaK, dna* and *grpE*), heat shock proteins (HSP) (*clpB, htpG*) and proteases (*clpX* and *hslV*). Guernec et al. (2013) ignored most of these genes in their microarray analysis due to the high intensity of their fluorescent signal. Nevertheless, they performed an evaluation of *dnaK* and *groEL* by qPCR, which revealed their increased transcription. Another GO category among up-regulated genes was “response to temperature stimulus” with HSP (*degP, hslR, htpX, ibpA* and *ibpB*) and σ factors (*rpoE, rpoH* and *rseA*). The GO categories “glucose metabolic process”, “response to drug” and “pyridine-containing compound metabolic process” included genes with less change in expression than those of the two previously mentioned categories. After treatments with carvacrol and, even more so, with citral, gene expression (Fig. 2) as well as membrane damage (Fig. 1) were lower than those observed after lethal heat treatments. Therefore, as already propounded in the case of PEF (Chueca et al., 2015), citral and carvacrol would likewise be two further compounds of great value in the avoidance of general stress cell response and resistance of bacteria in combined processes with heat.

Regarding the GO categories of differentially expressed genes, no categories were common to

all three treatments (carvacrol, citral and heat). As previously mentioned, carvacrol and heat shared certain up-regulated genes related to membrane stress, such as *pspB* and *pspG* (Table 5). As observed in the Venn diagram (Fig. 3), only one gene was down-regulated after the three treatments: *yghA*, an aldehyde reductase that contributes to the degradation of desired aldehyde end products of metabolic engineering (Rodriguez and Atsumi, 2014).

3.4 Response to carvacrol and citral exposure of single-deletion mutants for up-regulated genes

To evaluate the individual contribution of up-regulated genes, resistance to carvacrol and citral was tested in single-gene deletion mutants from the Keio collection (with BW25113 as parental strain). Main differences in BW25113's genotype in relation to MG1655's are: a) the deletion of *araBAD* and *rhaDAB*, b) replacement of a section of *lacZ* with four tandem *rrnB* terminators, and c) a frameshift mutation in *hsdR* which introduces a stop codon (Grenier et al., 2014). Notably, there were no differences between strain MG1655 and BW25113 affecting genes identified as showing transcription changes in response to the treatments applied.

Conditions for lethal treatments allowing an adequate discernment of mutants with statistically higher ($p \leq 0.05$) inactivation than the parental strain were selected. Cells were recovered in nonselective and selective media with sodium chloride and bile salts to detect cytoplasmic and outer membrane damage, respectively.

For genes up-regulated after carvacrol exposure, a lower ($p \leq 0.05$) cell envelope resistance to a 100 ppm carvacrol treatment during 10 min was observed in *ygaV, yjbO* and *pspC* mutants, compared to parental strain *E. coli* BW25113 (Fig. 4A). On the other hand, higher inactivation was not observed ($p > 0.05$) for up-regulated genes in mutants, when compared with BW25113, after a treatment of 200 ppm citral during 60 min. Only *ygaV* mutant was more resistant ($p \leq 0.05$) than parental strain when plated in selective recovery media added with bile salts, indicating a higher resistance of outer membrane in mutant cells against citral. This situation would indicate that whereas up-regulation of *ygaV* was correlated with citral treatment, it is not part of a protective/defensive response. Inactivation of the parental strain was around 1.5, 2 and

434 3.5 $\log_{10}$ cycles in non-selective and selective recovery media with sodium chloride and bile
435 salts, respectively (Fig. 4B). None of the mutant strains tested showed a statistically higher ($p >$
436 0.05) inactivation than BW25113. Surprisingly, after evidencing the MICs against carvacrol and
437 citral for their respective single-gene mutant strains, no differences were found for carvacrol,
438 whereas *sdhA, yejG* and *ygaV* mutants showed lower MIC against citral than the parental strain
439 (Table 6).
440 Brauner et al. (2016) described the difference between resistance, tolerance and persistence. Our
441 results reveal the role of *ygaV, yjbO* and *pspC* in *E. coli* survival to carvacrol as well as that of
442 *sdhA, yejG* and *ygaV* in *E. coli* growth with citral. Thus, although all of these genes were part of
443 *E. coli* response to exposure to those ICs as evidenced by transcriptomic analysis, they took part
444 through different mechanisms: one providing tolerance to carvacrol, the other enhancing
445 resistance of *E. coli* to citral. In addition, it might be possible that resistance and/or tolerance
446 against carvacrol and citral are mediated by more than one gene. Further experiments with
447 double (or multiple genes) knockout mutants would clarify this point.
448
449 **4 Conclusions**
450 To use ICs and/or EOs as natural antimicrobials for food preservation, it is important to know
451 the mechanisms by which these molecules exert their bactericidal power. This study is the first
452 to show quantitative information from the cellular transcriptome through microarray analysis of
453 stationary-phase *E. coli* after carvacrol and citral treatments. It should be noted that experiments
454 were carried out in an acid buffer. Therefore, further experiments to transcriptomic response of
455 cells in real food environments, such as in fruit juices, are needed.
456 After we selected treatments designed to damage almost the entire population, *E. coli* showed a
457 more significative transcriptomic response to the carvacrol than to the citral treatment. While
458 more genes were differentially expressed after citral treatment (156 genes) their statistical
459 differences placed them only in limit beyond $p \leq 0.05$. Carvacrol treatment resulted in 15 down-
460 regulated and 61 up-regulated genes, but with higher statistical reliability than those of citral.

Multidrug efflux pumps (*acrA*, *mdtM*), genes related with phage shock response (*pspA*, *pspB*, *pspC*, *pspD*, *pspF* and *pspG*), biosynthesis of arginine (*argC*, *argG*, *artJ*) and purine nucleotides (*purC*, *purM*) were the up-regulated genes that were most prominent after treatment with carvacrol.

Comparative transcriptomics of the response of *E. coli* to a heat treatment revealed a much weaker response to both ICs, indicating that inactivation by citral or carvacrol was not multitarget in nature.

On the one hand, deletion of *ygaV*, *yjbO* and *pspC*, three of the up-regulated genes after carvacrol treatments, caused a decrease in resistance of *E. coli* envelopes, as evidenced by the higher inactivation shown on selective recovery media with sodium chloride or bile salts. On the other hand, deletion of *sdhA*, *yejG* and *ygaV*, which were up-regulated after citral treatments, resulted in lower MIC for citral. Thus, the role of *ygaV*, *yjbO*, *pspC*, *sdhA*, *yejG* and *ygaV* in the mechanisms of *E. coli* inactivation by carvacrol and citral was confirmed.

This is an example of how transcriptomics can help expand knowledge in a field of increasing importance such as the use of EOs for minimally processed food. This paper may serve as a basis for future research into cell strategies, incorporating *a priori* knowledge of the mechanisms involved. In addition to evidencing damages in cell envelopes due to citral and carvacrol treatments, this project has also thrown light on the strategies deployed by the cells to survive low doses of citral and carvacrol.

5 Acknowledgements

This work was supported by CICYT (Spanish Interministerial Comission of Science and Technology, Projects AGL2012-32165 and AGL2015-69565-P), FEDER, the European Social Fund, from the Aragonese Office of Science, Technology and University Research; and the Spanish Ministry of Sports, Culture and Education: the latter provided B. Chueca with a grant to carry out this investigation. The authors also wish to thank Stanley Hanks (translator) for having revised and proofread the final version of this manuscript.

488

6 References

- Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., Pagán, R.,2011. The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. Innov Food Sci Emerg 12, 320-329.
- Ait-Ouazzou, Lorán, S., Arakrak, A., Laglaoui, A., Rota, C., Herrera, A., Pagán, R., Conchello, P. 2012. Evaluation of the chemical composition and antimicrobial activity of *Mentha pulegium, Juniperus phoenicea* and *Cyperus longus* essential oils from Morocco. Food Res Int 45(1), 313-319.
- Ait-Ouazzou, A., Espina, L., García-Gonzalo, D., Pagán, R., 2013. Synergistic combination of physical treatments and carvacrol for *Escherichia coli* O157:H7 inactivation in apple, mango, orange, and tomato juices. Food Control 32, 159-167.
- Ait-Ouazzou, A., Espina, L., Gelaw, T. K., de Lamo-Castellvi, S., Pagán, R., García-Gonzalo, D., 2013. New insights in mechanisms of bacterial inactivation by carvacrol. J Appl Microbiol 114, 173-185.
- Al Mamun, A. A., Lombardo, M. J., Shee, C., Lisewski, A. M., Gonzalez, C., Lin, D., Nehring, R.B., Saint-Ruf, C., Gibson, J.L., Frisch, R.L., Lichtarge, O., Hastings, P.J., Rosenberg, S.M., 2012. Identity and function of a large gene network underlying mutagenic repair of DNA breaks. Science 338, 1344-1348.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., Mori, H., 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2, 2006.0008.
- Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., Chalier, P., 2006. Antimicrobial activity of carvacrol related to its chemical structure. Lett Appl Microbiol 43, 149-154.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57, 289-300.

- Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., Shao, Y., 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277, 1453-1462.
- Bowman, J. P., Bittencourt, C. R., Ross, T., 2008. Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. Microbiol 154, 462-475.
- Brauner, A., Fridman, O., Gefen, O., Balaban, N. O., 2016. Distinguishing between resistance, tolerance and persistance to antibiotic treatment. Nat Rev Microbiol 14(5), 320-330.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol 94, 223-253.
- Carruthers, M. D., Minion, C., 2009. Transcriptome analysis of *Escherichia coli* O157:H7 EDL933 during heat shock. FEMS Microbiol Lett 295, 96-102.
- Chueca, B., Pagán, R., García-Gonzalo, D., 2014a. Differential mechanism of *Escherichia coli* inactivation by (+)-limonene as a function of cell physiological state and drug's concentration. PLOS ONE 9, e94072.
- Chueca, B., Pagán, R., García-Gonzalo, D., 2014b. Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction. Int J Food Microbiol 189, 126-131.
- Chueca, B., Pagán, R., García-Gonzalo, D., 2015. Transcriptomic analysis of *Escherichia coli* MG1655 cells exposed to pulsed electric fields. Innov Food Sci Emerg 29, 78-86.
- Clinical and Laboratory Standards Institute [CLSI], 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard–ninth edition. CLSI document M07-A9, Wayne, PA, Clinical and Laboratory Standards Institute.

- Condón, S., Palop, A., Raso, J., Sala, F. J., 1996. Influence of the incubation temperature after heat treatment upon the estimated heat resistance values of spores of *Bacillus subtilis*. Lett Appl Microbiol 22(2), 149-152.
- Coronnello, C., Tumminello, M., Miccichè, S., 2016. Gene-based and semantic structure of the Gene Ontology as a complex network. Phy A 458, 313-328.
- Darwin, A. J., 2005. The phage-shock-protein response. Mol Microbiol 57, 621-628.
- Darwin, A. J., Miller, V. L., 2001. The *psp* locus of *Yersinia enterocolitica* is required for virulence and for growth in vitro when the Ysc type III secretion system is produced. Mol Microbiol 39, 429-444.
- Espina, L., Somolinos, M., Ait-Ouazzou, A., Condón, S., García-Gonzalo, D., Pagán, R., 2012. Inactivation of *Escherichia coli* O157:H7 in fruit juices by combined treatments of citrus fruit essential oils and heat. Int J Food Microbiol 159, 9-16.
- Espina, L., Condón, S., Pagán, R., García-Gonzalo, D., 2014. Synergistic effect of orange essential oil or (+)-limonene with heat treatments to inactivate *Escherichia coli* O157:H7 in orange juice at lower intensities while maintaining hedonic acceptability. Food Bioprocess Tech 7, 471-481.
- Friedman, M., Henika, P. R., Mandrell, R. E., 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica*. J Food Protect 65, 1545-1560.
- Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling M., Dudoit S., Ellis B., Gautier L., Ge Y., Gentry J., Hornik K., Hothorn T., Huber W., Iacus S., Irizarry R., Leisch F., Li C., Maechler M., Rossini A. .J., Sawitzki G., Smith C., Smyth G., Tierney L., Yang J. Y., Zhang J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5(10), R80.

- Gorris, L. G. M., Smid, E. J., 2007. Natural antimicrobials for food preservation. In: Rahman, M. S. (Ed.), Handbook of Food Preservation, Second Edition, CRC Press, Boca Raton, Florida, pp. 237-254.
- Gould, G. W., 1989. Heat induced injury and inactivation. In: Gould, G. W. (Ed.), Mechanisms of action of food preservation procedures, Elsevier Applied Science, London, pp. 11-42.
- Grenier, F., Matteau, D., Baby, V., Rodrigue, S., 2014. Complete genome sequence of *Escherichia coli* BW25113. Genome Announc 2(5), e01038-14.
- Guernec, A., Robichaud-Rincon, P., Saucier, L., 2013. Whole-genome transcriptional analysis of *Escherichia coli* during heat inactivation processes related to industrial cooking. Appl Environ Microbiol 79, 4940-4950.
- Gueune, H., Durand, M. J., Thouand, G., DuBow, M. S., 2008. The *ygaVP* genes of *Escherichia coli* form a tributyltin-inducible operon. Appl Environ Microbiol 74, 1954-1958.
- Gunasekera, T. S., Csonka, L. N., Paliy, O., 2008. Genome-wide transcriptional responses of *Escherichia coli* K-12 to continuous osmotic and heat stresses. J Bacteriol 190, 3712-3720.
- Harcum, S. W., Haddadin, F. T., 2006. Global transcriptome response of recombinant *Escherichia coli* to heat-shock and dual heat-shock recombinant protein induction. J Ind Microbiol Biotechnol 33, 801-814.
- Hengge, R., 2011. The general stress response in Gram-negative bacteria. In: Storz, G., Hengge, R. (Eds.), Bacterial stress responses, Second Edition. ASM Press, Washington, DC, pp. 251–289.
- Jordan, S., Hutchings, M. I., Mascher, T., 2008. Cell envelope stress response in Gram-positive bacteria. FEMS Microbiol Rev 32, 107-146.

- Jovanovic, G., Lloyd, L. J., Stumpf, M. P., Mayhew, A. J., Buck, M., 2006. Induction and function of the phage shock protein extracytoplasmic stress response in *Escherichia coli*. J Biol Chem 281, 21147-21161.
- Kannan, G., Wilks, J. C., Fitzgerald, D. M., Jones, B. D., Bondurant, S.S., Slonczewski, J.L., 2008. Rapid acid treatment of *Escherichia coli*: transcriptomic response and recovery. BMC Microbiol *8*, 37.
- Kobayashi, H., Yamamoto, M., Aono, R., 1998. Appearance of a stress-response protein, phage-shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents. Microbiol 144 (Pt 2), 353-359.
- Kobayashi, R., Suzuki, T., Yoshida, M., 2007. *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. Mol Microbiol 66, 100-109.
- Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., Collins, J. J., 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130, 797-810.
- Liu, A., Tran, L., Becket, E., Lee, K., Chinn, L., Park, E., Tran, K., Miller, J.H., 2010. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother 54, 1393-1403.
- Mackey, B. M., 2000. Injured bacteria. In: Lund, B. M., Baird-Parker, T. C., Gould, G. W. (Eds.), The Microbiological Safety and Quality of Food, Aspen Publisher, Inc, Gaithersburg, Maryland, pp. 315-341.
- Oz, M., Lozon, Y., Sultan, A., Yang, K. H., Galadari, S., 2015. Effects of monoterpenes on ion channels of excitable cells. Pharmacol Ther 152, 83-97.
- Paul, S., Alegre, K. O., Holdsworth, S. R., Rice, M., Brown, J.A., McVeigh, P., Kelly, S.M., Law, C.J., 2014. A single-component multidrug transporter of the major

facilitator superfamily is part of a network that protects *Escherichia coli* from bile salt stress. Mol Microbiol 92, 872-884.

- Pleitner, A. M., Trinetta, V., Morgan, M. T., Linton, R. L., Oliver, H. F., 2014. Transcriptional and phenotypic responses of *Listeria monocytogenes* to chlorine dioxide. Appl Environ Microbiol 80, 2951-2963.
- Rodriguez, G. M., Atsumi, S., 2014. Toward aldehyde and alkane production by removing aldehyde reductase activity in *Escherichia coli*. Metab Eng 25, 227-237.
- Rosner, J. L., Slonczewski, J. L., 1994. Dual regulation of *inaA* by the multiple antibiotic resistance (*mar*) and superoxide (*soxRS*) stress response systems of *Escherichia coli*. J Bacteriol 176, 6262-6269.
- Rota, C., Carramiñana, J. J., Burillo, J., Herrera, A., 2004. *In vitro* antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. J Food Prot 67, 1252-1256.
- Salmelin, C., Vilpo, J., 2003. Induction of SOS response, cellular efflux and oxidative stress response genes by chlorambucil in DNA repair-deficient *Escherichia coli* cells (*ada*, *ogt* and *mutS*). Mut Res 522, 33-44.
- Sikkema, J., de Bont, J., Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem 269, 8022-8028.
- Smyth, G. K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Molec Biol 3, article 3.
- Somolinos, M., García, D., Condón, S., Mackey, B., Pagán, R., 2010. Inactivation of *Escherichia coli* by citral. J Appl Microbiol 108, 1928-1939.
- Ultee, A., Gorris, L. G., Smid, E. J., 1998. Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus*. J Appl Microbiol 85, 211-218.

643 • Visvalingam, J., Hernandez-Doria, J. D., Holley, R. A., 2013. Examination of the
644 genome-wide transcriptional response of *Escherichia coli* O157:H7 to cinnamaldehyde
645 exposure. Appl Environ Microbiol 79, 942-950.
646 • Wecke, T., Mascher, T., 2011. Antibiotic research in the age of omics: from expression
647 profiles to interspecies communication. J Antimicrob Chemother 66, 2689-2704.
648 • Wurpel, D. J., Totsika, M., Allsopp, L. P., Hartley-Tassell, L. E. Day, C.J., Peters,
649 K.M., Sarkar, S., Ulett, G.C., Yang, J., Tiralongo, J., Strugnell, R.A., Jennings, M.P.,
650 Schembri, M.A., 2014. F9 fimbriae of uropathogenic *Escherichia coli* are expressed at
651 low temperature and recognise Galbeta1-3GlcNAc-containing glycans. PLOS ONE 9,
652 e93177.
653

654 **Figure legends**

655 **Fig. 1.** Log_{10} cycles of inactivation of stationary phase cells of *Escherichia coli* MG1655 after
656 10-min treatments with 100 ppm of carvacrol (A), 50 ppm of citral (B) and heat treatment at
657 48°C (C) in citrate-phosphate buffer of pH 4.0. Survivors were recovered in non-selective
658 media (white bars) and in selective media with sodium chloride (grey bars) and bile salts (black
659 bars). Data are means±standard deviations (error bars).

660

661 **Fig. 2.** Volcano plot of transcriptional differences in *Escherichia coli* MG1655 after 10-min
662 treatments with 100 ppm of carvacrol (A) and 50 ppm of citral (B) in citrate-phosphate buffer of
663 pH 4.0. Individual differences are plotted as log_2 fold change vs. $-log_{10}$ adjusted *p*-value. Points
664 above the line at $p \leq 0.05$ indicate differential expression at a false discovery rate of 5%. The
665 number of up- or down- regulated genes ($p \leq 0.05$) is indicated.

666

667 **Fig. 3.** Comparison of levels of genome-wide expression in *Escherichia coli* MG1655 after 10-
668 min treatments with 100 ppm of carvacrol (CAR), 50 ppm of citral (CIT) and heat treatment at
669 48°C (HT) in citrate-phosphate buffer of pH 4.0. The number of differentially expressed genes
670 are shown as a Venn diagram. Quantities of up-regulated genes are shown in bold, and
671 quantities of down-regulated genes are shown in italics.

672

673 **Fig. 4.** Inactivation of stationary phase cells of *Escherichia coli* BW25113 after treatments with
674 100 ppm of carvacrol for 10 min (A) and 200 ppm of citral for 60 min (B) in citrate-phosphate
675 buffer of pH 4.0. Survivors were recovered in non-selective media (white bars) and in selective
676 media with sodium chloride (grey bars) and bile salts (black bars). Evaluated strains were the
677 parental strain BW25113 and its derived single-gene deletion mutants for the majority of up-
678 regulated genes revealed by transcriptomic analysis after IC treatments. Data are

679 means±standard deviations (error bars). * represents statistically significant differences ($p \leq$

680 0.05) between mutant strain and the parental strain for each recovery medium.

Tables

Table 1. Top 25 up-regulated genes (log_2-fold changes) in *Escherichia coli* MG1655 cells after a treatment with 100 ppm of carvacrol during 10 min. Microarray data were normalized, multiple testing correction was performed and spots with an adjusted *p*-value ≤ 0.05 were considered statistically significant.

Gene	bname	logFC	Description
acrA	b0463	1.87	acridine/multidrug efflux pump
argC	b3958	2.61	N-acetyl-gamma-glutamylphosphate reductase, NAD(P)-binding
argG	Z4534	2.00	argininosuccinate synthetase
artJ	b3243	2.71	arginine 3rd transport system periplasmic binding protein
cvpA	Z3575	1.65	membrane protein required for colicin V production
ECs4052	ECs4052	1.93	argininosuccinate synthetase
inaA	b2237	2.19	pH-inducible protein involved in stress response
mdtM	b4337	1.52	putative transport/multidrug efflux system protein
mtr	c_3914	2.96	Tryptophan-specific transport protein
ndh	b1109	1.74	respiratory NADH dehydrogenase 2/cupric reductase
pspA	b1304	4.57	regulatory protein for *psp* operon, inner membrane protein
pspB	b1305	4.42	DNA-binding transcriptional regulator of *psp* operon
pspC	b1306	3.21	DNA-binding transcriptional activator of *psp* operon
pspD	b1307	2.92	peripheral inner membrane phage-shock protein
pspF	b1303	1.60	*psp* operon DNA-binding transcriptional activator
pspG	b4050	4.63	phage shock protein G
purC	b2476	1.83	SAICAR synthetase
purM	b2499	2.01	AIR synthetase
ygaV	c_3216	1.75	hypothetical transcriptional regulator YgaV
yjbO	c_5019	4.54	hypothetical protein YjbO
znuC	b1858	1.52	zinc transporter subunit: ATP-binding component of ABC superfamily transport system
	c_1776	3.89	hypothetical protein
	c_0141	1.90	hypothetical protein
	c_0839	1.73	hypothetical protein
	b3695	1.50	regulator protein for *dgo* operon

687 **Table 2.** Top 25 up-regulated genes (log_2-fold changes) in *Escherichia coli* MG1655 cells after
688 a treatment with 50 ppm of citral during 10 min. Microarray data were normalized, multiple
689 testing correction was performed and spots with an adjusted *p*-value ≤ 0.05 were considered
690 statistically significant.

Gene	bname	logFC	Description
ECs0005	ECs0005	1.70	hypothetical protein
fadI	ECs3225	0.78	3-ketoacyl-CoA thiolase
fis	b3261	0.75	site-specific DNA inversion stimulation factor; DNA-binding protein; a trans activator for transcription
purH	Z5583	1.99	phosphoribosylaminoimidazolecarboxamide formyltransferase = AICAR formyltransferase IMP cyclohydrolase
pyrB	b4245	1.07	aspartate carbamoyltransferase, catalytic subunit
pyrI	b4244	1.41	aspartate carbamoyltransferase, regulatory subunit
rpmA	b3185	0.81	50S ribosomal subunit protein L27
ruvC	c_2277	0.79	crossover junction endodeoxyribonuclease
sad	b1525	1.44	putative aldehyde dehydrogenase
sdhA	Z0877	0.94	succinate dehydrogenase, flavoprotein subunit
vacJ	Z3610	0.80	lipoprotein precursor
yaaX	b0005	1.60	ORF, hypothetical protein
ybiJ	b0802	3.29	ORF, hypothetical protein
ybjM	b0848	1.43	predicted inner membrane protein
yejF	b2180	1.27	putative ATP-binding component of a transport system
yejG	b2181	1.28	ORF, hypothetical protein
ygaV	c_3216	1.72	hypothetical transcriptional regulator
yneI	c_1948	1.60	aldehyde-dehydrogenase like protein
Z0005	Z0005	1.60	ORF, hypothetical protein
Z1768	Z1768	1.14	unknown protein encoded by prophage CP-933N
Z5294	Z5294	0.87	ORF unknown function
	c_0008	1.31	hypothetical protein
	c_5343	1.01	hypothetical protein
	c_2891	0.87	hypothetical protein
	c_1908	0.86	hypothetical protein

691

692 **Table 3.** Common up-regulated genes (log_2-fold changes) in *Escherichia coli* MG1655 cells
693 after treatments with 100 ppm of carvacrol and with 50 ppm of citral during 10 min. Microarray
694 data were normalized, multiple testing correction was performed and spots with an adjusted *p*-
695 value ≤ 0.05 were considered statistically significant.

Gene	bname	logFC- carvacrol	logFC-citral	Description
rpmA	b3185	1.00	0.81	50S ribosomal subunit protein L27
yejF	b2180	0.94	1.27	putative ATP-binding component of a transport system
ygaV	c_3216	1.75	1.72	hypothetical transcriptional regulator
	c_1908	0.91	0.86	hypothetical protein

696

Table 4. Top 25 up-regulated genes (log_2-fold changes) in *Escherichia coli* MG1655 cells after heat treatment at 48°C during 10 min. Microarray data were normalized, multiple testing correction was performed and spots with an adjusted *p*-value ≤ 0.05 were considered statistically significant.

Gene	bname	logFC	Description
alaS	b2697	2.33	alanyl-tRNA synthetase
degP	b0161	2.11	periplasmic serine protease Do; HSP HtrA
dnaK	b0014	2.35	chaperone HSP70; DNA biosynthesis; autoregulated HSP
ECs3223	ECs3223	2.38	hypothetical protein
fxsA	b4140	2.42	ORF, hypothetical protein
greA	b3181	2.51	transcription elongation factor: cleaves 3 nucleotide of paused mRNA
groL	b4143	2.43	GroEL, chaperone HSP60, peptide-dependent ATPase
groS	b4142	3.07	GroES, 10 Kd chaperone binds to HSP60 in pres. Mg-ATP, suppressing its ATPase activity
gshA	b2688	3.86	gamma-glutamate-cysteine ligase
ibpA	b3687	3.39	HSP
ibpB	b3686	3.74	HSP
insG	b4278	3.33	IS4 hypothetical protein
sixA	b2340	2.21	ORF, hypothetical protein
smpA	b2617	2.76	small membrane protein A
uup	b0949	2.13	putative ATP-binding component of a transport system
ychS	b1228	2.93	ORF, hypothetical protein
yfeK	c_2954	3.17	hypothetical protein
yfeY	c_2966	3.16	hypothetical protein
Z3603	Z3603	2.40	ORF, hypothetical protein
	c_1257	3.50	putative conserved protein
	c_5225	3.05	hypothetical protein
	c_1016	2.74	hypothetical protein
	c_3703	2.71	transposase InsG for insertion sequence element IS4
	ECs1733	2.35	hypothetical protein
	c_0018	2.26	putative glutamate dehydrogenase

Table 5. Common up-regulated genes (log_2-fold changes) in *Escherichia coli* MG1655 cells after treatment with 100 ppm of carvacrol and heat treatment at 48°C during 10 min. Microarray data were normalized, multiple testing correction was performed and spots with an adjusted *p*-value ≤ 0.05 were considered statistically significant.

Gene	bname	logFC-carvacrol	logFC-heat	Description
pspB	b1305	4.42	0.80	DNA-binding transcriptional regulator of *psp* operon
pspG	b4050	4.63	1.94	phage shock protein G
yjbO	c_5019	4.54	1.72	hypothetical protein YjbO
	Z2476	1.18	0.86	ORF unknown function

707 **Table 6.** Minimal inhibitory concentration (MIC) (ppm) of citral and carvacrol in *Escherichia*

708 *coli* BW25113 and selected single-gene deletion strains.

Strains	Citral	Strains	Carvacrol
	MIC		MIC
BW25113	2,000	BW25113	300
Δ*purH*	2,000	Δ*arcA*	300
Δ*pyrB*	2,000	Δ*argC*	300
Δ*pyrI*	2,000	Δ*argG*	300
Δ*sad/nusB*	2,000	Δ*artJ*	300
Δ*sdhA*	**1,000**	Δ*inaA*	300
Δ*yaaX*	2,000	Δ*mtr*	300
Δ*ybiJ*	2,000	Δ*ndh*	300
Δ*ybiM*	2,000	Δ*pspA*	300
Δ*yejF*	2,000	Δ*pspB*	300
Δ*yejG*	**1,000**	Δ*pspC*	300
Δ*yneI*	2,000	Δ*pspD*	300
Δ*ygaV*	**1,500**	Δ*purC*	300
		Δ*purM*	300
		Δ*ygaV*	300
		Δ*yjbO*	300

709

Figure 1

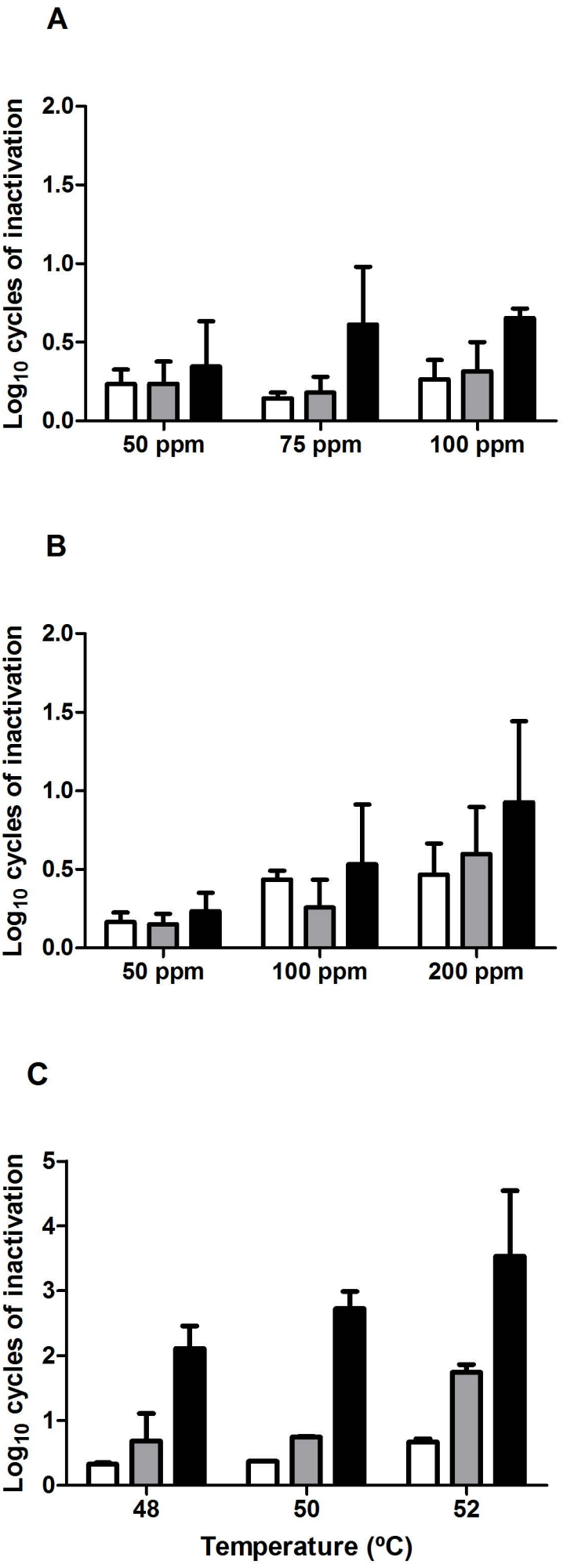

A
Log10 cycles of inactivation
2.0
1.5
1.0
0.5
0.0
50 ppm
75 ppm
100 ppm
B
Log10 cycles of inactivation
2.0
1.5
1.0
0.5
0.0
50 ppm
100 ppm
200 ppm
C
Log10 cycles of inactivation
5
4
3
2
1
0
48
50
52
Temperature (ºC)

Figure 2

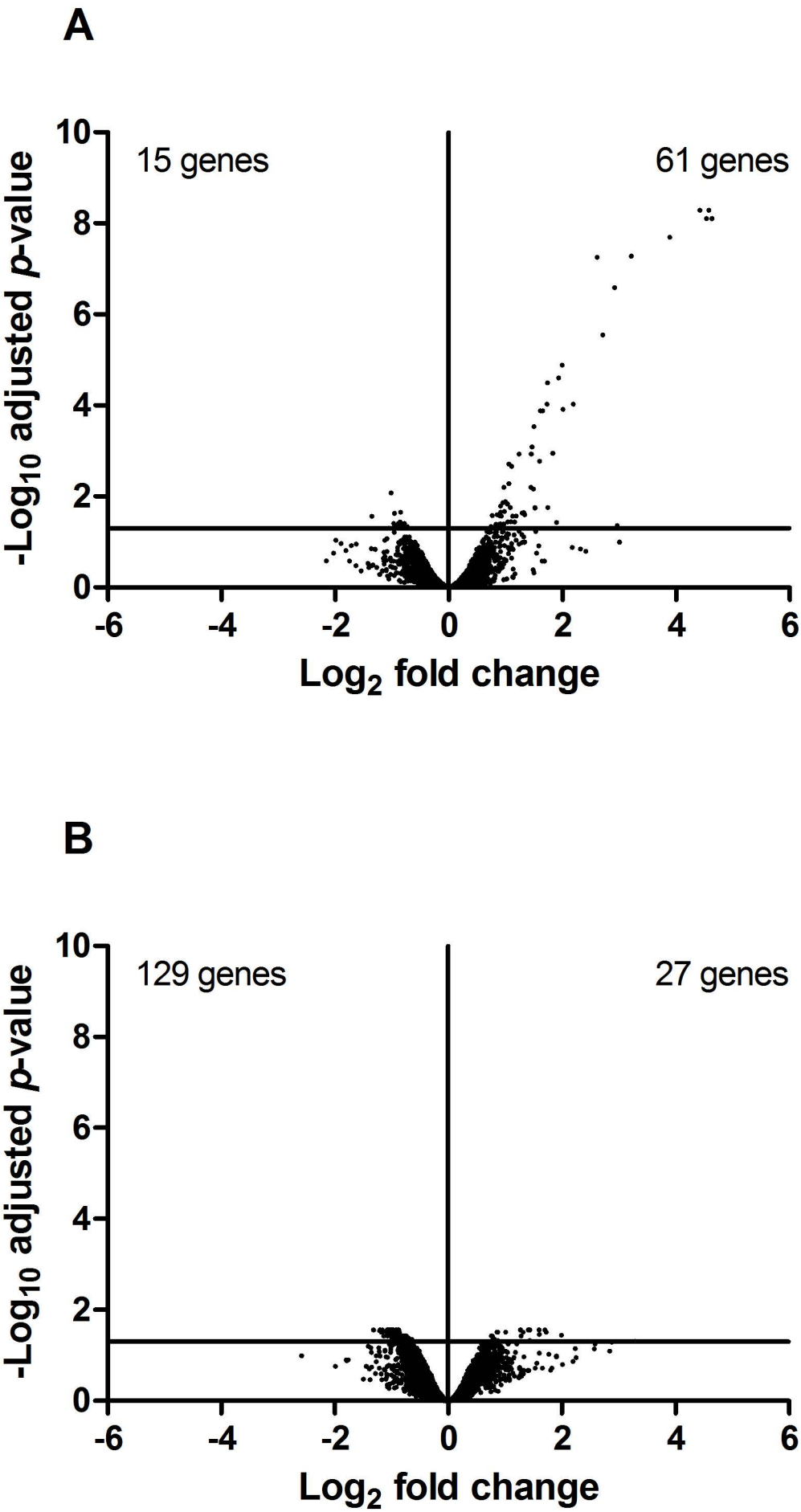

A
15 genes
61 genes
-Log10 adjusted p-value
Log2 fold change
B
129 genes
27 genes
-Log10 adjusted p-value
Log2 fold change

Figure 3

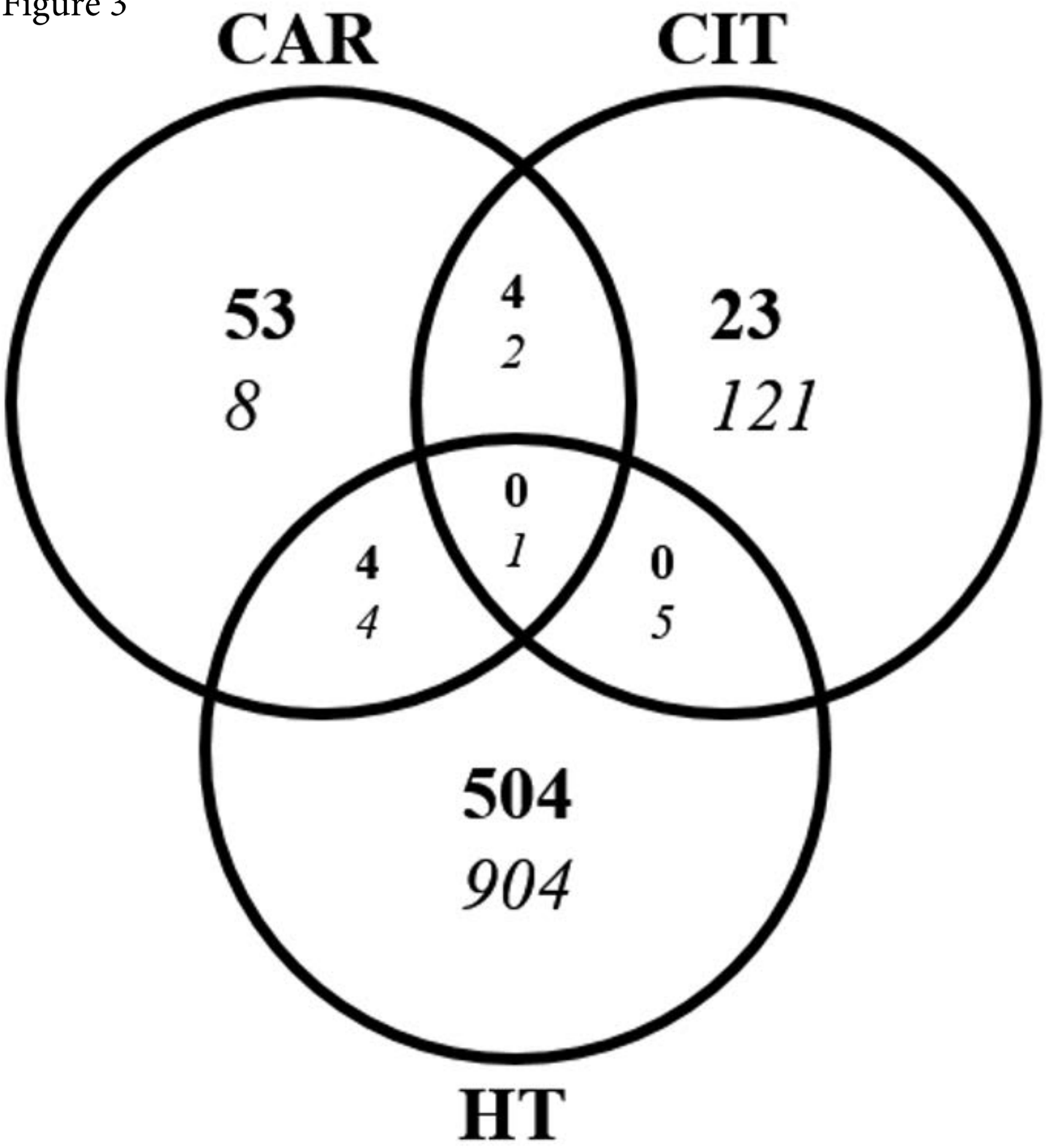
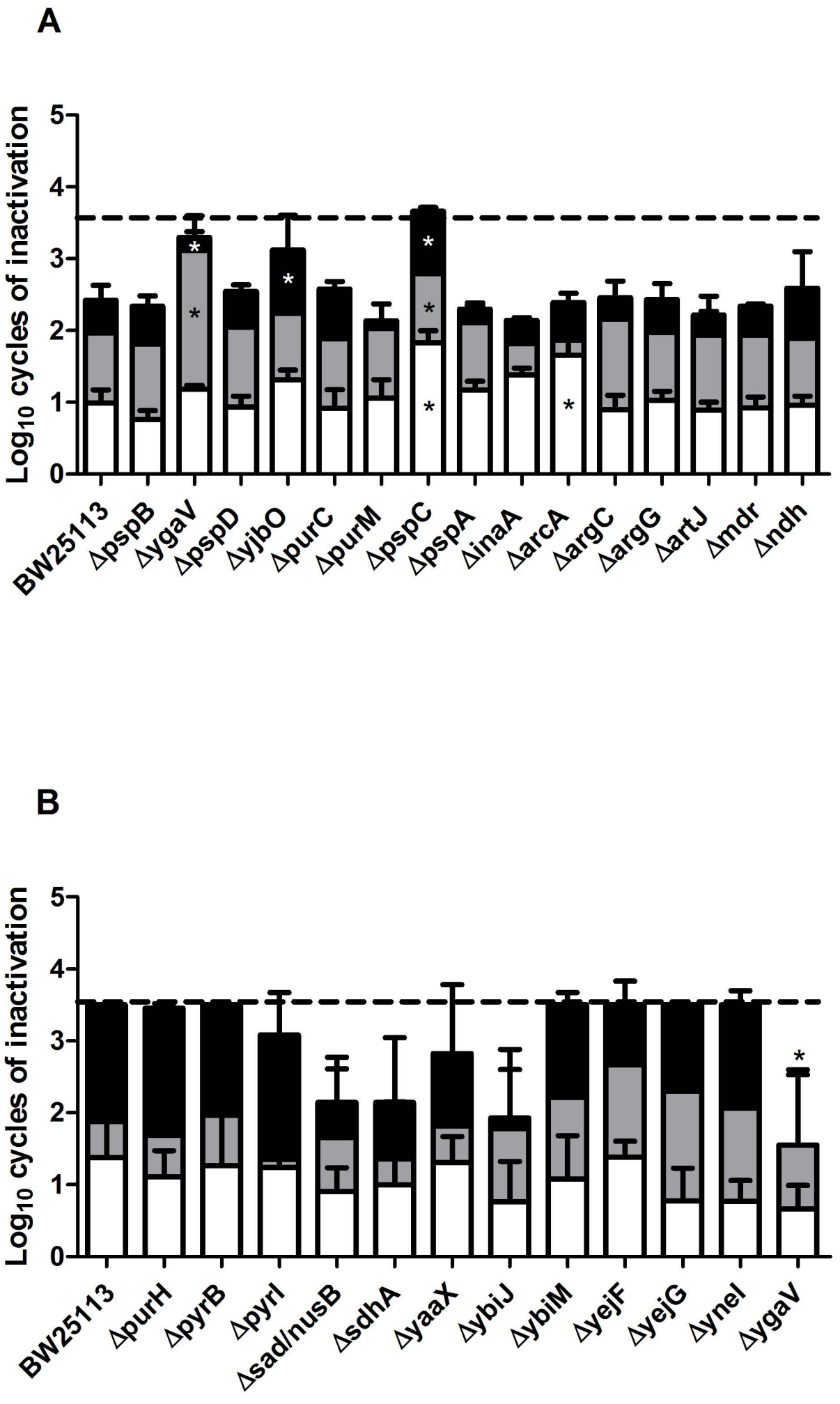


Figure 4


A
Log10 cycles of inactivation
0
1
2
3
4
5
BW25113
ΔpspB
ΔygaV
ΔpspD
ΔyjbO
ΔpurC
ΔpurM
ΔpspC
ΔpspA
ΔinaA
ΔarcA
ΔargC
ΔargG
ΔartJ
Δmdr
Δndh
B
Log10 cycles of inactivation
0
1
2
3
4
5
BW25113
ΔpurH
ΔpyrB
ΔpyrI
Δsad/nusB
ΔsdhA
ΔyaaX
ΔybiJ
ΔybiM
ΔyejF
ΔyejG
Δynel
ΔygaV