

1 Heavy Metal Susceptibility on *Escherichia coli* from Urine Samples from Sweden,
2 Germany and Spain

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13 Heavy metal resistance in *Escherichia coli* isolates

14 Key words: arsenic, heavy metal resistance, antibiotic resistance, *Escherichia coli*

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24 **Abstract**

25 Antimicrobial resistance is a major health care problem, with the intensive use of
26 heavy metals and biocides recently being identified as potential contributing factors to
27 the aggravation of this situation. This study investigated heavy metal susceptibility
28 and genetic resistance determinants in *Escherichia coli* isolated from clinical urine
29 samples from Sweden, Germany and Spain. A total of 186 isolates were tested for
30 minimal inhibition concentration to sodium arsenite, silver nitrate and copper (II)
31 sulphate. In addition, 88 of these isolates were whole genome sequenced for the
32 characterization of their genetic resistance determinants and epidemiology. For
33 sodium arsenite, the isolates could be categorized into a resistant and a non-resistant
34 group, based on MIC values: isolates of the resistant group exhibited the
35 chromosomal *ars* operon and belonged to non-B2 phylogenetic groups; in contrast,
36 within the B2 phylogroup, no *ars* operon was found and the isolates were susceptible
37 to sodium arsenite. Two isolates also harbored the silver/copper resistance
38 determinant *pco/sil* and belonged to sequence types ST10 (phylogroup A) and ST295
39 (phylogroup C). The ST295 isolate had a silver nitrate MIC ≥ 512 mg/L and
40 additionally produced extended spectrum beta-lactamases. To our knowledge, this is
41 the first study that describes the distribution of the arsenic resistance operon *ars*
42 within phylogroups of *E. coli* isolated from patients with urinary tract infections. The
43 arsenic operon *ars* was only present in all non-B2 clades, which have previously been
44 associated with the environment and commensalism in both humans and animals,
45 while B2-clades lacked the *ars* operon.

46 **Introduction**

47 Infections with multi-resistant bacteria have rapidly increased in both hospitals and

48 the community during the first decades of the 21st century. Extended-spectrum β -
49 lactamase (ESBL)-producing members of the *Enterobacteriaceae* family are part of
50 this development. They account for two thirds of the in-hospital mortality in Europe, a
51 proportion which is increasing (1). One of its most important members is *Escherichia*
52 *coli*, a common colonizer of the intestinal tracts of humans and animals and the
53 leading cause of urinary tract infections (UTIs) and Gram-negative sepsis (2). An
54 association between the use of heavy metals and the development of antibiotic
55 resistance has been recognized since at least the 1970s (3, 4). Heavy metals are used
56 as antiseptics, disinfectants, and preservatives in health care and consumer products
57 (5, 6), are also used in animal food production (7, 8), and accumulate as part of
58 industrial waste in soils (9, 10). Thus, these substances significantly contribute to
59 environmental pollution, with potential harm to wildlife and, consequently, humans
60 (9-11). The human microbiota is increasingly exposed to these chemicals through a
61 wide range of products, drinking water and the food chain (5, 11).
62 Genetic determinants leading to resistance to arsenic, copper or silver are widely
63 distributed in environmental bacteria, but also in human pathogens (6, 12). Likewise,
64 beta-lactamases are widespread within many bacterial taxa and, due to increased
65 antimicrobial selection pressure, some of these chromosomal genetic determinants
66 have become associated with mobile genetic elements. For example, the chromosomal
67 extended spectrum beta-lactamase CTX-M from *Kluyvera* sp. has been at least
68 mobilized once and then successfully spread to *E. coli* and *K. pneumonia*, causing the
69 ongoing CTX-M pandemic (13, 14). Similar to antimicrobial resistance, there are
70 reports of heavy metal resistant bacteria related to the (mis)use of these substances in
71 animal husbandry (8, 15) and hospital environments (16-18).
72 A great concern is the potential co-selection of resistance to antimicrobials caused by

73 exposure to heavy metals. There are reports of isolates with a combined resistance to
74 both antibiotics and heavy metals (6). In the human gut, heavy metal exposure levels
75 are sub-lethal for bacteria, making them possible resistance driving mechanisms (19).
76 Non-specific mechanisms such as reduced permeability/uptake and enhanced efflux
77 (20, 21) or shared mobile genetic elements (15, 22-24) that are able to mediate
78 resistance to both heavy metals and antimicrobials. However, although such co-
79 selection seems likely (15, 22, 23, 25), this is poorly understood. Worryingly, clinical
80 isolates with combined heavy metal and antimicrobial resistance have regularly been
81 found to be involved in hospital outbreak situations (18, 23, 26). It is hard to predict
82 what impact increased exposure to heavy metals has on the development and spread
83 of new potent clinical isolates, especially when resistance data for heavy metals is
84 only infrequently obtained.

85 Here we investigate the resistance to a several heavy metals and antibiotics
86 among *E. coli* isolated from urine samples, collected from patients at three hospitals
87 situated in different parts of Europe. Using whole genome sequence (WGS) analysis,
88 parts of the isolate collection was further characterized regarding genetic
89 antimicrobial resistance determinants, and clinically important epidemiological
90 markers.

91

92 **Material and methods**

93 *Bacterial isolates*

94 A total of 186 *E. coli* isolates from urine samples were included in the study. These
95 were collected during spring and early summer 2016 from three hospitals: Falun
96 (Sweden, May/June 2016); Bautzen (Germany, March/April 2016); and Gandía
97 (Spain, April – July 2016). Each participating clinic collected *E. coli* from female and

98 male patients successively until 100 female isolates were recovered. Since UTIs are
99 more common in females than in males, more female isolates were collected than
100 male isolates during the collection periods. To avoid an imbalance by gender, isolates
101 from females were randomly chosen to match the number of male isolates as follows:
102 30 isolates from each gender in Falun; 29 isolates from each gender in Bautzen; and
103 34 isolates from each gender in Gandía. The patient's age, gender and hospital ward
104 were recorded and the isolates anonymised; therefore no consent from the patients
105 was required.

106 Standard laboratory procedures and automated species identification systems
107 were used to identify the bacteria to the species level (BD Phoenix™ Automated
108 Microbiology System (BD, USA) in Gandía, MicroScan Walkway (Beckman Coulter,
109 USA) in Bautzen, Maldi TOF (Bruker Daltonics, USA) in Falun). Isolates were frozen
110 as glycerol stock at
111 -80 °C prior to further analysis.

112 *Susceptibility to heavy metals and antibiotics*

113 **Antibiotic susceptibility testing.** Antibiotic susceptibility testing was performed with
114 the disc diffusion method, as recommended by EUCAST (www.eucast.org). The
115 following antibiotics were included: ampicillin; piperacillin-tazobactam; mecillinam;
116 cefadroxil; cephalexin; cefepime; cefotaxime; ceftazidime; ceftibuten; cefuroxime;
117 ertapenem; meropenem; imipenem; aztreonam; ciprofloxacin; nalidixic acid;
118 gentamicin; tobramycin; amikacin; tigecycline; nitrofurantoin; trimethoprim;
119 trimethoprim-sulfamethoxazole; and chloramphenicol. The isolates were categorized
120 into susceptible, intermediate, or resistant, using the species-related breakpoints
121 defined by EUCAST. The first step criterion for testing for production of ESBL was
122

123 resistance to cephadroxil (inhibition zone < 12 mm) or cefalexin (inhibition zone
124 < 14 mm). To confirm ESBL-production phenotypically, the disc diffusion synergy
125 test with clavulanic acid and cefotaxime, ceftazidime and cefepime was used
126 (www.nordicast.org).

127 **Heavy metal susceptibility testing.** To test the susceptibility to heavy metals and
128 biocides, the minimal inhibition concentrations (MICs) were determined in a
129 microdilution assay (100 μ L), according to ISO 20776-1:2006 with the exception that
130 Iso-Sensitest broth (Oxoid, UK) was used. The following substances and
131 concentration ranges were included: sodium arsenite (4 – 2048 mg/L); silver nitrate
132 (4 – 128 mg/L); and copper(II) sulphate (128 – 4096 mg/L) (all Sigma-Aldrich,
133 USA). All isolates with elevated MIC-values for silver nitrate \geq 64 mg/L were
134 retested in the macro dilution format (1 mL) in glass tubes. Stock solutions were
135 prepared freshly at all times. Serial dilutions were inoculated with bacteria within 2 h
136 after their preparation. Following an overnight culture in 1.5 mL Luria-Bertani broth
137 (Sigma-Aldrich, USA) in room atmosphere at 35°C, a bacterial suspension resulting
138 in a final inoculum of 5×10^4 cfu for micro dilution and 5×10^5 cfu for macro dilution
139 was prepared. The plates and tubes were incubated for 18-20 h in room atmosphere at
140 35°C and the MIC-values were read as the lowest concentration yielding no visible
141 growth. *E. coli* ATCC 25922, *Enterobacter cloacae* CCUG 38138 and *Klebsiella*
142 *pneumoniae* ATCC 700603 were used as control strains.

143
144 *Whole genome analysis*

145 **DNA preparation and whole-genome sequencing.** A randomly chosen sub-
146 collection of the susceptibility tested isolates ($n = 96$) were analyzed by whole
147 genome sequencing (WGS). One colony of each isolate was incubated in 2 mL Luria-

148 Bertani broth for 8 hours at 37°C. DNA was prepared with a Wizard® Genomic DNA
149 Purification Kit (Promega, USA), according to the manufacturers recommendations
150 for Gram negative bacteria, with the exception that DNA was rehydrated with 10 mM
151 Tris-HCl (pH 8.0). The quality and quantity of the extracted DNA was assessed by gel
152 electrophoresis, spectrophotometry (Nanodrop, ThermoFisher) and Quant-iT dsDNA
153 BR assay and a Qubit instrument (Invitrogen, USA). After standardizing the DNA
154 extracts, the samples were transferred to Oxford Genome Center for library
155 preparation and WGS. Briefly, fragmented DNA was end-repaired, A-tailed, adapter-
156 ligated and amplified using Nextera DNA library Prep (Illumina, USA). Sequencing
157 was done on an Illumina HiSeq4000 platform, generating 150 bp paired-end reads.
158 **Sequence Analysis.** The read quality was assessed using FastQC software (v0.11.4,
159 <http://www.bioinformatics.babraham.ac.uk>) according to the developers'
160 recommendations. Species identification was performed using the ribosomal MLST
161 (rMLST) speciation tool available at pubmlst.org/rmlst. This tool searches for exact
162 matches to sequences defined in the rMLST allele library that have been derived from
163 more than 7,000 bacterial species. Allelic matches are cross-referenced with a large
164 curated set of bacterial isolates to determine the most likely species present in the
165 DNA sample. Illumina short reads were mapped to databases for resistance genes
166 (ARG-ANNOT, V2 (December 2015)) (27) for antibiotic resistance genes and a
167 database for mainly plasmid-borne heavy metal resistance genes that have been
168 thoroughly described (Supplementary table S1), using *srst2* (v0.2.0) (28).
169 The isolates were assigned to the main *E. coli* phylogenetic groups on the basis of
170 their clustering in a neighbor-joining tree from ribosomal MLST-alleles (rMLST).
171 Sequences were assembled using Velvet (v1.2.10) and VelvetOptimiser software
172 (v2.2.4) sampling all odd kmer lengths from 21 to 149. The default optimization

parameters were used, together with a minimum contig size of 200 bp and the scaffolding option switched off. Using the BIGSdb database software (29), multi-locus sequence typing was performed according to the seven gene Achtman scheme (30). A neighbor joining tree was constructed for rMLST (31) allele nucleotide sequences of the study isolates and the *E. coli* reference collection (ECOR, sequences were obtained from the ENA/SRA/DDBJ databases) (32, 33). Concatenated sequences for the rMLST scheme were retrieved, aligned with MAFFT (v7.271) (34) and the tree was calculated using PHYLIP (v3.695) (35). Paralogous loci were excluded (BACT000060, BACT000065), resulting in 51 concatenated ribosomal loci for the rMLST. The dataset was then bootstrapped 500 times with PHYLIP SEQBOOT, followed by calculations of distance matrices with PHYLIP DNADIST and neighbor joining trees with PHYLIP NEIGHBOR, and a consensus tree using PHYLIP CONSENSE.

Draft genomes were annotated with the annotation software Prokka (v1.12-beta) (36) with default settings followed by a parsing of the output files for the *ars* operon (coding sequences) for each isolates using the module SeqIO in Biopython (37) (parsing script available on request). For *ars* positive isolates, a neighbor joining tree was calculated based on concatenated sequences for *ars*RBC and rMLST in accordance to the procedure described for the rMLST tree above. Both trees were compared after constructing a tanglegram with the Dendroscope software (v3.5.9, www.ab.informatik.uni-tuebingen.de/software/dendroscope).

Data handling and statistics

The results from the susceptibility testing to the antimicrobial substances and from the short read mapping (resistance determinants) were analyzed with focus on co-

198 resistance and influence of gender, age and origin. In addition, the frequency of
199 resistance determinants and phenotypes were evaluated with regard to phylogenetic
200 groups and sequence types.
201 Associations between the variables were measured using Pearson correlation (phi
202 coefficient for binary variables). For all variable-pairs with correlation or phi
203 coefficients of > 0.5 or < -0.5 , the correlation was checked for meaningfulness. For
204 meaningful associations, hypotheses were formulated, the odds ratio (OR) was
205 calculated and statistical significance was evaluated by Fisher's exact test. Results
206 with $OR > 2$ or $OR < 0.5$ and a $p < 0.05$ as statistical significance level were included.
207 Phylograms were visualized using EvolView (<http://www.evolgenius.info>),
208 histograms and gene synteny using the statistical software R (v3.3.3, R Foundation for
209 Statistical Computing, Vienna, Austria, www.R-project.org, packages ggplot2 and
210 genoPlotR). The location of the *ars* operon on the *E. coli* chromosome was visualized
211 using the BLAST Ring Image Generator BRIG (v0.95)(38) with the finished
212 chromosome sequence of *E. coli* MG1655 (Genbank accession U00096.3) as
213 reference.

214

215 **Data Availability**

216 All 88 paired end reads are available from the ENA/SRA/DDBJ databases under the
217 project reference PRJEB17631, for detailed accession numbers see supplementary
218 table S4.

219

220 **Results**

221 *Susceptibility testing*

222 **Antimicrobials.** Resistance to the tested antibiotics was rare with the exception of

ampicillin, trimethoprim, co-trimoxazole, and ciprofloxacin. For these antibiotics, the lowest rates of susceptible isolates were found in Spanish isolates. A total of 19 isolates (9.9%) produced ESBL type enzymes. Nine isolates were from Spain, three from Germany, and seven from Sweden. Eleven of these isolates were included in the whole genome sequence analysis. Of these, all isolates carried ESBL of CTX-M type: CTX-M-1 ($n=1$), CTX-M-9 or CTX-M-9-like ($n=2$), CTX-M-15 ($n=5$), CTX-M-27 ($n=1$), CTX-M-55 ($n=1$), and both CTX-M-15 and CTX-M-27 ($n=1$).

Additionally, OXA-1 enzymes were found in three isolates with CTX-M production and one isolate without ESBL-phenotype (Supplementary table S2).

MIC-distributions of heavy metals. The MIC ranges for the 186 isolates analyzed were as follows: sodium arsenite 8 to 2048 mg/L, silver nitrate 8 to >512 mg/L, and copper(II) sulphate 512 to 2048 mg/L (Table 1). For sodium arsenite, the study isolates could be categorized into two groups with a cutoff at 128 mg/L, 67 isolates had a MIC > 128 mg/L and 119 isolates had a MIC \leq 128 mg/L. For silver nitrate, isolate WTCHG-295 had a MIC > 512 mg/L when retested in macro dilution (Supplementary figures S1.a-c).

Summary of the whole genome sequenced isolate collection

Genome assembly. Whole genome sequencing was performed on a sub-collection of 96 isolates. High-quality short reads for *E. coli* were obtained for 88 isolates, with an average coverage of 108 [standard deviation (SD): ± 23]. Draft genomes were obtained for all sequenced isolates, using kmer values from 99 to 129 [median of 121], resulting in a median contig number of 162 [min – max: 49 – 550] and a median N50 of 208,148 [min – max: 59,334 - 681,908]. The average genome length was 5,139,676 [SD: $\pm 198,236$].

248 **Phylogenetic groups and sequence types.** Out of the 88 whole genome sequenced
249 isolates, 55 isolates (62.5%) were assigned to phylogroup B2, 13 isolates (15%) to
250 phylogroup D and 18 isolates to (20%) B1, F, C, E or A. The most frequent sequence
251 types were ST131 (phylogroup B2) ($n = 15$), ST73 (B2) ($n = 13$), ST69 (D) ($n = 6$),
252 ST95 (B2) ($n = 5$), ST1193 (B2) ($n = 5$), ST127 (B2) ($n = 4$) and ST141 (B2) ($n = 3$).
253 Among the CTX-M-producing isolates, the most frequent sequence types were ST131
254 (B2) (CTX-M-15 ($n = 4$), CTX-M-27 ($n = 1$), CTX-M-15 and -27 ($n = 1$); and for the
255 remaining ESBL-producing isolates ST38 (D, CTX-M-9), ST156 (B1, CTX-M-1),
256 ST115 (D, CTX-M-9), ST23 (C, CTX-M-55) and ST295 (C, CTX-M-15). The
257 sequence types were equally distributed among countries, patient gender, and age. No
258 statistically significant associations of antimicrobial susceptibility to heavy metals
259 were observed, however, both *pco/sil* and *ars* resistance determinants showed an
260 association with non-B2 phylogroups.

261 *Genetic and phenotypic resistance to heavy metals*

262 All isolates with sodium arsenite MIC-values of more than 128 mg/L exhibited the
263 chromosomal *ars* operon *arsRBC* or *arsRDABC* ($n = 36$). In contrast, all study
264 isolates that lacked the *arsRB* determinants had MIC-values below 128 mg/L. None of
265 the isolates investigated harbored plasmid-borne *ars* operons. The complete
266 *arsRDABC* operon was found in isolates belonging to the ST69 complex ($n = 7$) and
267 in one isolate assigned to ST1836 (WTCHG_243), both these sequence types belong
268 to phylogroup D. The determinants *arsRBC* were found in all other non-B2 groups.
269 These findings were confirmed with the isolates within the ECOR-strain collection,
270 with the exception that no *ars*-operon was found in ECOR-42 (ST64) (Figure 1).

272 Two isolates were positive for silver resistance determinant *sil/pco*. One

273 belonged to ST10 (A) (WTCHG-248) and the other (WTCHG-295) to ST295 (C), the
274 latter of which also harbored CTX-M-15 enzymes and was positive for the quinolone
275 efflux pump *qnrS*. The MIC-values for silver nitrate in microdilution were 16 mg/L
276 for both, but isolate WTCHG-295 had a MIC > 512 mg/L when retested in
277 macrodilution. Isolate WTCHG-245 even grew after one passage in > 512 mg/L silver
278 nitrate.

279

280 *ars operon*

281 A closer examination of the genetic context of the *ars* operon showed, that in *E. coli*
282 MG1655, the *ars* determinants were located around 275 kbp from the replication
283 origin (Figure 2). The gene content of the operon and the surrounding genes showed
284 association with phylogenetic groups, which has been shown in Figure 3 for one
285 representative isolate per phylogroup. In B2 phylogroup isolates, the region between
286 *gor* and its following open reading frame (ORF) up until *slp* contained only the
287 arsenic reductase *arsC*. Despite the fact that the *ars* operon of the isolates belonging
288 to phylogroups B2, ST69 (D) and all other sequence types of phylogroup D/F was
289 different, all of these isolates possessed the genetic determinants *hemSR* and *hmuTUV*
290 around 5 kbp downstream of the *ars* operon, genes that are thought to be involved in
291 iron transport (Supplementary Table S3). Within this study, phylogroups E and C were
292 underrepresented, but the composition of the genetic environment of the *ars* operon of
293 these phylogroups strongly resembled those from the neighboring groups of the
294 rMLST tree. Phylogroup E isolates either possessed the gene order of B1 or B2, and
295 phylogroup C isolates either the gene order of phylogroup A or B1 isolates (Figure 3).
296 The phylogenetic categorization based on concatenated ribosomal sequences and that
297 derived from the *arsRBC* genes resulted in identical phylogroup assignments for most

298 isolates (Figure 4).

299

300 Discussion

301 In the present study, heavy metal resistance in *E. coli* from urine samples was strongly
302 associated with phylogenetic clades, which have previously been associated with the
303 environment and commensalism, and thus, reflect the selective pressure the bacteria
304 are exposed to. A particularly strong association was found for arsenic, where isolates
305 could be categorized into two sub-groups according to their arsenic susceptibility. The
306 isolates in the more resistant group possessed the chromosomal *ars* operon and
307 belonged to non-B2 phylogenetic groups within *E. coli*, which have previously been
308 associated with environment and commensalism, whereas the arsenic susceptible
309 group did not. Although less frequent, resistance to silver mediated by the
310 copper/silver resistance determinant *pco/sil* in *E. coli* also occurred only in non-B2
311 phylogroups.

312 Arsenic is a heavy metal that is ubiquitous in the environment, where bacteria are
313 exposed to it in varying levels. Resistance to arsenic is therefore common in a wide
314 range of bacteria and can be found in many species within the Enterobacteriaceae
315 family (6). Within the study isolates, 36% had an arsenic-resistant phenotype, with a
316 suggested ECOFF-value at 128 mg/L. This phenotypic arsenic resistance was
317 associated with the occurrence of the chromosomal *ars*-operon. Interestingly,
318 although having been previously described mainly in plasmids (39-41), there were no
319 plasmid-borne arsenic resistance genes in these isolates. Furthermore, isolates with
320 increased tolerance to arsenic all belonged to non-B2 phylogroups. Due to
321 environmental pollution, humans and the gut microbiota are exposed to increasing
322 levels of arsenic through drinking water and food chain (9, 11); however, while the

enzymes mediated by the bacterial *ars*-operons reduce toxicity of their ligands As(III) and As(V), human exposure is mainly to arsenic bound in organic compounds (11). Furthermore, clearance of absorbed arsenic from the human body is mainly via the renal route, with only minimal hepatobiliary excretion (11). Therefore, it is likely, that the gut microbiota is exposed to comparatively low arsenic levels, which additionally are bound in compounds that are not ligands of the enzymes encoded by the *ars*-operon.

E. coli belonging to phylogroup B2 are known to be associated with extraintestinal infections, and thus, are well adapted to the human host (2). Hence, it is consistent that the lack of tolerance to arsenic in *E. coli* belonging to the B2 phylogroup does not lead to a survival disadvantage and thus the deletion of *arsR* and *arsB* might have been beneficial to this group. In contrast, our findings suggest that *E. coli* belonging to commensal and environmental phylogroups, with a higher risk of exposure to Ars(III) or Ars(IV) redox states, may well have a survival advantage from arsenic resistance factors encoded by the *ars*-operon (9). Interestingly, there was a small group of isolates within phylogroup D that in addition to *arsRBC* also carried *arsD* and *arsA*. Both *arsD* and *arsA* are thought to give competitive benefits in environments with high arsenic levels (42) and increased fitness compared to isolates with only *arsRBC* genes (43); however, among our study isolates, there was no significant difference regarding MIC for sodium arsenite between isolates with and without *arsD* and *arsA*.

All isolates possessed the arsenate reductase encoded by *arsC* which catalyzes the reduction of arsenate to the less toxic arsenite (9). The genetic environment of *arsC* in combination with the finding that the phylogeny of the concatenated sequences of ribosomal genes and *arsRBC* displayed the same phylogeny, supported the conclusion

348 that *arsR* and *arsB* have been lost in phylogroup B2, rather than acquired in non-B2
349 isolates. While the deletion of the arsenite transporter *arsB* and its transcriptional
350 repressor *arsR* seemed to be a beneficial event for phylogroup B2 *E. coli* isolates, the
351 enzyme *arsC* remained in all clades. Interestingly, the location of the *ars* operon on
352 the *E. coli* chromosome is in close proximity to the origin of replication. The position
353 of genes on the chromosome has previously been shown to influence gene
354 expression,(44) and proximity to the replication origin potentially increases
355 expression (45). Thus, the proximity to the replication origin may indicate a particular
356 biological relevance of *ars* genes to the bacteria. Although *arsC* was found in all
357 isolates, it remains unclear whether that can be explained by the ubiquitous
358 occurrence of arsenic in the environment, or whether *arsC* has an additional, yet
359 unknown biological function.

360 While arsenic is one of the most ubiquitous of all environmental toxic compounds,
361 silver is rapidly bound to proteins, halogens or it is reduced to atomic silver, and thus
362 less abundant in its toxic form compared to arsenic. This is reflected in the
363 dissemination of resistance systems to silver ions: the plasmid-borne silver resistance
364 determinants *sil/pco* were regularly found where silver compounds are used or
365 released (46-48), and less frequently observed in *E. coli* populations from remote
366 areas (22). This is of particular interest, as silver compounds are still used as
367 antimicrobials in hospitals and patient care. The interest in copper as antimicrobial
368 has also renewed against the background of increasing antibiotic resistance. In
369 contrast to arsenic and silver, copper is essential for cell physiology, and thus
370 sophisticated systems to maintain homeostasis are present in bacterial cells. (49) The
371 plasmid-borne *pco* operon has previously been shown to increase tolerance to copper
372 ions, (50) but not to copper alloys, which are increasingly popular as antimicrobial

373 surfaces materials in hospital environments (51). Copper resistant bacteria have been
374 described as an emerging problem within animal husbandry, where copper is widely
375 used as food additive (52). However, while silver resistant isolates are frequently
376 isolated from patients, to our knowledge, this has not been described for copper
377 resistance.

378 Arsenic, silver and copper are potential resistance driving factors in *in-vitro* studies
379 (53, 54). This is also clinically relevant, since isolates with *sil/pco* determinants
380 regularly co-produce ESBL enzymes (15, 22, 23). The studies referred above
381 specifically highlighted the role of horizontal gene transfer through plasmids, and thus
382 co-selection for spread of resistance determinants. Similarly to previous findings, one
383 of the *sil/pco*-positive isolates from this study also produced CTX-M-15 enzymes.

384 The present study emphasizes the role of clones in spread of resistance: phylogroup
385 B2 has previously been found being less resistant to antimicrobials (55), which is in
386 line with our findings regarding arsenic susceptibility. Worryingly, environmental
387 pollution with arsenic has a significant influence on the gut microbiota, leading to a
388 shift towards increased resistance (56). One can only speculate about the contribution
389 of this selective pressure to the risk of development of new virulent and multi-
390 resistant clones.

391 In conclusion, the distribution of arsenic and silver resistance in *E. coli* is
392 concentrated among *E. coli* phylogroups associated with the environment, rather than
393 specific pathotypes. Thus, the substantial environmental pollution in some areas of the
394 world with arsenic, silver and antimicrobials may pose a risk for the development of
395 isolates carrying multiple resistance genes. The phylogenetic relationships described
396 in the present study may contribute to a better understanding of the resistance
397 potential and the selection mechanisms of pathogenic *E. coli* in human and

398 veterinarian settings.

399

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408

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577 **Appendix**578 **Figures**579 **Tables**

580 **Table 1.** Results from MIC-testing for biocides and heavy metals on study isolates
581 and three reference strains.

	MIC (mg/L) range	M50 (mg/L)	M90 (mg/L)	MIC ranges (mg/L) Reference strains ¹		
	Study isolates (n = 186, <i>E. coli</i>)			<i>E. coli</i> (ATCC 25922)	<i>E. cloacae</i> (CCUG 38136)	<i>K. pneumonia</i> (ATCC 700603)
Sodium arsenite	8 -2048	64	1024	32-128	32-128	1024
Silver nitrate	8 – >512	32	32	16-32	16-32	16-32
Copper (II) sulphate	512 – 2048	1024	1024	1024-2048	1024-2048	1024-2048

582 ¹ The presented MIC ranges for all reference strains are based on micro dilution
583 assays only.

584

585 **Figures**

586 **Figure 1.** Neighbor joining tree based on 51 ribosomal MLST allele sequences for
587 study isolates and the ECOR strain collection. Colored leaves in the tree indicate
588 phylogenetic groups (F, D, B2, E, B1, C, A). Columns from left to right: clonal
589 complex and sequence types according to seven gene MLST (Achtman scheme), MIC
590 for sodium arsenic (red rectangle indicates the MIC-value for respective isolate, grey

591 rectangles indicate place holders, blank lines mean no data available), *ars* operon
592 (purple - *arsR*; blue - *arsB*; orange - *arsC*; yellow - *arsD*; red - *arsA*), arrows indicate
593 orientation in draft genome (➔ forward, ➠ reverse).

594

595 **Figure 2.** Illustration of the location of the *ars* operon on the chromosome of *E. coli*
596 MG1655. The image was constructed using BRIG. Inner to outer circle: scale (kbp);
597 GC skew of *E. coli* MG1655; positions of ribosomal genes, macrodomains Ori, Right,
598 Ter and Left and less structured regions according to Valens *et. al.* (57)

599

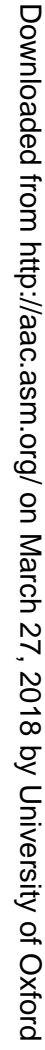
600 **Figure 3.** Gene synteny of the *ars* operons and their genetic environment for five
601 representative isolates per phylogroup: WTCHG_213 (A), WTCHG_203 (B1),
602 WTCHG_211 (D), WTCHG_284 (D), WTCHG_272 (B2).

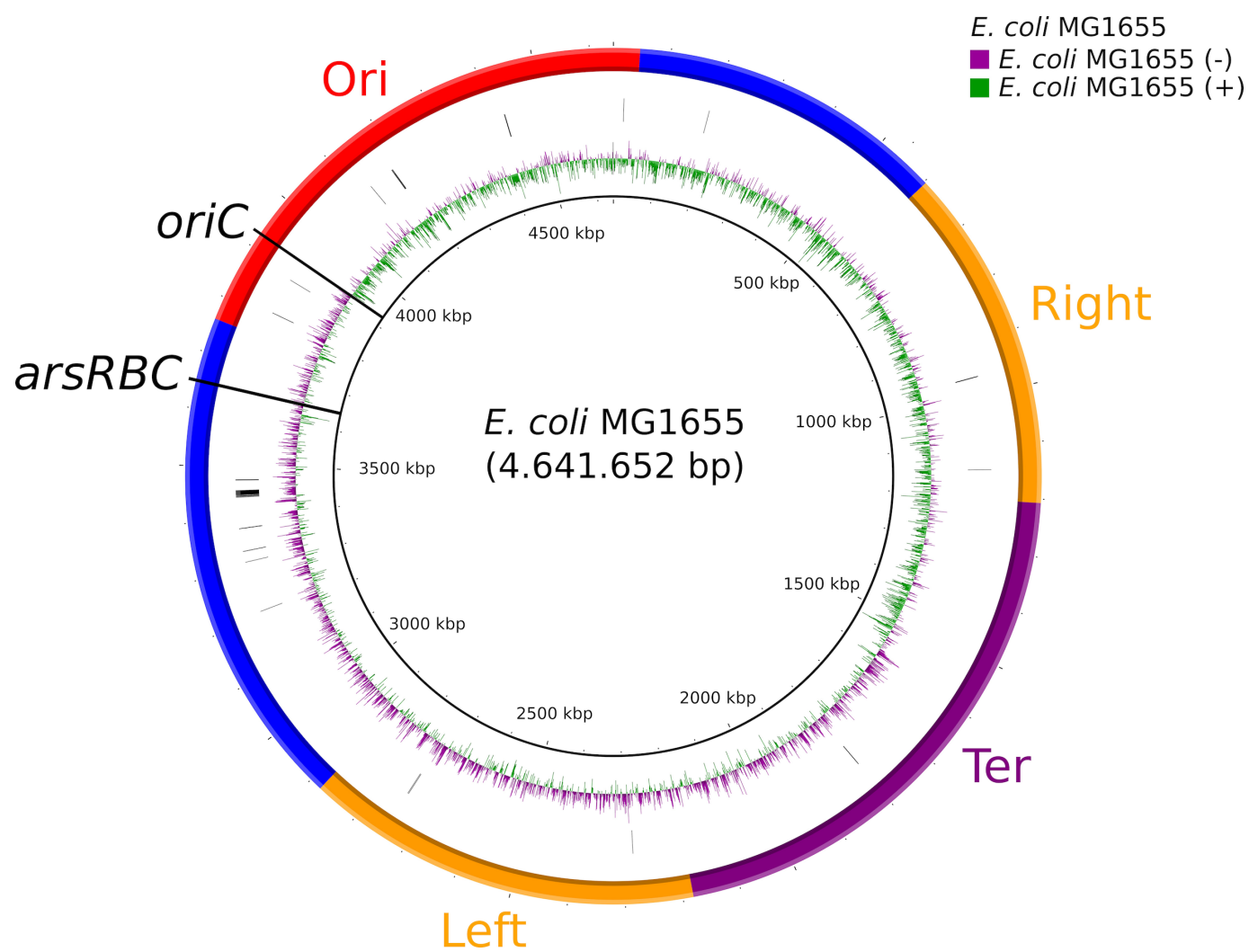
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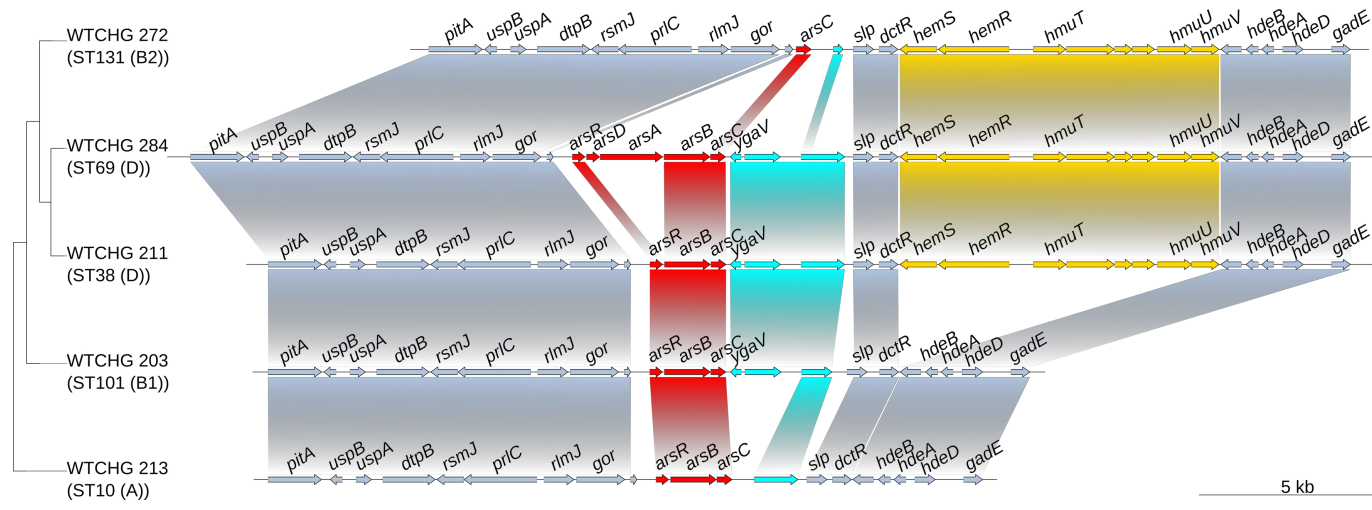
604 **Figure 4.** Tangle plot of neighbor joining trees for rMLST and *arsRBC*. The rMLST
605 tree is based on 51 ribosomal MLST allele sequences (left side) and concatenated
606 *arsRBC* sequences (right side) for *ars* positive isolates from the study isolate
607 collection and the ECOR strain collection. Colors and letters indicate phylogenetic
608 groups (F, D, F, E, B1, A).

609

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rMLST

