Short communication: Heat-resistant *Escherichia coli* as potential persistent reservoir of extended-spectrum β -lactamases and Shiga toxin-encoding phages in dairy

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ABSTRACT

Here we report the isolation of heat-resistant *Escherichia coli* from raw milk cheeses. Detection of the heat-resistance markers *clpK* and *orfI* by PCR was followed by phenotypical confirmation of increased heat-resistance. These strains were Shiga toxin-negative and, although several were found to be multidrug resistant, no plasmids encoding extended-spectrum β -lactamases (ESBL) were found in any of the isolates. The aim of this study was to assess the potential of these strains to acquire ESBL plasmids and a modified Shiga toxin-encoding phage. Only 4 ESBL-encoding, heat-sensitive *E. coli* strains were isolated from 1,251 dairy samples (2/455 raw milk and 2/796 raw milk cheese samples). One incompatibility group FII plasmid (CTX-M-14, 79.0 kb) and 3 incompatibility group I1 plasmids (CTX-M-15, 95.2, 96.1, and 97.8 kb) were fully sequenced and de novo assembled. All 4 plasmids are readily transferred to heat-resistant *E. coli* isolates in plate matings (9.7×10^{-5} to 3.7×10^{-1} exconjugants per recipient) and, to a lesser extent, in milk (up to 7.4×10^{-5} exconjugants per recipient). Importantly, the plasmids are stably maintained during passaging in liquid media without antimicrobial pressure. The heat-resistant isolate FAM21805 was also shown to be capable of acting as donor of all 4 ESBL plasmids. In addition, 3 of 11 tested ESBL exconjugants of heat-resistant strains were lysogenized by the modified Shiga toxin-encoding phage 933W $\Delta stx::gfp::cat$. The higher fraction of heat-resistant *E. coli* (93 of 256 isolates) compared with the estimated 2% previously predicted based on genomic prevalence of heat resistance genes seems to indicate a selection advantage in the raw milk

cheese production environment. The combination of 2 factors may lead to said advantage: increased survival during thermization of raw milk (heating to subpasteurization temperatures) and increased survival rates during cheese ripening. Should these strains acquire ESBL-encoding plasmids, Shiga toxin-encoding phages, or both, these genetic elements would profit from the selection advantage of their host and become more abundant in this particular environment, which in turn could lead to an increased threat to consumers of raw milk products.

Key words: heat resistance, horizontal gene transfer, *Escherichia coli*, extended spectrum β -lactamase, dairy

Short Communication

Bojer et al. (2010) first described a novel plasmid-borne *clp* ATPase in a persistent, unusually heat-resistant nosocomial *Klebsiella pneumoniae* isolate and designated it *ClpK*. Shortly thereafter, a chromosomal heat-resistance cluster, including a close homolog of *clpK* and the heat-resistance marker *orfI* (named *thr* for thermotolerance), was described in *Cronobacter sakazakii* ATCC 29544, and subsequent screening revealed the presence of *orfI* and its strict correlation with increased decimal reduction values at 58°C (D_{58}) in other *Enterobacteriaceae*, including *Escherichia coli* (Gajdosova et al., 2011). Another study performed comparative genomic analyses of 29 *E. coli* strains and identified a ~14 kb locus of heat resistance (**LHR**), with >99% sequence identity to the previously described clusters in *Klebsiella pneumoniae* and *Cronobacter sakazakii*; the LHR was found to be present in approximately 2% of published *E. coli* whole genomes and genome shotgun sequences (Mercer et al., 2015). In dairy products, in vitro characterization of *E. coli* with regard to cheese production-relevant stresses identified several heat-resistant strains isolated from raw milk cheeses (Peng

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et al., 2012). Further studies of these strains confirmed increased heat-resistance in milk at subpasteurization temperatures, as employed during thermization, which is relevant because food safety is increased whereas natural enzymes and the flora of raw milk remain intact to provide the characteristic taste and quality of raw milk cheese (Peng et al., 2013b). Heat-resistant strains also showed increased survival rates during ripening of semihard raw milk cheese (Peng et al., 2013a). The aim of the present study was to identify further heat-resistant *E. coli* isolates from the dairy environment and assess their potential to receive and act as a potential persistent reservoir of antimicrobial resistances and virulence genes (encoding Shiga toxins).

In the course of this study, *E. coli* isolates were PCR screened for heat-resistance markers *clpK* (Bojer et al., 2010) and *orfI* (Gajdosova et al., 2011). Double PCR-positive strains were phenotypically confirmed by heat-inactivation experiments in Luria-Bertani Lennox broth (**LB**; 10 g/L of peptone, 5 g/L of yeast extract, 5 g/L of NaCl, pH 7.0). Strains exhibiting a reduction in colony-forming units of less than 1 log within 30 min at 55°C were considered phenotypically heat-resistant. Antimicrobial resistance (**AMR**) profiles were determined for 18 agents by disc diffusion experiments according to CLSI (2009), with a focus on β -lactams (Oxoid, Pratteln, Switzerland). The antimicrobials tested were gentamicin, kanamycin, streptomycin, chloramphenicol, tetracycline, nalidixic acid, ciprofloxacin, trimethoprim, sulfamethoxazol/trimethoprim (19/1), ampicillin (**AMP**), cefoxitin, cephalothin, cefuroxime, cefotaxime (**CTX**), cefepime, aztreonam, amoxicillin/clavulanic acid (20/10), and ertapenem. Phylogenetic groups were determined by quadruplex PCR (Clermont et al., 2013) and multilocus sequence typing was performed with the 7-allele scheme previously described (Wirth et al., 2006; University of Warwick, 2016).

Plasmids of strains encoding extended-spectrum β -lactamases (**ESBL**) were isolated using the PureYield Plasmid Maxiprep System (Promega, Dübendorf, Switzerland) according to the manufacturer's instructions. Plasmids were sequenced by Illumina MiSeq paired-end library (2 $\times$ 250 bp reads, 550 bp insert size; Microsynth, Balgach, Switzerland) and mate-pair library (2 $\times$ 300 bp, median insert size 3 kb, up to 17 kb; Nextera mate pair sample preparation protocol, made in-house). Nextera adapters were removed using NxTrim (O'Connell et al., 2015) and sequences were de novo assembled with SPAdes v3.6.0 (Nurk et al., 2013). After manual circularization, the sequences were confirmed by mapping the raw reads back to the finished sequence and by an additional check with the error-detection tool NxRepair (Murphy et al., 2015). The average sequence coverage of the assembled plas-

mids was between 200 and 5,000 fold. Final assemblies were automatically annotated using the RAST server (Overbeek et al., 2014; RAST, 2015), and manually refined using CLC Genomics Workbench (v8.0.3, Qiagen, Hombrechtikon, Switzerland) and BLAST analysis at NCBI (BLAST, 2016). Refined annotations were exported as GenBank files, sequin format tables created using Artemis (v16.0.0; Rutherford et al., 2000), and submitted using the Sequin submission tool (v14.00; Sequin, 2016).

Horizontal gene transfer (**HGT**) experiments were done in plate matings, as previously described (Toomey et al., 2009) with slight modifications. Equal volumes (500 μ L) of donor and rifampicin (**RIF**)-resistant recipient overnight cultures grown in presence of AMP (100 μ g/mL) and RIF (200 μ g/mL), respectively, were mixed, centrifuged (12,000 $\times g$, 2 min, 25°C), and had the supernatant completely removed. Cells were resuspended in 50 μ L of dilution solution (8 g/L of NaCl, 1 g/L of peptone) and spotted on predried LB agar plates (without antimicrobials). Donor and recipient alone served as controls. After 24 h of static incubation at 37°C, spots were scraped off, cells were resuspended in 3 mL of dilution solution, and plated in duplicate (separate dilution series) on LB agar with suitable combinations of antimicrobial agents to select for donors (LB_{AMP}, 100 μ g/mL), recipients (LB_{RIF}, 200 μ g/mL), and exconjugants (LB_{AMP/RIF}, 100/200 μ g/mL). Three biological replicates were performed for each donor or recipient combination and all controls. After overnight incubation at 37°C, colonies were enumerated and exconjugant frequencies per recipient were calculated. Three exconjugant colonies of each biological replicate were PCR confirmed for presence of the heat-resistance marker gene *clpK*, thus confirming the identity of the recipient, even though frequencies of spontaneous rifampicin resistant mutants of donors were several orders of magnitude lower than exconjugant frequencies, if detected at all. The HGT experiments in milk were done in the same way with the following alterations: overnight cultures (without antimicrobials) were adjusted to a final concentration of 10⁷ cfu/mL into 25 mL of homogenized, UHT-treated milk, mixed by inversion, and statically incubated for 24 h at 37°C. All antimicrobials were obtained from Sigma-Aldrich (Buchs, Switzerland).

We tested the stability of ESBL plasmids in their wild-type hosts and in the exconjugants derived from *E. coli* strains FAM21805 and K-12 MG1655. The strains were serially passaged without antimicrobial pressure in LB medium at 37°C. Cultures were diluted 1 in 1,000 every 24 h for the first 72 h followed by a final dilution of 1 in 1,000 and an incubation of 96 h. Samples were plated in duplicate (separate dilution series) on

LB and LB_{AMP}; the experiment was done in 3 biological replicates.

The chloramphenicol-resistant *E. coli* K-12 MG1655 (933W Δ *str::gfp::cat*; Quirós et al., 2015) served as the donor of the modified phage 933W in plate matings. We used the previously generated 11 pFAM22321 ex-conjugants as recipients for the phage. Putative lysogens (selected with LB_{AMP}/chloramphenicol/RIF, 100/25/100 µg/mL) were PCR tested for the presence of *clpK* (as described above) and the chloramphenicol resistance cassette (*cat*) of the modified phage (Serra-Moreno et al., 2006). Also, *wrbA* was confirmed as the phage insertion site by PCR of the left and right junctions between the phage and bacterial genome (Serra-Moreno et al., 2007).

A total of 256 *E. coli* raw milk cheese isolates were screened for heat-resistance markers *clpK* and *orfI*, 93 (36.3%) of which were *clpK*- and *orfI*-positive (24 and 9 isolates were single positives, respectively). Of the double PCR-positive isolates, 4 were found not to be clearly heat-resistant as judged by heat-inactivation experiments. The PCR for double-positive strains thus predicted heat resistance in our phenotypic test correctly in 95.7% (89/93) of isolates tested. None of the strains screened encode Shiga toxin. Antimicrobial resistance is one of the greatest threats to public health; specifically, ESBL-producing Enterobacteriaceae are considered a major threat (CDC, 2013) and ESBL are the single most important AMR mechanism of gram-negative bacteria (Bush and Jacoby, 2010). We determined the AMR profiles of 44 double PCR-positive, phenotypically confirmed, heat-resistant strains. Nine of the 18 antimicrobial agents chosen were β -lactams due to the importance of this family of antimicrobials. Five heat-resistant strains (11.4%) are multidrug resistant (**MDR**), defined as resistant to 3 or more classes of antimicrobials, and 9 (20.5%) are resistant to at least 1 antimicrobial agent, with tetracycline resistance being the most frequent (8 strains, 18.2%). A subset of 30 strains (including all MDR strains) is given in Table 1. None of the heat-resistant strains isolated in our study show ESBL phenotypes. However, heat-sensitive, Shiga toxin-negative, ESBL-encoding *E. coli* were isolated from both raw milk (2 isolates, 455 samples, 431 *E. coli* isolates; CHROMagar *E. coli*, Labo-Life, Pully, Switzerland) and raw milk cheeses (2 isolates, 796 samples, 178 *E. coli*-positive samples, CHROMagar ECC, Labo-Life). These were all MDR strains in addition to the broad resistance to β -lactams (Table 1).

Plasmids encoding ESBL were found, sequenced, and de novo assembled for all 4 ESBL-encoding strains isolated in our study (Table 2). They each encode a narrow-spectrum TEM-1 β -lactamase and an aminoglycoside N(3')-acetyltransferase (*aac3-III*) in addi-

tion to a CTX-M family ESBL. Plasmid pFAM22321 belongs to incompatibility group (**Inc**) FII and encodes CTX-M-14, whereas the other 3 ESBL plasmids are nearly identical IncII plasmids, which harbor CTX-M-15; CTX-M-15 and -14 (in descending order) are the 2 most commonly found ESBL in humans. In food animals, CTX-M-14 is the second most common, whereas CTX-M-15 generally plays a minor role and has only been found recently (Endimiani et al., 2012, Geser et al., 2012, Seiffert et al., 2013). Both CTX-M enzymes are commonly associated with incompatibility groups F and II, among others (Carattoli, 2009). All IncII plasmids in this study feature the IncI shuffle region, which includes alternate PilV C-terminal regions (Brouwer et al., 2015). Plasmid pFAM22871_2 and pFAM23292_2 both encode 7 different tip adhesins (*shfA*, *shfA'*, *shfB*, *shfB'*, *shfC*, *shfC'*, and *shfD'*), albeit in 2 different arrangements, whereas pFAM23293_1 only encodes the adhesion variants *shfA*, *A'*, *C*, and *C'*. All 3 plasmids have *shfA'* as the tip of pilV in the consensus arrangement of the assembly. No reference sequence was required to assemble the shuffle regions, thus the arrangements given can be considered the predominant ones for each plasmid under the conditions used for cultivation and extraction. The 3 IncII plasmids are highly similar to pJIE174 (EU418931), a CTX-M-15-encoding plasmid of a patient urine *E. coli* isolate from Westmead Hospital, Sydney, Australia (phylogenetic group D, ST2495; Zong et al., 2015).

We have obtained complete sequences of additional plasmids for 3 of our ESBL isolates (Table 2). FAM22871 harbors an additional IncFIA/FIB/FII plasmid, pFAM22871_1, which encodes a TEM-1 β -lactamase as well as streptomycin, tetracycline, and sulfonamide resistance genes. For FAM23292, we found an IncFIA/FIB/FII plasmid, pFAM23292_1, the only AMR gene of which is a TEM-1 β -lactamase. None of the sequenced plasmids encodes antibacterial biocide or metal resistance genes. This is important to note, as such resistances can contribute to the spread and maintenance of resistance plasmids in absence of antimicrobials (Pal et al., 2014). The sequences of 3 plasmids have been deposited at GenBank: pFAM22321 (CTX-M-14, TEM-1, accession no.: KU288634), pFAM22871_1 (TEM-1, MDR, accession no.: KU355873), and pFAM22871_2 (CTX-M-15, TEM-1, accession no.: KU355874).

We used FAM22321 as a donor of its ESBL plasmid with 11 heat-resistant *E. coli* strains and *E. coli* K-12 MG1655 as recipients to broadly illustrate that heat-resistant *E. coli* isolated from dairy are amenable to horizontal gene transfer (Figure 1, A). The other 3 ESBL plasmids (pFAM22871_2, pFAM23292_2, and pFAM23293_1) were mated with recipients FAM21805

and K-12 MG1655 (Figure 1, A). As proof of principle, HGT in milk was assessed with FAM21805 as a recipient and all 4 ESBL strains as donors (Figure 1, B). In a last step, all 4 FAM21805 exconjugants were used as ESBL plasmid donors with FAM23012 as a recipient (Figure 1, C). Exconjugant frequencies in plate matings were high, ranging from 9.7×10^{-5} to 3.7×10^{-1} exconjugants per recipient. We found statistically significant differences among various combinations of donor and recipient, as indicated in Figure 1 (one-way ANOVA, Holm-Sidak, $\alpha = 0.05$). Exconjugant frequencies in milk are several orders of magnitude lower than in plate matings with the same recipient (up to 7.4×10^{-5} exconjugants per recipient). With FAM22321 as a donor, HGT in milk was not efficient enough for quantitative evaluation, but exconjugants have been obtained and confirmed without prior enrichment nonetheless. For the other 3 donors, FAM22871, FAM23292, and FAM23293, exconjugant frequencies were 6.0×10^2 , 7.1×10^2 , and 1.5×10^3 fold lower in milk than in the corresponding plate mating, respectively. Conjugation with strain FAM22321 always resulted in significantly lower exconjugant frequencies compared with at least one other ESBL donor for the same recipient strain. Even when the donor of all 4 ESBL plasmids was the same strain, FAM21805, significantly fewer exconjugants were obtained with pFAM22321 than with pFAM23293_1 (Figure 1, C).

As strains FAM22871 and FAM23292 each harbor a plasmid encoding a narrow-spectrum β -lactamase (TEM-1) in addition to the ESBL plasmid, we assessed whether one or both are transferred during plate matings. A biological triplicate of HGT experiments with all 4 ESBL strains into recipient strain FAM21805 was carried out by parallel plating with ampicillin (100 μ g/mL) and cefuroxime (100 μ g/mL) as β -lactams. The MIC of cefuroxime for TEM-1-producing *E. coli* isolates is well below the employed concentration (Wu et al., 1994), and selection pressure is thus sufficient to distinguish strains harboring CTX-M-14 or -15 from those harboring TEM-1 only. Exconjugant frequencies evaluated on LB_{AMP/RIF} and LB_{cefuroxime/RIF} were not significantly different from each other (2-sided *t*-test, $P \geq 0.40$). Also, 20 exconjugant colonies per HGT experiment and replicate (60 in total for each donor) were replicated from LB_{AMP/RIF} onto LB_{cefuroxime/RIF} and brilliance ESBL agar (Oxoid). All colonies regrew, confirming that ESBL plasmids had been transferred in each instance tested. Possible transfer of the TEM-1-encoding plasmids was also assessed. For pFAM22871_1, a screening of exconjugants for tetracycline resistance (LB_{tetracycline}, 15 μ g/mL) was done; only 1 of 60 exconjugants was tetracycline resistant and received pFAM22871_1. The TEM-1-encoding plasmid pFAM23292_1 features no

additional antimicrobial resistances we could use for selection. Therefore, specific primers for its TEM-1 region (p61.1_TEM-1_reg_F: 5'-GACAGGAAGCAAAGCT-GAAAGG-3' and p61.1_TEM-1_reg_R: 5'-GTGC-CGTCCAAGATCATGATCC-3') and a primer pair specific for the TEM-1 region of pFAM23292_2 (p24.2_TEM-1_reg_F: 5'-TTTTCTCTCCACAGGCCTCG-3' and p24.2_TEM-1_reg_R: 5'-CTCCTGCTGTAGTG-TAACCTGC-3') were used to screen for presence of each plasmid in exconjugants (annealing temperature: 64°C). Only the ESBL plasmid pFAM23292_2 was found in exconjugants (20 colonies screened); thus, whereas transfer of the narrow-spectrum β -lactamase-encoding plasmids of our isolates has been observed in the case of pFAM22871_1, the total HGT frequencies in our experiments are almost exclusively due to transfer of ESBL plasmids. The selection using ampicillin for determination of ESBL exconjugants with the other strains as recipients is thus feasible.

The high frequencies of exconjugants observed in plate matings are reason to expect isolation of ESBL-encoding, heat-resistant *E. coli* strains in the future, especially if these plasmids are maintained in absence of antimicrobial pressure. In general, plasmids can be maintained by several mechanisms: high copy number (and stochastic distribution to daughter cells), toxin-antitoxin (TA) systems, or partitioning proteins (Million-Weaver and Camps, 2014). One or more TA systems have been found on all plasmids except pFAM23293_2 and pFAM23293_3 (small plasmids of 5.1 and 3.2 kb encoding no AMR, Table 2). As reviewed in Wen et al. (2014), if the encoding plasmid is lost then the TA system could act as postsegregation killing mechanism. The 3 highly similar CTX-M-15-encoding plasmids also harbor the partitioning proteins ParA-ParB, which physically distribute the plasmid into both daughter cells and thus maintain it (Bignell and Thomas, 2001; Table 2). The ratio of ampicillin-resistant colony-forming units to total colony-forming units did not decrease during the entire propagation in liquid medium without antimicrobial pressure for any isolate or exconjugant, even though the total colony-forming units decreased statistically significantly on d 7 compared with at least 1 previous time point for all wild-type strains and exconjugants (one-way ANOVA, Holm-Sidak, $\alpha = 0.05$, Figure 2). For plasmid stability experiments of exconjugants with FAM22871 and FAM23292 as donors, we confirmed that only the ESBL plasmid had been transferred (by checking tetracycline resistance by plating and the TEM-1 regions by PCR, respectively). The 4 ESBL plasmids were stably maintained in all *E. coli* strains under the conditions tested. These experimental findings are in agreement with the *in silico* evidence of mechanisms for plasmid maintenance.

Table 1. *Escherichia coli* strains used in this study

Strain	Serovar	Phylogenetic group	MLST ²	Misc. ³	Antibiotic resistance profiles ⁴	Dairy origin	Reference
FAM19195	O8:H21	B1		Heat-resistant	str	Raw milk cheese	(Peng et al., 2012, 2013b)
FAM21805	O68:H14	A		Heat-resistant		Raw milk cheese	(Peng et al., 2012, 2013b)
FAM21807	O68:H14	A		Heat-resistant	str	Raw milk cheese	(Peng et al., 2012)
FAM21808	O11:H11	A		Heat-resistant	str	Raw milk cheese	(Peng et al., 2012)
FAM21843	O178:H12	A		Heat-resistant	STR, TMP	Raw milk cheese	(Peng et al., 2012, 2013b)
FAM21845	O68:H14	A		Heat-resistant	GEN, KAN, STR, TET, TMP, SXT, AMP	Raw milk cheese	(Peng et al., 2012)
FAM22636		A		Heat-resistant	str	Raw milk cheese	Present study
FAM22639		A		Heat-resistant	str	Raw milk cheese	Present study
FAM22791		A		Heat-resistant		Raw milk cheese	Present study
FAM22808		A		Heat-resistant	GEN, KAN, STR, CHL, TET, TMP, SXT, AMP	Raw milk cheese	Present study
FAM22891		B1		Heat-resistant		Raw milk cheese	Present study
FAM22936		A		Heat-resistant	str	Raw milk cheese	Present study
FAM22940		B1		Heat-resistant	amp, cef	Raw milk cheese	Present study
FAM22947		A		Heat-resistant		Raw milk cheese	Present study
FAM22954		A		Heat-resistant		Raw milk cheese	Present study
FAM22961		A		Heat-resistant		Raw milk cheese	Present study
FAM22962		A		Heat-resistant		Raw milk cheese	Present study
FAM22963		A		Heat-resistant		Raw milk cheese	Present study
FAM23012		A		Heat-resistant	TET	Raw milk cheese	Present study
FAM23014		A		Heat-resistant	str	Raw milk cheese	Present study
FAM23016		A		Heat-resistant	str	Raw milk cheese	Present study
FAM23030		A		Heat-resistant		Raw milk cheese	Present study
FAM23031		A		Heat-resistant	str	Raw milk cheese	Present study
FAM23078		A		Heat-resistant	str	Raw milk cheese	Present study
FAM23092		A		Heat-resistant	TET	Raw milk cheese	Present study
FAM23093		A		Heat-resistant	TET	Raw milk cheese	Present study
FAM23101		A		Heat-resistant	GEN, KAN, TET, TMP, SXT, AMP	Raw milk cheese	Present study
FAM23106		A		Heat-resistant	str	Raw milk cheese	Present study
FAM23109		A		Heat-resistant	STR, TET, AMP	Raw milk cheese	Present study
FAM23113		A		Heat-resistant	KAN, STR, CHL, TET, AMP, cef, amc	Raw milk cheese	Present study
K-12 MG1655							DSM 18039
FAM22321		A	ST4483	ESBL phenotype; TEM-1, CTX-M-14	GEN, KAN, STR, CHL, TET, NAL, CIP, TMP, SXT, AMP, CEF, CXM, CTX, atm	Raw milk	Present study
K-12 MG1655 (pFAM22321)				Exconjugant	GEN, AMP, CEF, CXM, CTX, amc		Present study
FAM21805 (pFAM22321)				Heat-resistant, exconjugant	GEN, AMP, CEF, CXM, CTX		Present study
FAM22871		E	ST69, STC69	ESBL phenotype; TEM-1, CTX-M-15	GEN, KAN, STR, TET, AMP, CEF, CXM, CTX, ATM	Raw milk	Present study
K-12 MG1655 (pFAM22871_2)				Exconjugant	GEN, AMP, CEF, CXM, CTX, atm		Present study

Continued

Table 1 (Continued). *Escherichia coli* strains used in this study

Strain	Serovar	Phylogenetic group	MLST ²	Misc. ³	Antibiotic resistance profiles ⁴	Dairy origin	Reference
FAM21805 (pFAM22871_2)				Heat-resistant, exconjugant	GEN, AMP, CEF, CXM, CTX, atm		Present study
FAM23292		E	ST362	ESBL phenotype; TEM-1, CTX-M-15 Exconjugant	GEN, KAN, STR, TET, AMP, CEF, CXM, CTX, ATM	Raw milk cheese	Present study
K-12 MG1655 (pFAM23292_2)				Exconjugant	GEN, AMP, CEF, CXM, CTX, ATM		Present study
FAM21805 (pFAM23292_2)				Heat-resistant, exconjugant	GEN, AMP, CEF, CXM, CTX, atm		Present study
FAM23293		E	ST362	ESBL phenotype; TEM-1, CTX-M-15 Exconjugant	GEN, CHL, TET, AMP, CEF, CXM, CTX, ATM, AMC	Raw milk cheese	Present study
K-12 MG1655 (pFAM23293_1)				Exconjugant	gen, AMP, CEF, CXM, CTX, ATM		Present study
FAM21805 (pFAM23293_1)				Heat-resistant, exconjugant	GEN, AMP, CEF, CXM, CTX, atm		Present study
K-12 MG1655 (933W)				933W $\Delta str::gfp::cat$ lysogenized	CHL		(Quirós et al., 2015)
K-12 MG1655 (pFAM22321, 933W)				Exconjugant, 933W $\Delta str::gfp::cat$ lysogenized	GEN, CHL, AMP, CEF, CXM, CTX, atm, amc		Present study
FAM21805 (pFAM22321, 933W)				Heat-resistant, exconjugant, 933W $\Delta str::gfp::cat$ lysogenized	GEN, CHL, AMP, CEF, CXM, CTX		Present study
<i>E. coli</i> DV155	E		ST4007	Control for E PCR			(Vogt et al., 2014)

¹Determined by quadruplex and group C- and E-specific PCR (Clermont et al., 2013).²Seven-allele multilocus sequence typing (MLST) scheme (University of Warwick, 2016).³*clpK*- and *orfE*-positive by PCR and phenotypically heat-resistant; representative exconjugants used for determination of antimicrobial resistance profiles and plasmid stability assays. Further exconjugants of the horizontal gene transfer experiments are not listed. Misc. = miscellaneous information on strains; ESBL = extended-spectrum β -lactamases; TEM = β -lactamases, named TEM after patient (Temoneira); CTX-M = ESBL conferring cefotaxime (CTX) resistance, first described in Munich (M); 933W = Shiga toxin encoding phage; *str* = Shiga toxin gene; *gfp* = gene encoding green fluorescent protein; *cat* = chloramphenicol resistance cassette.⁴Uppercase letters denote a resistant phenotype, lowercase letters an intermediate phenotype. Antimicrobials tested: gentamicin (GEN), kanamycin (KAN), streptomycin (STR), chloramphenicol (CHL), tetracycline (TET), nalidixic acid (NAL), ciprofloxacin (CIP), trimethoprim (TMP), sulfamethoxazole/trimethoprim (19/1) (SXT), ampicillin (AMP), cefoxitin (FOX), cephalothin (CEF), cefuroxime (CXM), cefotaxime (CTX), cefepime (FEP), aztreonam (ATM), amoxicillin/clavulanic acid (20/10) (AMC), and ertapenem (ETP).

Table 2. Sequenced plasmids of ESBL-encoding *Escherichia coli* strains in this study

Strain	Plasmid (size, kb)	Inc/pST ¹	Most similar plasmid (size, kb; Accession No.) ²	β -lactamases ³	Other antimicrobial resistances ⁴	Plasmid maintenance ⁵	Accession No.
FAM22321	pFAM22321 (79.0)	IncFII/–	pFOS-HK151325 (69.8; JX627737)	<i>bla</i> _{CTX-M-14} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>hok/sok</i> , <i>pemIK</i>	KU288634
FAM22871	pFAM22871_1 (141.5)	IncFIA, FIB, FII/–	pSH163_120 (120.5; JN983046)	<i>bla</i> _{TEM-1}	<i>aph(3'')-Ia</i> , <i>sul2</i> , <i>strA</i> , <i>strB</i> , <i>tetA</i> , <i>tetC</i> , <i>tetR</i> , <i>aac3-III</i> , tunicamycin resistance	<i>ccdAB</i> , <i>hok/sok</i> , <i>vapBC</i> , <i>yacAB</i> , <i>parAB</i> , <i>pndAC</i>	KU355873
	pFAM22871_2 (97.8)	IncII/pST37	pJIE174 (96.5; EU418931.1)	<i>bla</i> _{CTX-M-15} <i>bla</i> _{TEM-1}			KU355874
FAM23292	pFAM23292_1 (137.0)	IncFIA, FIB, FII/–	pCTXMI5 (138.7; KP789020.1)	<i>bla</i> _{TEM-1}		<i>ccdAB</i> , <i>parAB</i> , <i>pemIK</i>	
	pFAM23292_2 (96.1)	IncII/pST37	pJIE174 (96.5; EU418931.1)	<i>bla</i> _{CTX-M-15} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>parAB</i> , <i>pndAC</i>	
FAM23293	pFAM23293_1 (95.2)	IncII/pST37	pJIE174 (96.5; EU418931.1)	<i>bla</i> _{CTX-M-15} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>parAB</i> , <i>pndAC</i>	
	pFAM23293_2 (5.1)	–/–	pBS512_5 (5.1; CP001060.1)				
	pFAM23293_3 (3.2)	–/–	pO104:H7 (3.2; KM085453.1)				

¹Plasmid incompatibility (Inc) groups based on PlasmidFinder version 1.3 (<https://cge.cbs.dtu.dk/services/PlasmidFinder>; Carattoli et al., 2014), and plasmid sequencing type (pST) determined using PubMLST (<http://pubmlst.org/plasmid>; Jolley and Maiden, 2010).

²Defined by BLASTn max score (BLAST, 2016).

³ β -lactamases were manually curated and compared with reference sequences deposited at www.lahey.org/studies.

⁴Other antimicrobial resistance genes determined by BLASTp $\geq 96\%$ identity (BLAST, 2016); the entire nt sequence was also screened by BLASTx against the BacMet biocides and metal resistance protein database (<http://bacmet.biomedicine.gu.se>, last update January 16, 2014; Pal et al., 2014).

⁵*parA/parB* is a plasmid-partitioning system, all others are toxin-antitoxin systems.

Having demonstrated that heat-resistant *E. coli* from dairy are capable of efficiently acquiring and maintaining ESBL plasmids, we finally wanted to investigate possible lysogenization by the modified Shiga toxin-encoding phage 933W. Lysogenization of exconjugants FAM21805 (pFAM22321), FAM23012 (pFAM22321), and FAM23093 (pFAM22321) was possible in plate matings (PCR-positive for *clpK*, *cat*, and left and right junctions), yet frequencies were too close to the detection limits to yield quantitative data (average detection limits of lysogens per recipient: 3.5×10^{-8} , 1.1×10^{-7} , and 1.5×10^{-7}). The *clpK*-negative, AMP-, chloramphenicol-, or RIF-resistant colonies were detected as well; these colonies likely were spontaneous RIF-resistant mutants of K-12 MG1655 (933W), which received pFAM22321 from the intended recipient strains.

In this study, we found new heat-resistant *E. coli* isolates from raw milk cheeses. We found them at a higher rate (93 of 256 isolates) than the approximate 2% prevalence of the LHR, which includes *clpK*, in published whole-genome sequences and genome shotgun sequences (Mercer et al., 2015). As the sampling of the current study was done with anonymized samples in collaboration with private diagnostics laboratories, the observed frequency of 36.3% heat-resistant *E. coli* possibly does not reflect an exact prevalence value. Still, a true prevalence of more than 2% is very likely. A possible explanation for this observation is that the milk for production of raw milk cheeses is often treated by thermization of raw milk (heating to subpasteurization temperatures). This increases food safety, but does not denature alkaline phosphatase, and thus distin-

Figure 1. Exconjugant frequencies per recipient. (A) Wild-type strains carrying extended-spectrum β -lactamases (ESBL)-encoding plasmids FAM22321, FAM22781, FAM23292, and FAM23293 as donors in plate matings. FAM22321 was used as donor for 11 heat-resistant *Escherichia coli* strains to demonstrate their amenability to horizontal gene transfer and K-12 MG1655 as control. (B) All 4 ESBL-encoding strains as donors in milk. (C) All 4 FAM21805 exconjugants as donors of ESBL-encoding plasmids in plate matings. Bars indicate averages and SD; those not sharing a letter (a–c) are statistically significantly different from each other. Analyzed by means of one-way ANOVA, Holm-Sidak, $\alpha = 0.05$. Groups compared in ANOVA are separated by horizontal dashed lines.

Figure 2. Stability of ESBL plasmids in wild-type strains and exconjugants of FAM21805 and K-12 MG1655 without antimicrobial selection. (A) Stability of plasmids in wild-type extended-spectrum β -lactamases (ESBL) strains. (B) Stability of FAM21805 exconjugants. (C) Stability of K-12 MG1655 exconjugants. Cultures were diluted 1 to 1,000 each day and incubated at 37°C without antimicrobial pressure. The last incubation step was done for 4 d, leading to some decrease in total colony-forming units per milliliter on d 7 (statistically significant to at least one previous time point for all strains tested; one-way ANOVA, Holm-Sidak, $\alpha = 0.05$). No statistically significant differences were found in the ampicillin (amp)-resistant fraction over the time course for any of the strains tested (one-way ANOVA, Holm-Sidak, $\alpha = 0.05$). Error bars represent SD.

guishes these cheeses from those made with pasteurized milk by retaining the unique quality and taste. Several heat-resistant *E. coli* isolates included in our study have previously been shown to exhibit increased decimal reduction values (D-values) at these subpasteurization temperatures in realistic settings (Peng et al., 2013b). In combination with increased survival rates during cheese ripening (Peng et al., 2013a), this may explain the apparently more frequent isolation of heat-resistant *E. coli* found in raw milk cheeses. They may be enriched during the entire cheesemaking process compared with their heat-sensitive counterparts. Fortunately, none of the heat-resistant strains was found to be pathogenic or harbor ESBL-encoding plasmids. Only 4 ESBL-encoding *E. coli* strains were isolated from 1,251 samples in our laboratory so far (455 raw milk and 796 raw milk cheese samples). Geser et al. (2012) also found low prevalence of ESBL-producing *E. coli* in bulk tank milk (0/100 samples) and mastitis milk (1 of 67 samples encoding CTX-M-14 and TEM-1). None of our ESBL isolates belonged to the invasive *E. coli* sequence type ST131, which is mostly associated with the CTX-M-15 ESBL and contributes significantly to its worldwide spread. In fact, only 1 of the 4 ESBL strains isolated in the current study belongs to a sequence type commonly associated with ESBL-producing *E. coli*. Strain FAM22871 (CTX-M-15) belongs to ST69, which is usually associated with CTX-M-14 (Ewers et al., 2012). Strains FAM23292 and FAM23293 have been isolated from raw milk cheeses of the same dairy 2 mo apart; both belong to phylogenetic group E and are ST362. They are distinguished by the small plasmids pFMA23293_2 and pFAM23293_3, which were not found in FAM23292. All 4 ESBL isolates are capable of conjugating their ESBL plasmids at high rates into heat-resistant *E. coli*, where they are maintained without antimicrobial pressure and remain mobile. This, in conjunction with the likely selection advantage of heat-resistant *E. coli* during raw milk cheese production, could result in an increased abundance of ESBL plasmids in this system. The strains carrying the ESBL plasmids may then act as donors, as demonstrated with FAM21805 exconjugants. This may happen in the cheese production setting or within the human gastrointestinal tract after consumption, where bacterial densities are high and conjugation is aided by close spatial proximity. This could result in carriage of these plasmids by healthy individuals, with the continuous possibility of pathogens acquiring these plasmids, should an infection be contracted (Machado et al., 2013). As we found for pFAM22321 exconjugants of strains FAM21805, FAM23012, and FAM23093, Shiga toxin phages might also enter the population of heat-resistant *E. coli* and could profit from a selection advantage. Any kind of reservoir of ESBL or MDR

plasmids or Shiga toxin-encoding phages, especially if positively selected in a given environment, is worrisome, as it may contribute to the spread of critically important AMR and toxin genes in *Enterobacteriaceae*.

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