TITLE: The effect of exposure to chlorhexidine residues at "during use" concentrations on antimicrobial susceptibility profile, efflux, conjugative plasmid transfer and metabolism of *Escherichia coli*

Wesgate R[1], Fanning S[2, 3, 4], Hu Y[2, 3] and Maillard J-Y[1]*

[1] School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, UK

[2] UCD-Centre for Food Safety, School of Public Health, Physiotherapy & Sports Science, University College Dublin, Ireland

[3] Key Laboratory of Food Safety Risk Assessment, Ministry of Health, China National Center for Food Safety Risk Assessment (CFSA), Beijing, China

[4] Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Chlorine Gardens, Belfast BT9 5DL, United Kingdom

* corresponding author:

Prof Jean-Yves Maillard: School of Pharmacy and Pharmaceutical Sciences, Cardiff University Redwood Building, King Edward VII avenue, Cardiff CF10 3NB, UK

telephone: + 44(0)2920879088

e-mail: MaillardJ@cardiff.ac.uk

ABSTRACT (260)

There is no standardised protocol to predict the levels of microbicide concentrations that are left on surfaces as a result of the use of these products, nor is there a standardised method to predict the potential risk that such levels pose to emerging antibacterial resistance. The ability to distinguish between selection and adaption processes for antimicrobial resistance in bacteria, and the impact of different concentrations of microbicide exposure have not been fully investigated to date. This study considers the effect of chlorhexidine digluconate (CHX) exposure at low concentration on selected phenotypes of *Escherichia coli* and relates findings to risk of emerging antimicrobial resistance.

A concentration of 0.006 mg/ml CHX is a realistic during-use exposure concentration measured on surfaces. At this concentration it was possible for CHX-susceptible bacteria to survive, adapt through metabolic alterations, to exhibit a transient decrease in antimicrobial susceptibility, and express stable clinical cross-resistance to front line antibiotics. Efflux activity was present naturally in tested isolates and it increased in the presence of 0.00005 mg/ml CHX but ceased with 0.002mg/ml CHX. Phenotypic microarray assays highlighted a difference in metabolic regulation at 0.00005 mg/ml and 0.002 mg/ml CHX, more changes occurred after growth with the latter concentration. Metabolic phenotype changes were observed for substrates involved with the metabolism of some amino acids, co-factors and secondary metabolites. It was possible for one isolate to continue transferring ampicillin resistance in the presence of 0.00005 mg/ml CHX, whilst 0.002 mg/ml CHX prevented conjugative transfer. In conclusion, *E. coli* phenotype responses to CHX exposure are concentration dependent, with realistic residual CHX concentrations resulting in stable clinical cross-resistance to antibiotics.

INTRODUCTION

The European Biocidal Product Regulation (1) aims to standardise and monitor the introduction to the market and use of biocidal products (BP). Included in the "conditions for granting an authorisation" section (1), it is stipulated that the responsible regulatory body must be notified if the product manufacturer is aware or becomes aware that there is potential for the development of resistance to the active substance. It is also mandatory that the "biocidal product has no unacceptable effects on the target organisms" in particular "unacceptable resistance or cross-resistance" and that the "chemical diversity of the active substances is adequate to minimise the occurrence of resistance in the target harmful organism". Despite these clear requirements, there is not as yet a standardised method to evaluate and predict the risk of the development of resistance to microbicides. Neither is there an adequate understanding of how environmental concentrations of microbicide, remaining after disinfection effect surviving target organisms. Knapp and colleagues (2) proposed a decision tree-based method that utilises MICs and MBCs combined with antibiotic susceptibility profiling and stability testing to predict potential microbicide driven resistance when applied *in situ*. The method focused on "in-use" concentrations of chlorhexidine and benzalkonium chloride and investigated surviving bacteria for any changes in susceptibility profile to antimicrobial agents including the microbicide or biocidal formulation (2).

Biocidal formulated products are generally applied with the active ingredient at a concentration considerably higher than necessary to kill targeted microorganisms. The concentration of the active ingredient that is applied at the point of initial decontamination is termed "in-use" concentration (3). Biocidal product activity depends upon a number of factors such as concentration of actives, contact time,

formulation excipients, pH, organic challenge, temperature, target microorganisms (4). Product dilution upon usage and surface will contribute to lower residual concentration on surfaces which may result in concentrations below the minimal inhibitory concentration (MIC) for given microorganisms (5).

CHX is typically incorporated into surface disinfectant products at 20 mg/ml, and is often marketed with claims of residual activity of up to 6 hours and even 48 hours when applied to the skin surface (6). The concentration of chlorhexidine in products typically varies from 0.1 mg/ml in ophthalmic preparations to 40 mg/ml in surgical scrubs (7). The Scientific Committee on Emerging and Newly Identified Health Risks (8) stated, “Despite the regulatory requirements to study the environmental stability of individual products, data on the fate and concentrations of microbicides in the environment are sparse”. Although CHX may provide a degree of antimicrobial activity at a low concentration, there is little information available on CHX concentration remaining on surfaces following application and on the effect of residual microbicide concentrations on emerging resistance in bacteria.

This investigation aims to expose a range of previously characterised *Escherichia coli* to a concentration of CHX typically found as a residual on surfaces after application, and to measure any changes in antimicrobial susceptibility profile that arise, while providing some understanding of the associated bacterial mechanisms.

RESULTS

Measurements of any changes in antimicrobial susceptibility following exposure to CHX-

There was an average 99.97% decrease in CHX concentration once the solution was removed from the glass surface at time zero. The average CHX concentration recovered over time was recorded as 0.006 ± 0.002 mg/ml (Fig. 1). Baseline MIC and MBC data showed that the CHX concentration recovered from the glass surface was within the range of the CHX MIC and MBC for the test strains (Table S1). There was no statistical correlation between drying time and CHX concentration (P=0.62; Pearsons correlation analysis, r^2=0.07).

When exposed to CHX 20 mg/ml for 30 s in a suspension test, no viable bacteria were recovered (Fig. 2). However, exposure to lower concentrations (0.002 or 0.007 mg/ml resulted only in a 2-3 $\log_{10}$ reduction in *E. coli* 13P5 or IB2 viability regardless of the contact time (up to 5 min) (Fig. 2). There was no significant difference ($P \geq 0.0001$) in bacteria killing following exposure to 0.002 or 0.007 mg/ml CHX. Pre-exposing isolates to 0.0075 mg/ml CHX did not affect ($P \geq 0.0001$) their susceptibility to the different CHX concentrations tested (Fig. 2)

When exposed directly to residual CHX concentrations remaining on surfaces (0.0047-0.0075 mg/ml) for 5 min, a significant (> 5 $\log_{10}$) reduction in bacterial viability was observed (Table 1). When viable bacteria where recovered after a 5 min exposure time, changes in MIC/MBC were observed primarily from surfaces with a 24 and 168 h CHX drying time (Table 2). The highest fold increase in MIC (32-fold) was observed in *E. coli* ATCC®25922 and the CTX-M-14 isolates (IL3,IL4

and 1B2) on surface dried for 168 hours, while the highest MBC increase (62-fold) was observed with 1L3 and 1B2 on surfaces dried for 168 hours.

CHX MBC increased further for *E. coli* 13P5 following one additional passage in CHX 0.002 mg/ml. However, MBC decreased to the baseline value following 5 passages in CHX 0.002 mg/ml and remained at baseline value after 10 passages (Fig. 3). There were no significant changes ($P \geq 0.0001$) in MBC for *E. coli* 1B2 following passaging in CHX 0.002 mg/ml (Fig. 3). When change in MIC was considered changes were considered, there were no significant differences in MIC ($P \geq 0.0001$) before and following passaging either isolates in CHX 0.002 mg/ml (Fig. S1).

Escherichia coli ATCC®25922 was found to be susceptible to all antibiotics tested, whilst the isolates showed different antibiotic susceptibility pattern (Table S2). CTX-M-14 isolates were clinically resistant to tetracycline, trimethoprim and trimethoprim-sulfamethoxazole, CTX-M-15 and TEM-20 isolates were resistant to ampicillin, cefpodoxime and cephalothin (Table S2). In addition, *E. coli* 1B2 (CTX-M-14) was also to streptomycin, and *E. coli* 13P5 (CTX-M-15) was also resistant to tetracycline, streptomycin nalidixic acid, trimethoprim and trimethoprim-sulfamethoxazole (Table S2).

Exposure to CHX 0.0047 or 0.0075 mg/ml resulted in amoxicillin/clavulanic acid, or amoxicillin / clavulanic acid and cefoxitin resistance in *E. coli* 13P5 and in amoxicillin / clavulanic acid resistance in *E. coli* 1B2 following exposure to 0.0047 mg/ml CHX (Table 3). Clinical resistance to amoxicillin / clavulanic acid remained stable in *E. coli* 13P5 following passaging, but resistance to cefoxitin was lost (Table 3). In contrast, *E. coli* 1B2 developed additional clinical resistance to ampicillin,

amoxicillin / clavulanic acid, cefpodoxime and cephalothin following 5 passage in CHX 0.0002 mg/ml or in broth.

Conjugation assay

Exposure to 0.00005 mg/ml CHX did not show a statistically significant change (P=0.730) in transfer rates in *E. coli* 13P5, as measured by the rescue of the ampicillin phenotype from the initial transfer rate of 1.34×10^{-5}. Exposure 0.002 mg/ml CHX appeared to halt conjugation (Table S3).

Effect of exposure to CHX residues on efflux

Bacterial exposure to CHX at 0.00005 mg/ml resulted in active efflux regardless of the isolate as indicated with no change in relative fluorescence (Fig. 4). The addition of CCCP causes the level of fluorescence to increase ($P \leq 0.0001$) confirming the fact that efflux pumps may be responsible for the extrusion of EtBr.

Effect of CHX residues on microbial metabolism

Overall, bacterial exposure to a concentration of 0.002 mg/ml CHX had a greater impact on metabolism than when exposed to 0.005 mg/ml CHX (Table 4). Bacterial growth in the presence of CHX 0.00005 mg/ml decreased reduced the ability of *E. coli* 13P5 to metabolise salicin, 6.5% [w/v] NaCl and 4% [w/v] urea (Fig. S2).
The metabolism of methylene diphosphonic acid increased in the presence of 0.00005 mg/ml CHX. Growth in the presence of CHX 0.002 mg/ml decreased the bacterial metabolism when exposed to 6% [w/v] NaCl and 5% [w/v] urea. However, the metabolism at pH 4.5 in the presence of L-serine and at pH 9.5 in the presence of L-phenylethlamine was found to increase (Fig. S2). *Escherichia coli* 13P5 was

less efficient at metabolising salicin when exposed to 0.00005 mg/ml than when exposed to 0.002 mg/ml or not exposed at all. Growth in the presence of CHX (0.00005 and 0.002 mg/ml) increased the ability of *E. coli* 13P5 to metabolise at L-alanine at pH 4.5, L-phenylethylamine at pH 9.5 and 6% [w/v] NaCl and betaine when compared to bacteria un-exposed to CHX (Table 4).

When one considers the metabolic pathway mapping of *E. coli* (K12), all of the changes observed in this study were located in the domains of amino acid metabolism, carbohydrate metabolism, metabolism of co-factors and vitamins and the biosynthesis of secondary metabolites (Fig. 6).

DISCUSSION

The amount of CHX recovered from surfaces (an average of 0.006 ± 0.002 mg/ml) represents the “during use” exposure concentration, a realistic prediction of true-to-life conditions of product use during its application (3). “During use” exposure differed from “low concentration” which often reflects a concentration value below the MIC, which is not necessarily representative of a concentration that applies in practice. It was noticeable there was some variability in the concentration measured at recovery (range 0.0047 to 0.0097 mg/ml) over a 168 h period, although the increasing concentration of CHX did not appear to have an impact on the MIC/MBC nor antibiotic susceptibility. Furthermore, measured CHX residual concentrations were between the MIC/MBC and 10-fold lower the MIC/MBC for the isolates tested. These residual CHX concentrations were associated with greater changes in CHX susceptibility profile.

Some biocidal manufacturers make claims about their formulations including having a “residual biocidal activity”. Related to this is the suggestion that a sub-inhibitory concentration of a biocidal product may exert a selective pressure sufficient to drive bacterial adaptation. (4,9-16). In this study, CHX concentrations similar or 10-fold lower than the MIC/MBC were capable of selecting for changes in MIC/MBC to the biocidal active as well as altering bacterial susceptibility phenotypes to one or more antimicrobial compounds. These findings support the hypothesis that residual CHX concentrations remaining on abiotic surfaces can select for antibiotic resistance (Table 2). Further, the stability of these clinical antibiotic susceptibility changes further narrows the chemotherapeutic options for treatment (Table 3).

To gain a better understanding of the impact of a low sub-inhibitory CHX concentrations in selecting for changes in bacterial susceptibility, we investigated two concentrations, 0.0005 mg/ml which provided an environment conducive to an adaptive tolerance, while having a low antibacterial impact, and 0.002 mg/ml exerting a greater selective condition and enhanced pressure for metabolic changes. Noticeable differences were recorded when bacteria were exposed to the two concentrations tested. CHX concentrations of 0.00005 mg/ml resulted in an increase in efflux pump activity, as measured by EtdBr accumulation assays. In contrast CHX 0.002 mg/ml appeared to supress efflux although such observation is likely linked to the bactericidal effect of this concentration (data not shown). *E. coli* possesses multiple efflux transporters primarily belonging to the he hydrophobic and amphiphilic efflux RND (HAE-RND) family (17). Indeed, CCCP has been used to screen RND efflux pumps activity although it is a proton motive force (PMF) inhibitor and is likely to have an impact on other bacterial PMF-driven functions (18).

Here we showed that a RND pump is likely involved in *E. coli* in response to CHX exposure and potentially contributing to an decrease in antibiotic susceptibility (Table 3). Deletion of *acrA* or *tolC* has been shown to render E. coli cells more susceptible to aminoglycosides about also to ampicillin (18). We did not perform any MIC testing in the presence of CCCP which would have indicated the potential impact of RND transporter. Future study will look at the expression of specific RND transporters when E. coli is exposed to residual CHX concentration.

There was also a clear disparity in the number of metabolic changes observed following prior exposure to these two concentrations of CHX, with CHX 0.002 mg/ml appearing to exhibit a greater effect on bacterial metabolism (Table 4). All of the metabolic changes observed were located in the domains of amino acid metabolism, carbohydrate metabolism, metabolism of co-factors and vitamins, and the biosynthesis of secondary metabolites (Fig. 6). These findings agree with those of reported by Condell et al. (9) where the largest number of up-regulated genes after CHX exposure were involved in general cell metabolism. A concentration of 4% [w/v] urea and salicin were metabolised equally either with exposure to 0.002 mg/mL CHX, or in the absence of it, but decreased following exposure to 0.00005 mg/mL CHX. Upon exposure to 0.002 mg/ml CHX a 1.67 $\log_{10}$ reduction in viability for this bacterium was recorded, which in this case could have resulted from a surviving sub-population of bacteria showing the same level of salacin metabolism (19-22). The ability to utilise salicin (a secondary metabolite β-glucoside) as a carbon source has previously been shown to provide a competitive advantage referred to as a Growth Advantage in Stationary Phase (GASP) in *E. coli* (23,24). The *bgl* operon encodes the genes that function for β-glucoside catabolism and is a silent operon that has been implicated in the upregulation of proteins associated

with transport functions or enzymes involved in cellular metabolism. The presence of the *bgl* operon appears to activate additional metabolic functions owing to enhanced ability to access nutrient substrates (23,24). Furthermore, an increase in other carbon sources L-alanine, L-serine and phenylethylamine were identified. A combination of the increased utilisation of amino acids and carbohydrates suggest that CHX induces a membrane related stress response in *E. coli* 13P5. It has been suggested that surviving bacterial cells are directing mechanisms primarily towards cell membrane processes, such as changes in outer membrane structure and signalling functions (9,25). The impact of low concentration of CHX on metabolism and a potential mechanistic link to decreased susceptibility to CHX, but also unrelated compounds, is interesting and need to be further investigated.

It has been shown that in the case of antibiotics and microbicides, this selection process might be relevant at very low concentrations (12,13,25-28). Antibiotic concentration-specific outcomes selecting for mutations relating to increased resistance have been highlighted (29-32), supporting the concept of a distinct difference in the selection process deriving from low- and high-concentrations of antibiotics. A similar comparative study has not yet been performed for microbicides.

In this study, we established the residual CHX concentration remaining on surfaces following the use of a solution of 20 mg/ml CHX. Importantly, bacteria surviving exposure to these residual concentrations were not less susceptible to the bactericidal effect of CHX compared to bacteria that were not exposed (Fig. 2). We also observed that exposure to different sub-MIC CHX concentrations (0.0005 mg/ml or 0.002 mg/ml) produced different response in isolates and the main two isolates 13P5 (ST10; CTX-M-15) and 1B2 (ST1629; CTX-M-14) had different

response to these CHX sub-MIC. A decreased susceptibility (MIC and MBC) to CHX and antibiotics was observed, although a decreased CHX MIC was not stable. Although RND efflux may be involved (Fig. 4), differences in metabolism were also observed, which enforces the postulate that decreased antimicrobial susceptibility following exposure to a microbicide is multi-factorial in bacteria (9,33-35).

In conclusion, following CHX application to surface, residual concentration of CHX may be present in the environment at levels conducive to bacterial adaptation through metabolic changes which may be associated in decreased CHX susceptibility and an increase in clinical resistance to antibiotics of importance to human health. The practical impact of such observations associated with CHX use would need be ascertain in situ, by monitoring environmental isolates where CHX products are used.

MATERIALS AND METHODS:

Microorganisms

This study investigated seven environmental isolates of *E. coli* (provided by S. Fanning; University College Dublin) and a reference strain *E. coli* ATCC®25922 that was used for comparison (Table 5). All strains were cultured in Müller-Hinton broth (MHB; Fisher Scientific, Loughborough, UK) and incubated at 37 ± 1°C for 18-24 h. When necessary all bacterial strains were cultured on MH agar (MHA) plates and stored in the fridge at 4-6°C for up to one month. Overnight MHB bacterial cultures were centrifuged at 5000 x *g* for 15 min at 20 ± 1°C. The supernatant recovered was discarded and the bacterial pellet was then re-suspended in 10 ml phosphate buffer saline (PBS; Fisher Scientific, Loughborough, UK). Turbidity of these test suspensions was adjusted spectrophotometrically ($OD_{600\ nm}$) using sterile MHB to

achieve a turbidity equivalent to approximately 1 -2 × 10^8 CFU/ml. Enumeration of test suspensions was carried out using the drop counting method (36).

Determination of surface dried CHX concentration

One mL of 20 mg/l chlorhexidine digluconate (CHX; Fischer Scientific, UK) was pipetted into a flat-bottomed glass McCartney bottle. Then 1 ml of CHX was removed with a pipette (time 0 h) and the remaining residue left to dry at room temperature (21°C) in a biological safety level-2 cabinet for 6, 24, 48, 96 or 168 hours. After the appropriate drying time 1 ml of sterile de-ionised water (diH_20) was added to the bottle and CHX residue was re-suspended using a vortex mixer and magnetic stirrer for 1 minute. This solution was aspirated and dispensed into a glass autosampler vial for HPLC analysis (Thermo Scientific, UK). The mobile phase was a 1:1 ratio of water and acetonitrile (HPLC grade, Sigma Aldrich, UK) with 0.5% [v/v] trifluroacetic acid (HPLC grade, Sigma Aldrich, UK). Retention rate was 6 minutes. An initial calibration curve was performed with a CHX standard stock (20 mg/ml) halving concentrations running from 0.5 mg/ml to 0.001 mg/ml.

Modified carrier test and bacterial survival after exposure.

Modified carrier tests were performed to measure bacterial cell survival after exposure to surface dried residual concentrations of CHX. One ml of 20 mg/ml CHX was pipetted into a glass flat-bottomed McCartney bottle. Then 1 ml was removed by pipette at 0 h and the bottle left to dry at room temperature (21°C) for 6, 24 or 168 hours. After the appropriate drying time 20 µl of standardised washed inoculum (1-2 x 10^8 cfu/ml) was added to the bottom of the McCartney bottle and left for an exposure time of either 5 min or 24 h. Following exposure, 1 ml De-Engley

(DE) neutraliser (Fisher Scientific, Loughborough, UK) was added to the bottle and the inoculum was re-suspended using a vortex mixer for 1 min. The reduction in viability (log_{10}) after exposure was determined. In addition, 100 µl sample was removed from the test vial after neutralisation, placed into 10 ml MHB and incubated for 18-24 hours 37°C in order to perform additional susceptibility testing.

Minimal Inhibitory and minimal bactericidal concentrations

MIC and minimal bactericidal concentration (MBC) were measured using the BS EN ISO: 20776-1 (37) broth microdilution method before and after exposure to CHX. Each plate was incubated for 24 hours at 37°C and results were recorded based on positive or negative visible growth.

Antibiotic susceptibility testing (AST)

An adjusted bacterial inoculum (1 x 10^4 CFU/ml) from samples taken before and after exposure to CHX was spread onto MHB agar plate. Antibiotic containing discs (Beckton Dickinson, UK) were placed onto the agar surface and plates incubated for 24 h at 37°C. Zones of inhibition were recorded, and breakpoints calculated in accordance to the (38) EUCAST (2020) protocol.

Inactivation kinetics

Inactivation kinetics were only investigated with *E. coli* 1B2 and 13P5. We tested both isolates that were pre-exposed to 0.0075 mg/ml CHX (corresponding to a 168 h drying time on surfaces) and isolates that were not pre-exposed to CHX. One ml of standardised bacteria culture (1 x 10^9 CFU/ml) was mixed with 1 ml PBS. Eight ml of CHX at 20 mg/ml, 0.002 mg/ml or 0.007mg/ml was added to the bacterial suspension

and vortexed for 30 s. *E. coli* 1B2 and 13P5 were exposed to CHX for contact times of 0.5, 1, 3 and 5 min at room temperature.

These concentrations were chosen to represent the in-use concentration (20 mg/ml), the concentration found left on a surface (0.006 ± 0.002 mg/ml) and the concentration below the MIC value (0.002 mg/ml) in order to exert a selective pressure but not necessarily kill all microbial cells. Following these contact times 1 ml of each test suspension was added to 9 ml DE neutralising agar and vortexed for 30 s. A volume of 100 µl of the neutralised mixture was diluted in 900 µl PBS and surviving bacteria were enumerated in duplicate on MHB using the drop counting method. Plates were incubated at 37°C for 24 h and CFU/ml were calculated. Inactivation kinetics were plotted using the $\log_{10}$ CFU/ml recovered over time.

Effect of exposure to CHX residues on efflux.

Change in efflux were only investigated with *E. coli* 1B2 and 13P5. Overnight bacterial cultures were adjusted to 1 x 10^8 CFU/ml in 20 ml sterile MHB. Suspensions were incubated at 37°C in a shaking incubator (120 rpm) until the mid-log growth phase was reached ($OD_{600\ nm}$ of 0.2-0.3; 2 to 3 hours approx.). Bacterial cells were centrifuged at 5,000 x g and the resulting pellet washed with diH2O**.** Suspension was adjusted to a final $OD_{600\ nm}$ of 0.4. One ml of each bacterial isolate suspension was removed and boiled (95°C) for 10 min to be used as a positive control. Fifty µl of an ethidium bromide (EtBr; Sigma-Aldrich, UK) stock solution (10 mg/ml) was added to each well of a 96 well microtitre plate to give a final concentration of 0.005 mg/ml. Fifty µl of carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich, UK), an efflux pump inhibitor (EPI), was added to appropriate wells to give a final concentration of 0.1 mM. One hundred µl of either

boiled bacteria cells or test bacteria cells were added to the plate and 50 µl of PBS was added to those wells without EPI. The final volume in all wells was 250 µl. Plates were then scanned in a Tecan microplate reader (Tecan M200 Infinite PRO, Life Sciences, UK) at 37°C for an initial 10 min to obtain baseline fluorescence values. After 10 min, 50 µl CHX (0.00005 mg/ml) was injected to each well using the automated injector module equipped to the Tecan microplate reader. The plates were then scanned in the Tecan microplate reader for an additional 50 min. Background controls consisting of microbicides or EPI and EtBr with no bacteria, were run alongside experimental sets and used to normalise data accounting for any background fluorescence omitted by inhibitory compounds used.

Phenotype stability testing

Phenotype stability was only investigated with *E. coli* 1B2 and 13P5. Stability in microbicide and antibiotic susceptibility changes observed after microbicide exposure was assessed through successive passaging of surviving bacteria in microbicide-free broth or broth supplemented with 0.002 mg/ml CHX (2). Daily (24 h) passages were performed over a 10-day period and microbicide MIC, MBC and antibiotic susceptibility profiles were measured after passage 1, 5 and 10.

Effect of CHX residues on microbial metabolism

Change in metabolism were only investigated with *E. coli* 1B2 and 13P5. CHX (0.00005 mg/ml) was chosen to provide a low antibacterial effect whilst providing an environment for an adaptive tolerance, while 0.002 mg/ml CHX was chosen to create a less favourable environment providing a more pronounced selective condition and exerting an enhanced pressure for metabolic changes.

E. coli 13P5 was grown to its 3rd generation with three subsequent sub-cultures onto MHA agar (incubated at 37°C; 16-18h). One loopful of colonies was taken from a MHA plate, streaked onto a new MHA plate and incubated at 37± 2°C for 18-24 h. Two independent biological replicates were grown for each test; the two replicates were tested on a separate day (n=8). The 3rd generation was sub-cultured onto three MHA agar plates containing no CHX, 0.00005 mg/ml or 0.002 mg/ml CHX and incubated at 37± 2°C for 16-18h. A preliminary test was then performed in order to ensure that the isolate would grow on the agar plates in the presence of these concentrations of CHX.

A range of phenotype microarray (PM) plates were selected for the assay (Table 6). Before being loaded into the PM microplates (Biolog, Inc., Hayward, CA, United States), 13P5 was incubated for 16-18 h on MHA plates containing no CHX, 0.00005 mg/ml CHX or 0.002 mg/ml CHX. After incubation in the presence/absence of CHX, several colonies were selected with a sterile plastic culture loop and suspended into Inoculating Fluid-0 (IF-0; Biolog, Inc., Hayward, CA, United States) until a cell density of 42% transmittance ($T_{42\%}$) was reached in a turbidimeter (Biolog, Inc., Hayward, CA, United States).

For plates PM1-PM2, 15 ml $T_{42\%}$ cell suspension was mixed with 75 ml of Biolog redox dye mix A (1:5 dilution) in order to create a final cell suspension of $T_{85\%}$. For PM 3-8, 680 µl 2 M-sodium succinate/200 µM ferric citrate solution was added to 68 ml of the $T_{85\%}$ cell suspension. One hundred µl of each mixture was pipetted into each well of the appropriate microplate. All PM plates were incubated in an OmniLog reader at 37°C for 72 h. Readings were recorded every 15 min and data was analysed in OmniLog PM software (Biolog, Inc). Each experiment was

performed in duplicate on two separate days with independent bacterial cultures. Data obtained from PM experiments were collated and analysed in OmniLog PM software (39,40).

Conjugation assay

To determine the conjugative transfer of the ampicillin (AMP) resistance determinant, the liquid mating method was followed as described by Lambrecht et al. (41). *E. coli* 13P5 was selected for further investigation due to its ampicillin resistance and its ability to recover and grow after exposure to CHX 0.002 mg/ml or 0.00005 mg/ml for 5 min CHX. The recipient *E. coli* J35R (S. Fanning; University College Dublin) was chosen for its chromosomally encoded rifampicin resistance. For each test three independent biological replicates were performed. A single colony for each replicate was inoculated into 5 mL MHB for 16-18h at 37°C. The donor, *E. coli* 13P5 was grown in the presence of ampicillin (100 µg/ml) and the recipient (*E. coli* J35R) was grown in the presence of rifampicin (100 µg/ml). Cultures were centrifuged at 5,000 x g, pellet washed with PBS and re-suspended in 5 ml MHB. Bacterial suspensions were diluted 10-fold in MHB with CHX to obtain an exposure concentration of 0.00005 mg/ml or 0.002 mg/ml. A control was performed with no CHX. Initial mating concentrations ranged from 2.30×10^7 CFU/ml to 7.30×10^7 CFU/ml for the donor strain (*E. coli* 13P5) and 1.17×10^8 CFU/ml to 7.30×10^8 CFU/ml for the recipient strain (*E. coli* J35R). Donor and recipient strains were mixed in a ratio of 1:5. Liquid mating was performed for 4 hours at room temperature (25°C), after which, bacteria were enumerated using the spread plating technique. Enumerated mating suspensions were plated onto media containing ampicillin (100 µg/ml) for donors and transconjugants, rifampicin (100

μg/ml) for recipient for transconjugants, or double selective plates containing ampicillin (100 μg/ml) and rifampicin (100 μg/ml) for transconjugants. Plates were incubated overnight 16-18h at 37°C and colonies counted. The limit of detection for enumeration was 1 CFU/ml. The limit of quantification was ≥10 colonies/plate. Transfer ratios were calculated as the number of transconjugants divided by the number of recipients, defined below:

$$Transfer\ ratio = \frac{number\ of\ transconjugants}{number\ of\ recipients}$$

Statistical analysis

Pearson's correlation analysis was used to determine the relationship between surface drying time and the concentration of CHX determined via HPLC. One-Way and Two-way Analysis of Variance (ANOVA) were used when comparing differences between single and multiple factors respectively.

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TABLE 1 Bacterial reduction in viability following a 5 min contact time with CHX concentrations found on surfaces after drying for different length of time (based on Fig. 1)

E. coli	**Drying time hours (CHX concentration mg/ml)**[1]			
	0 (0.0049)	**6 (0.0097)**	**24 (0.0047)**	**168 (0.0075)**
ATCC®25922 (n=3)[2]	>5.82 ± 0.15	>5.82 ± 0.15	>5.82 ±0.15	>5.83 ±0.08
CTX-M-14				
1L3 (n=3)	>5.81 ± 0.44	>5.81 ± 0.44	>5.81 ± 0.44	5.34 ± 0.49
1L4 (n=3)	>5.93 ± 0.29	5.08 ± 0.41	>5.93 ± 0.29	>5.99 ± 0.05
1B2 (n=5)	>6.00 ± 0.21	>6.00 ± 0.21	5.62 ± 0.54	5.46 ±0.47
CTX-M-15				
13P4 (n=3)	>5.67 ± 0.40	>5.67 ± 0.40	5.55 ± 0.41	5.49 ± 0.68
13P5 (n=5)	>5.89 ± 0.13	5.76 ± 0.28	5.58 ± 0.44	5.58 ± 0.63
TEM-20				
25P5 (n=3)	>5.78 ± 0.26	>5.78 ± 0.26	>5.78 ± 0.26	>5.81 ± 0.01
25OS1 (n=3)	>5.80 ± 0.30	>5.80 ± 0.30	>5.80 ± 0.30	>5.92 ± 0.30

[1] > indicates no viable bacteria recovered. The limit of detection was 1 x 10^2 CFU/ml

[2] n: number of biological replicates

TABLE 2 Changes in susceptibility for bacteria surviving a 5 min exposure to CHX concentrations measured on surfaces after drying. **a)** MIC and **b)** MBC values (mg/ml) for bacteria. Fold increase in MIC or MBC is shown in bracket.

a) Minimal inhibitory concentration (MIC) measurements

		CHX concentration mg/ml			
	Baseline MIC	**0.0049**	**0.0097**	**0.0047**	**0.0075**
	***E. coli* ATCC®25922**				
	0.005	0.04 (8-fold)*	-	0.04 (8-fold)*	0.16 (32-fold)*
CTX-M-14	***E. coli* 1L3**				
	0.005	-	0.04 (8-fold)*	0.04 (8-fold)*	0.16 (32-fold) #
	***E. coli* 1L4**				
	0.005	-	0.04 (8-fold)*	-	0.16 (32-fold)*
	***E. coli* 1B2**				
	0.005	-	-	0.04 (8-fold)#	0.16 (32-fold)#
	0.01	-	-	0.08 (8-fold)	0.16 (16-fold)
	0.02	-	-	0.04 (4-fold)	0.16 (8-fold)
CTX-M-15	***E. coli* 13P4**				
	0.02	-	-	-	-
	***E. coli* 13P5**				
	0.01	-	0.04 (4-fold)*	0.08 (8-fold)*	0.1 (10-fold)*
TEM-20	***E. coli* 25P5**				
	0.005	-	-	0.04 (4-fold)*	-
	***E. coli* 25OS1**				
	0.005	-	-	-	-

- No recoverable growth post-exposure

* observed in 1/3 repeat

observed in ½ repeats

b) Minimal bactericidal concentration (MBC) measurements

		CHX concentration mg/ml			
	Baseline MBC	**0.0049**	**0.0097**	**0.0047**	**0.0075**
	***E. coli* ATCC®25922**				
	0.005	0.04 (8-fold)*	-	0.08 (16-fold)*	0.16 (32-fold)*
CTX-	***E. coli* 1L3**				
	0.005	-	0.04 (8-fold)*	0.04 (8-fold)*	0.16 (32-fold)*

	0.005	-	-	-	0.32 (62-fold)*
	***E. coli* 1L4**				
	0.005	-	0.04 (8-fold)*	-	0.16 (32-fold)*
	***E. coli* 1B2**				
	0.005	-	-	0.08 (16-fold)[#]	0.31 (62-fold)[#]
	0.01	-	-	0.08 (8-fold)	0.16 (16-fold)
CTX-M-15	***E. coli* 13P4**				
	0.005	-	-	-	-
	***E. coli* 13P5**				
	0.01	-	0.04 (4-fold)*	0.08 (8-fold)*	0.16 (16-fold)*
TEM-20	***E. coli* 25P5**				
	0.005	-	-	0.04 (4-fold)*	-
	***E. coli* 25OS1**				
	0.005	-	-	-	-

- No recoverable growth post-exposure

* observed in 1/3 repeat

[#] observed in ½ repeats

TABLE 3 Clinically relevant changes in antibiotic susceptibility phenotype according to EUCAST (2020) breakpoint values for *E. coli* before and after a 5 min initial exposure to, and passage in, CHX or broth only.

***E. coli* 13P5**				
Initially exposed to	**CHX 0.0047 mg/ml**		**CHX 0.0075 mg/ml**	
Passage in	CHX	Broth	CHX	Broth
Initial exposure	**AMC**		**AMC, FOX**	
Passage 1	**AMC**	-	**AMC**	-
Passage 5	**AMC**	**AMC**	**AMC,IPM***	**AMC**
Passage 10	**AMC**	**AMC**	**AMC**	**AMC**
***E. coli* 1B2**				
Initially exposed to	**CHX 0.0047 mg/ml**		**CHX 0.0075 mg/ml**	
Passage in	CHX	Broth	CHX	Broth
Initial exposure	-		**AMC**	
Passage 1	-	**AMC***	**AMP***	**AMC**
Passage 5	-	**AMP,AMC*,CIP, CPD, CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CIP, CPD,CF**
Passage 10	**CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CPD,CF**

- : No change in antibiotic susceptibility observed; **BOLD**: Change in phenotype from clinically sensitive to clinically resistant

Ampicillin (AMP), amoxicillin/clavulanic acid (AMC), cefpodoxime (CPD), cephalothin (CF), ciprofloxacin (CIP), imipenem (IMP), cefoxitin (FOX)

* only observed in ½ repeat.

TABLE 4 Summary of CHX exposure at concentrations of 0.00005 mg/m or 0.002 mg/ml on bacterial metabolism, resulting in either increase (🡅), decrease (🡇) or no change (-) following

	Compared to no CHX		**Compared to each other**	
	0.00005 (mg/ml)	0.002 (mg/ml)	0.00005 (mg/ml)	0.002 (mg/ml)
Salicin	🡇	–	🡇	🡅
Methylene diphosphonic acid	🡅	–	🡅	🡇
pH 4.5 + L-Alanine	🡅	🡅	=	=
pH 4.5 + L-Serine	-	🡅	=	=
pH 9.5 + Phenylethylamine	🡅	🡅	=	=
5.5% [w/v] NaCl	=	🡇	🡅	🡇
6% [w/v] NaCl	=	🡇	🡅	🡇
6% [w/v] NaCl and Betaine	=	🡇	=	=
6% [w/v] NaCl and Creatinine	=	🡇	=	=
4% [w/v] Urea	🡇	-	🡇	🡅
5% [w/v] Urea	🡇	🡇	-	-

TABLE 5 Summary of ESBL producing *E. coli* isolates and their resistance features

ISOLATE	MLST	ESBL	Plasmid(s) (kb) isolated from transconjugants
UCD-CFS ECP-1L3	**ST23**	CTX-M-14	200-, 120-
UCD-CFS ECP-1B2	**ST1629**	CTX-M-14	110-
UCD-CFS ECP-1L4	**ST23**	CTX-M-14	130-
UCD-CFS ECP-13P5	**ST10**	CTX-M-15	80-
UCD-CFS ECP-13P4	**ST10**	CTX-M-15	70-
UCD-CFS ECP-25OS1	**ST34 (ST10 Cpix)**	TEM-20	120-, 60-
UCD-CFS ECP-25P5	**ST10**	TEM-20	110-, 50-

TABLE 6 Features associated with the selected phenotype microarray (PM) plates

PM PLATE number	SUBSTRATE CLASS
PM1	Carbon sources
PM2A	Carbon sources
PM3B	Nitrogen sources
PM4A	Phosphorous and sulfur source
PM6	Peptide nitrogen sources
PM7	Peptide nitrogen sources
PM8	Peptide nitrogen sources
PM9	Osmolytes
PM10	pH

FIG 1 Concentration of CHX (initially set at 20 mg/m) recovered after drying this solution directly on a glass surface. Error bars are standard deviation of the mean. Dashed lines depict the range of MIC and MBC values for all *E. coli* isolates tested (see Table S1). Note the abscissa distance for the histogram is not proportional to increasing drying time. (data based on 3 biological repeats)

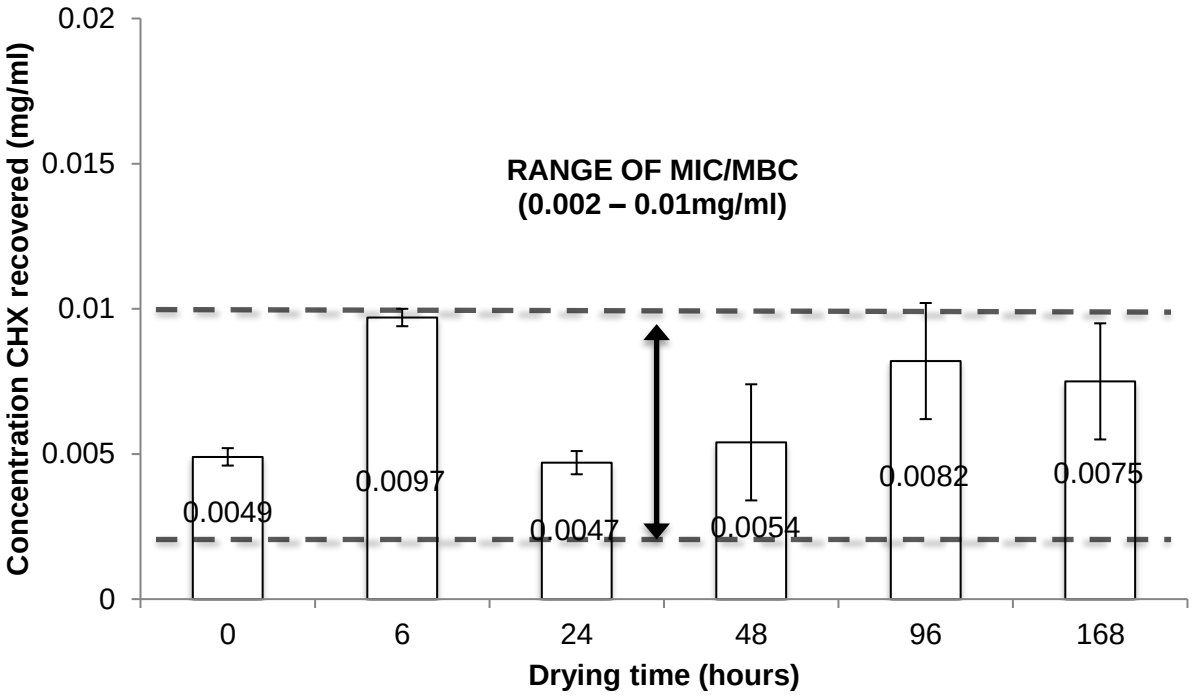
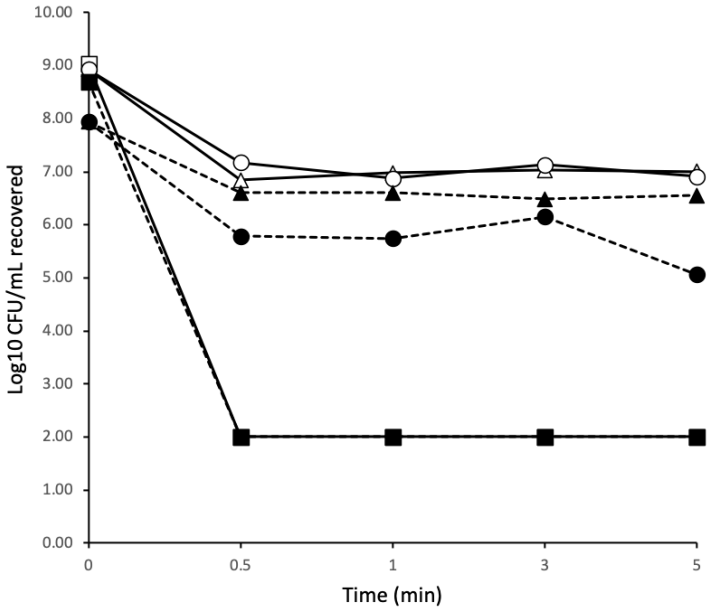


FIG. 2 Inactivation kinetics of *E. coli* **a)** 1B2 and **b)** 13P5 in the presence of CHX concentrations of 0.002 (circle), 0.007 (triangle) or 20 mg/ml (square). Isolates were pre-exposed to no CHX (white) or 0.0075 mg/ml CHX (black). (data based on 3 biological repeats)

a)



b)

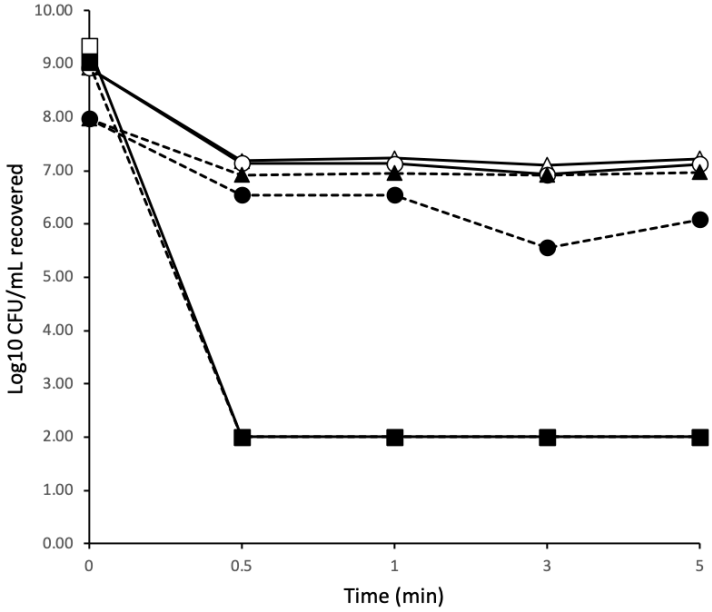
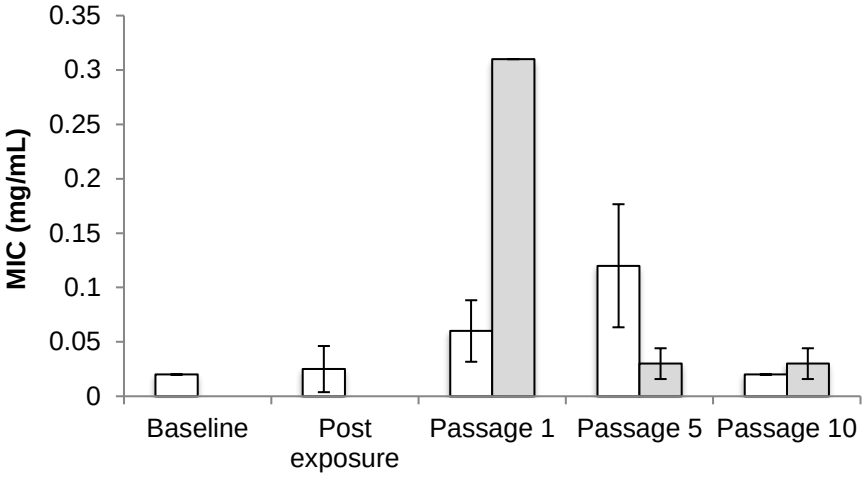


FIG 3 Average MBC values for *E. coli* 13P5 and 1B2 before and after 1, 5 and 10 passages in CHX 0.002 mg/ml (grey) or in broth only (white). Isolates were originally exposed to 0.0075 mg/ml CHX (corresponding to CHX concentration recovered from surfaces after 168 h). **a)** *E. coli* 13P5 and **b)** *E. coli* 1B2. Standard deviation of the mean is shown. (data based on 3 biological repeats)

a)



b)

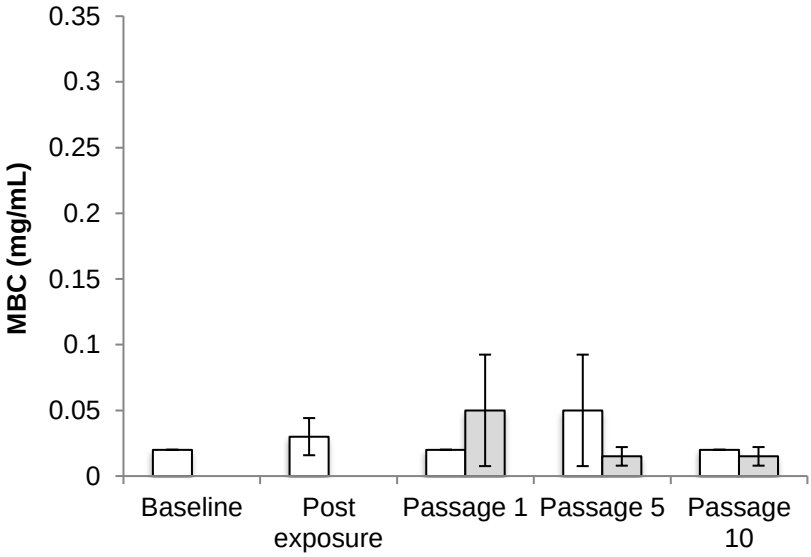
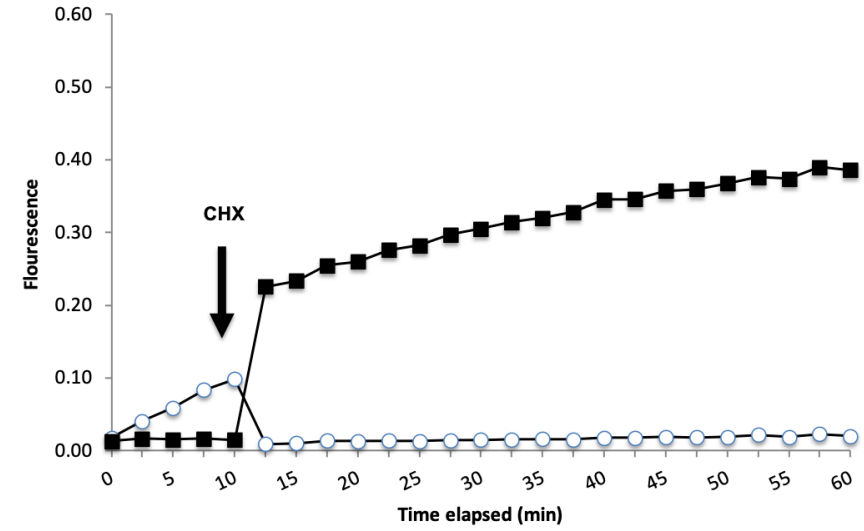
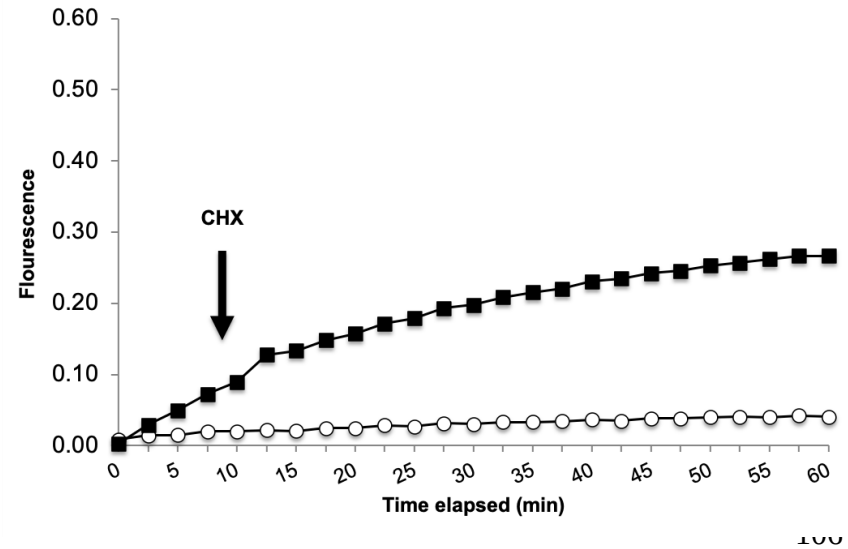


FIG 4 Trace graphs showing relative fluorescence values recorded for *E. coli* exposed to CHX after 10 min, and with or without the addition of CCCP. CHX 0.00005 mg/ml (circle), CHX 0.00005 mg/ml + CCCP (square). **a)** *E. coli* ATCC25922; **b)** *E. coli* IB2 and **c)** *E. coli* 13P5

a)



b)



c)

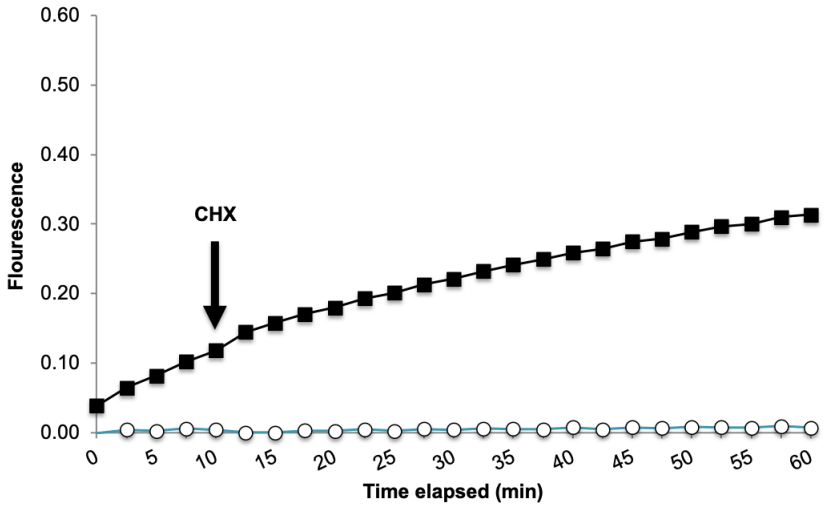
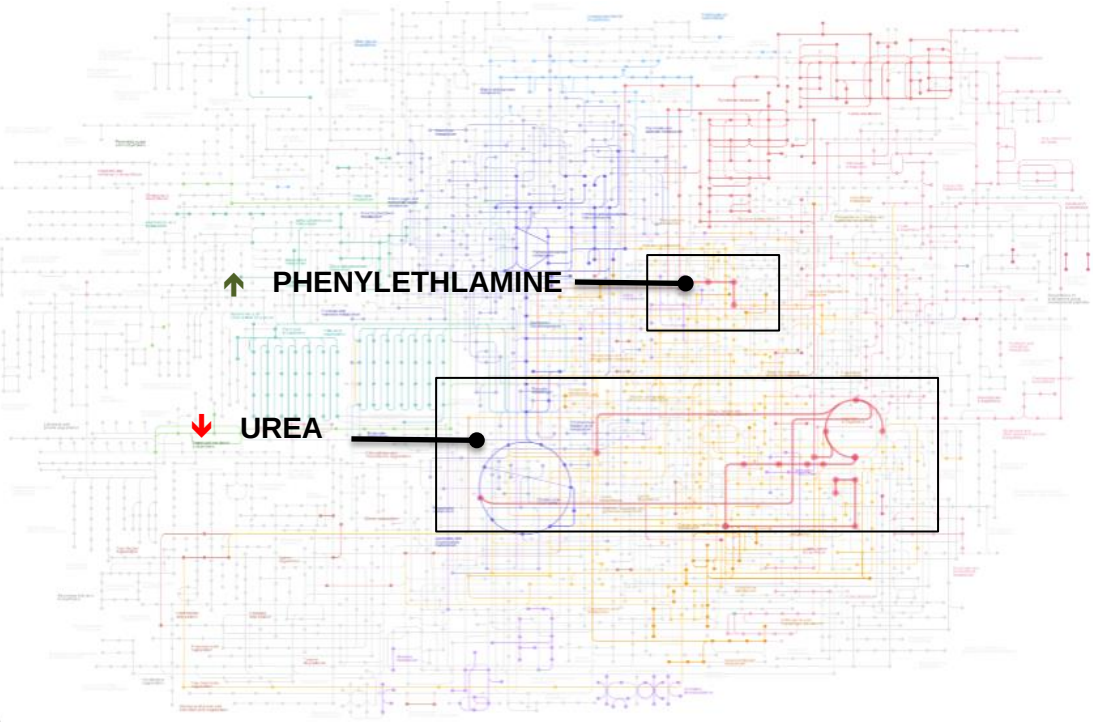

0.60
0.50
0.40
0.30
0.20
0.10
0.00
Flourescence
CHX
0
5
10
15
20
25
30
35
40
45
50
55
60
Time elapsed (min)

FIG 6 Metabolic network of *E. coli* K12 (produced with: https://www.genome.jp/kegg; accessed on: 23.07.20). Location of pathways that include substrates involved in observed metabolic changes (n=2). **a)** Comparison between bacteria not exposed to CHX and exposed to 0.00005 mg/ml CHX. **b)** Comparison between bacteria not exposed to CHX and exposed to 0.002 mg/ml CHX.

↑= increased metabolism of substrate ↓= decreased metabolism of substrate

a)



b)