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Transferable heat-resistance in *E. coli* food isolates

J. Hummerjohann, PhD, Head of Research Group & CH-NRL

STEC meeting, EU-RL, Rome, Oct 12-13th, 2017

Discovery of factors mediating heat-resistance

- Novel, **plasmid-borne Clp ATPase, ClpK**, mediating increased heat-resistance in nosocomial *K. pneumoniae* discovered (Bojer et al. 2010)
- **Chromosomal cluster** with homolog of *clpK* discovered in *Cronobacter sakazakii* ATCC 29544, hints for presence in other Eb. (Gajdosova et al. 2011)
- *ClpK* positive **dairy isolates** show **increased survival at sub-pasteurization temperatures** and during cheese ripening (Peng et al. 2013a,b)
- ~14kb **locus of heat-resistance (LHR)** encoding *clpK* found in approximately **2% of published *E. coli* genomes &** studies on foodborne *E. coli*, Salmonella & Enterobacter (Mercer et al. 2015 & 2017)
- Genome Sequence of **thermotolerant, LHR+ foodborne Salmonella enterica Serovar Senftenberg** ATCC 43845 (Nguyen et al, 2017)
- We found *clpK* positive, heat-resistant strains **high frequencies** in raw milk cheeses

Heat-resistant *E. coli*: new co-selection mechanism & relevance for dairy industry

- FAM21805 proved to be extraordinarily heat-resistant

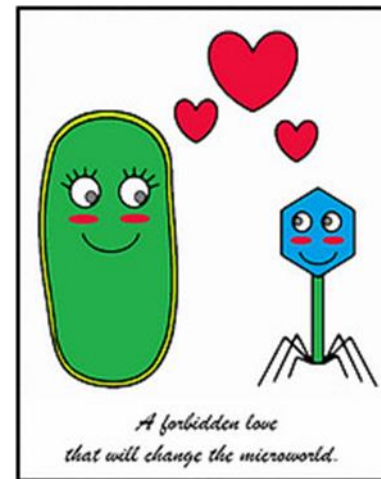
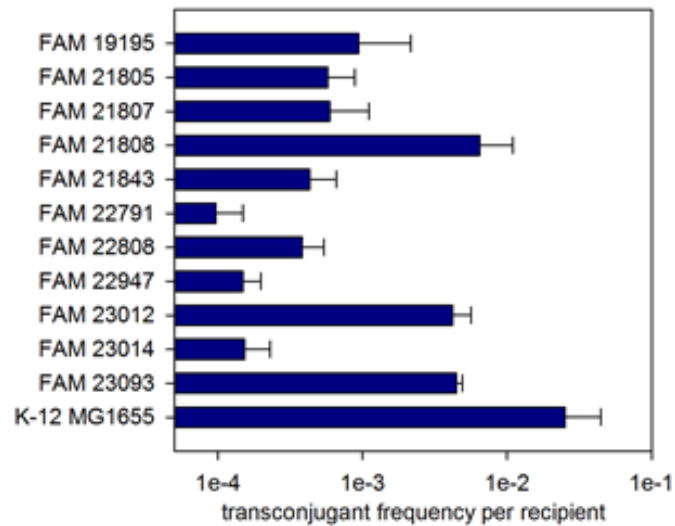
Isolate	Serotype	D-value (at 65 °C) ¹	<i>stx</i>	<i>clpK</i>
FAM21846	O16:H21	4.6 ± 0.2 s	-	-
FAM21843	O178:H12	33.2 ± 3.0 s	-	+
FAM21805	O68:H14	143.9 ± 79.4 s	-	+

¹S. Peng, J. Hummerjohann, R. Stephan, and P. Hammer, J Dairy Sci, 2014, 97:6623

- A potential persistent reservoir of ESBL and Shiga toxin-encoding phages in dairy
Marti et al (2016), J Dairy Sci.,99(11):8622-8632.
- 1 ESBL-positive heat-resistant *E. coli* dairy isolate

Transconjugation & transduction frequencies

***E. coli* dairy isolates with ESBL+ plasmid pFAM22321 & Lysogenisation by Shiga toxin phage 933W (*stx2::Cm^R*)**

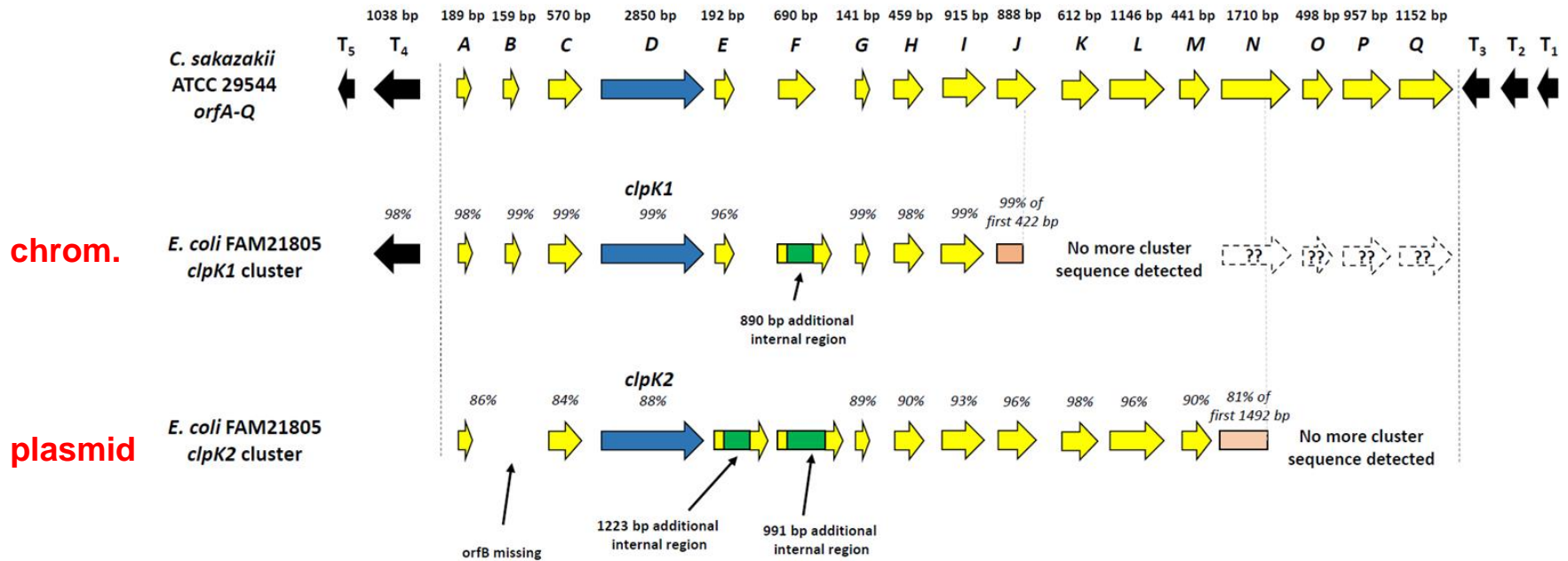


=> Proof of principle shown:

Heat-resistant *E. coli* dairy isolates could gain horizontally transferred genes to alter into ESBL-positive, heat-resistant STEC

Marti et al, JDS 2016

Heat-resistance *clpK*-clusters are mobile



E. Boll, R. Marti, J. Hummerjohann, & C. Struve
Statens Serum Institut, Copenhagen, and Agroscope, Bern,
2017 **Frontiers in Microbiology**

LHR2s are transferrable to STEC and EAEC and confer heat resistance

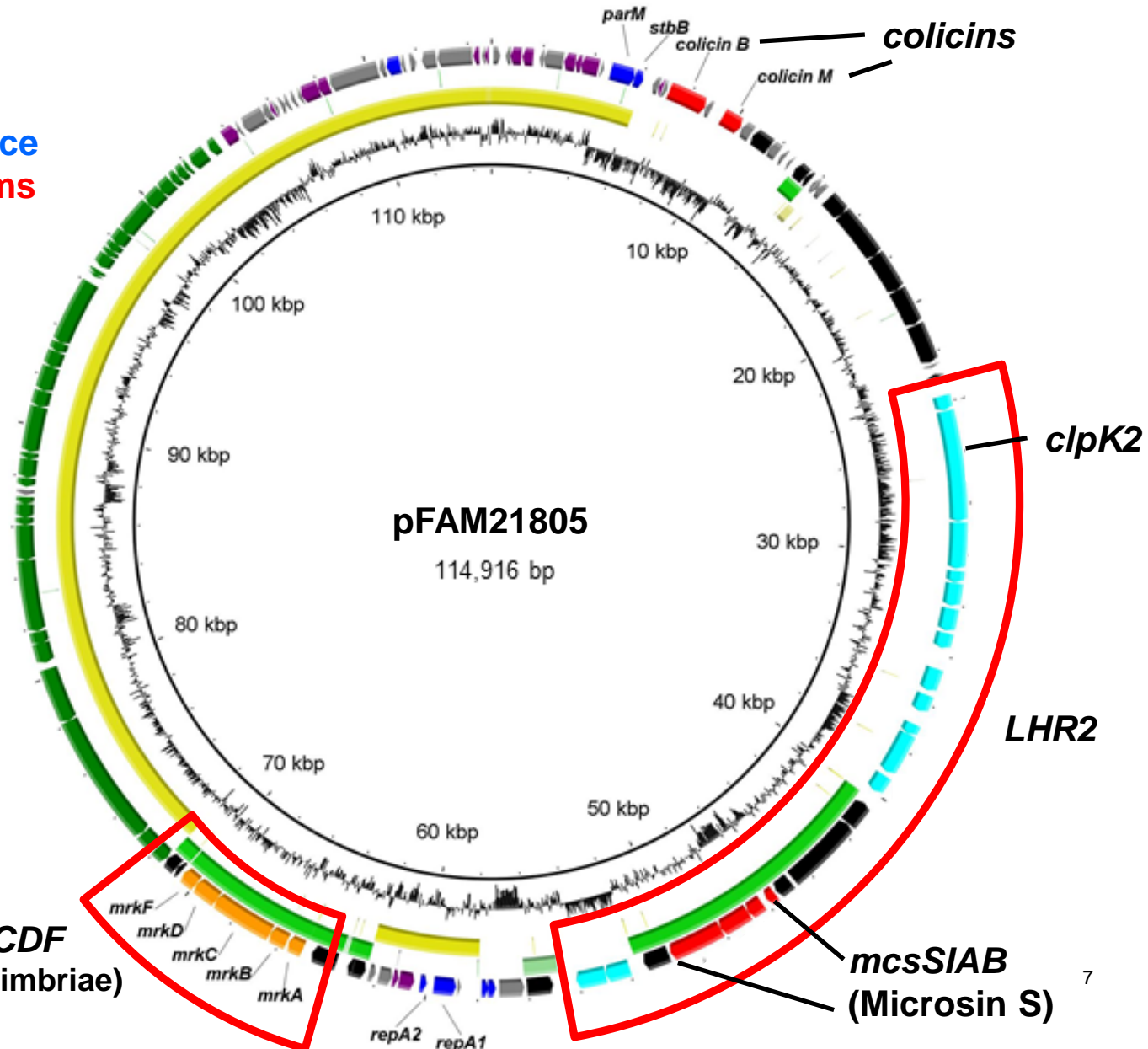
Relative survival after 45min. at 55°C;

	Strain	Relative survival ¹		
		Average	SD ²	p-value ³
STEC	FAM22873 wild-type	5.64E-04	1.64E-04	-
	FAM22873 Δ LHR2 (21805)	9.49E-04	1.08E-03	0.322
	FAM22873 <u>LHR2</u> (21805)	4.28E-02	2.24E-02	0.028
	FAM23288 wild-type	9.03E-04	2.04E-04	-
	FAM23288 Δ LHR2 (21805)	4.79E-04	5.38E-04	0.124
	FAM23288 <u>LHR2</u> (21805)	1.02E-02	8.12E-03	0.029
EAEC	55989 wild-type	3,60E-04	1,54E-04	-
	55989 Δ LHR2 (21805)	5,05E-04	1,98E-04	-
	55989 <u>LHR2</u> (21805)	3,91E-01	1,23E-01	0.007
	C227-11 φ cu	3,23E-04	4,89E-04	-
	C227-11 φ cu Δ LHR2 (21805)	1,20E-04	1,08E-04	-
	C227-11 φ cu <u>LHR2</u> (21805)	4,27E-01	1,39E-01	0.001

Boll, Marti et al. 201

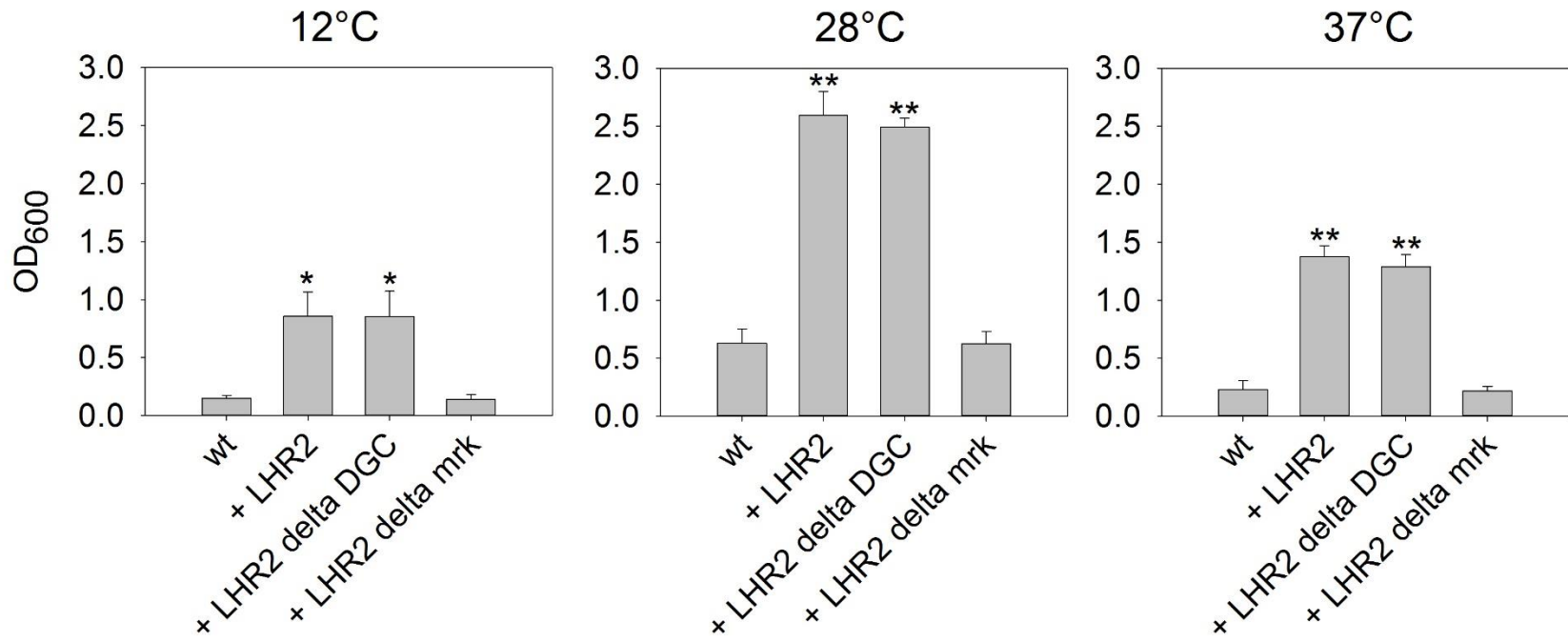
LHR2 on conjugative plasmid in FAM21805

Hypothetical proteins
Plasmid replication/maintenance
Toxin-antitoxin systems
LHR2 genes
Adhesins
Mobile elements



(Boll, Marti et al. 2017)

pFAM21805 increases biofilm formation of transconjugants



- Biofilm formation increased at every temperature tested
- Dependent on *mrk* locus, not diguanylate cyclase

(Boll, Marti et al. 2017)

Biofilm forming potential - Crystal violet assays (PS surface)

heat resistant *E. coli*

Strain	LB			LBnoS			RPSM			ABTCAA		
	12°C	28°C	37°C	12°C	28°C	37°C	12°C	28°C	37°C	12°C	28°C	37°C
FAM 19195	-	IV	-	-	III	-	-	III	III	I	IV	II
FAM 21805	I	IV	II	-	III	I	-	II	III	I	III	III
FAM21805 (LHR1&2)				-	II	I	-	III	III	I	III	II
				I	-	-	-	III	III	-	I	I
				I	IV	I	-	III	III	IV	IV	III
FAM 21845	IV	III	III	IV	IV	III	II	IV	III	IV	IV	IV
FAM21845 (MDR)				I	II	I	-	II	II	I	II	III
				III	I	II	-	III	II	II	III	III
				II	II	II	-	III	III	I	II	II
FAM 22808	I	IV	I	-	IV	I	-	III	III	II	III	II
FAM 22891	III	II	I	III	II	I	-	III	III	III	II	IV
FAM 22936	IV	II	II	IV	I	I	-	II	II	III	III	III
FAM 22940	-	I	I	-	-	-	-	III	III	I	II	II
FAM 22947	II	I	II	I	I	II	-	IV	IV	II	III	II
FAM 22954	II	IV	II	II	IV	II	I	III	III	III	IV	IV
FAM 22961	II	III	I	-	III	-	I	III	III	III	IV	II
FAM 22962	-	-	-	-	-	-	-	III	II	-	IV	II
FAM 22963	III	III	I	III	III	II	I	III	III	IV	IV	II
FAM 23012	IV	III	III	III	II	II	-	II	II	III	III	IV
FAM 23014	III	III	II	III	III	III	-	III	III	III	III	III
FAM 23016	III	III	II	III	IV	II	I	II	II	IV	IV	III
FAM 23030	II	III	I	III	IV	I	I	II	III	IV	IV	II
FAM 23031	I	I	I	I	I	-	-	II	III	I	III	I
FAM 23078	-	I	I	-	I	-	-	-	II	I	IV	III
FAM 23092	III	III	III	III	II	III	-	II	II	II	III	IV
FAM 23093	IV	III	II	III	III	II	-	I	II	II	III	III
FAM 23101	III	III	II	IV	III	II	-	III	II	IV	III	IV
FAM 23106	-	I	-	-	I	-	-	II	II	I	IV	III
FAM 23109	I	II	II	II	II	II	I	II	II	III	IV	III
FAM 23113	III	III	II	IV	II	II	-	II	III	III	III	III
FAM 21846	-	I	II	-	III	I	-	-	II	I	III	III
FAM 22942	I	IV	IV	I	IV	III	-	II	II	II	IV	IV
FAM 22956	I	IV	IV	I	III	IV	-	III	III	II	III	III
FAM 22996	-	-	-	-	-	-	-	I	III	-	-	-
FAM22321	-	I	II	-	I	I	-	II	III	-	-	I
FAM22871	I	IV	I	I	IV	I	-	II	I	I	IV	II
FAM22321 & FAM22871 (ESBL)				II	II	II	-	III	II	III	IV	III
				IV	IV	IV	-	II	II	II	IV	II
				I	I	I	-	II	II	III	IV	III
DN50	-	IV	I	-	IV	I	-	-	-	I	IV	II

Sequence of FAM21845

Genetic location	Size, GC, #CDS	AMR genes	Other resistance genes	Biofilm relevant operons
FAM21845 chromosome	4'901'989, 51.0%, 4'812	<u>β-lactam</u> : <i>ampC</i> <u>other</u> : RND efflux pumps	<u>Arsenic</u> : <i>arsRBC</i> , <i>arsRDABC</i> <u>Silver</u> : <i>silRSE</i> , <i>silCFBAP</i> <u>Copper</u> : <i>cusCFBA</i> , <i>cusRS</i> <i>pcoABCDRSE</i> <u>other</u> : See Table S3	<u>Cellulose synthesis</u> : <i>bcsABZC</i> , <i>bcsEFG</i> <u>Curli synthesis</u> : <i>csgDEFG</i> , <i>csgBAC</i> <u>PGA synthesis</u> : <i>pgaABCD</i> <u>Colanic acid synthesis</u> : <i>wza</i> to <i>wcaL</i>
pFAM21845_1	147'225, 51.7%, 174	<u>Tetracycline</u> : <i>tet(B)</i> <u>Aminoglycosides</u> : <i>strA</i> (2x), <i>strB</i> (2x), <i>aph(3')-Ic</i> , <i>aph(4)-Ia</i> , <i>aac(3)-Iva</i> , <i>aadA1</i> <u>Trimethoprim</u> : <i>dfrA1</i> <u>Sulphonamide</u> : <i>sul1</i> <u>β-lactam</u> : <i>bla_{TEM-1}</i> <u>β-lactam</u> : <i>bla_{TEM-1}</i>	<u>Mercury</u> : <i>merR</i> , <i>merTPCADE</i> Disinfectants: <i>qacEΔ1</i>	
pFAM21845_2	54'159, 44.9% 57			Type III fimbriae: <i>mrkABCDF</i>

- pFAM21845_1&2 transferrable in HGT, _2 significantly increases BF formation
- No increase in benzalkonium chloride resistance in HGT (*qacEΔ1*, MIC 32 mg/l)

Output

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

Marti, Muniesa, Schmid, Ahrens, Naskova & Hummerjohann
J Dairy Sci. 2016 Nov;99(11):8622-8632.

- **Turn Up the Heat—Food and Clinical *Escherichia coli* Isolates Feature Two Transferrable Loci of Heat Resistance**

Boll*, Marti*, Hasman, Overballe-Petersen, Stegger, Kim, Knøchel, Krogfelt, Hummerjohann and Struve (*equal contr)

Frontiers Microbiology, doi: 10.3389/fmicb.2017.00579

- **Biofilm formation potential of heat-resistant *Escherichia coli* dairy isolates and full genome sequence of MDR heat resistant strain FAM21845**

Marti, Schmid, Kulli, Schneeberger, Naskova, Knøchel, Ahrens, Hummerjohann

Appl. Env. Microbiol 2017, 83(15). pii: e00628-17. doi: 10.1128/AEM.00628-17.

Conclusions:

Combining the dark side of *E. coli*

- Sequenced **4 ESBL encoding plasmids** from dairy isolates are readily transferred to and maintained in heat-resistant *E.coli*
- Modified Shiga toxin-encoding phage **lysogenized** heat-resistant ESBL exconjugants
- **Locus of heat resistance (LHR)** is located on chromosome and/or an plasmids, both can be transferred to other isolates incl STEC & EAEC
- **Biofilm** production may be an further factor for co-selction

ESBL- and other AMR harboring, Shiga toxin-encoding, heat-resistant and biofilm-forming *E. coli* **could arise and potentially act as a persistent reservoir** of these factors in food production environment or elsewhere

Acknowledgements



**Erik Boll
Carsten Struve
Karen Krogfelt**

UNIVERSITY OF
COPENHAGEN



Susanne Knøchel



**Universität
Zürich^{UZH}**

R. Stephan



B Universitat
de Barcelona

Maite Muniesa



**M. Schmid & C. Ahrens
U. Zürcher & T. Berger**



Thank you for your attention



Agroscope good food, healthy environment

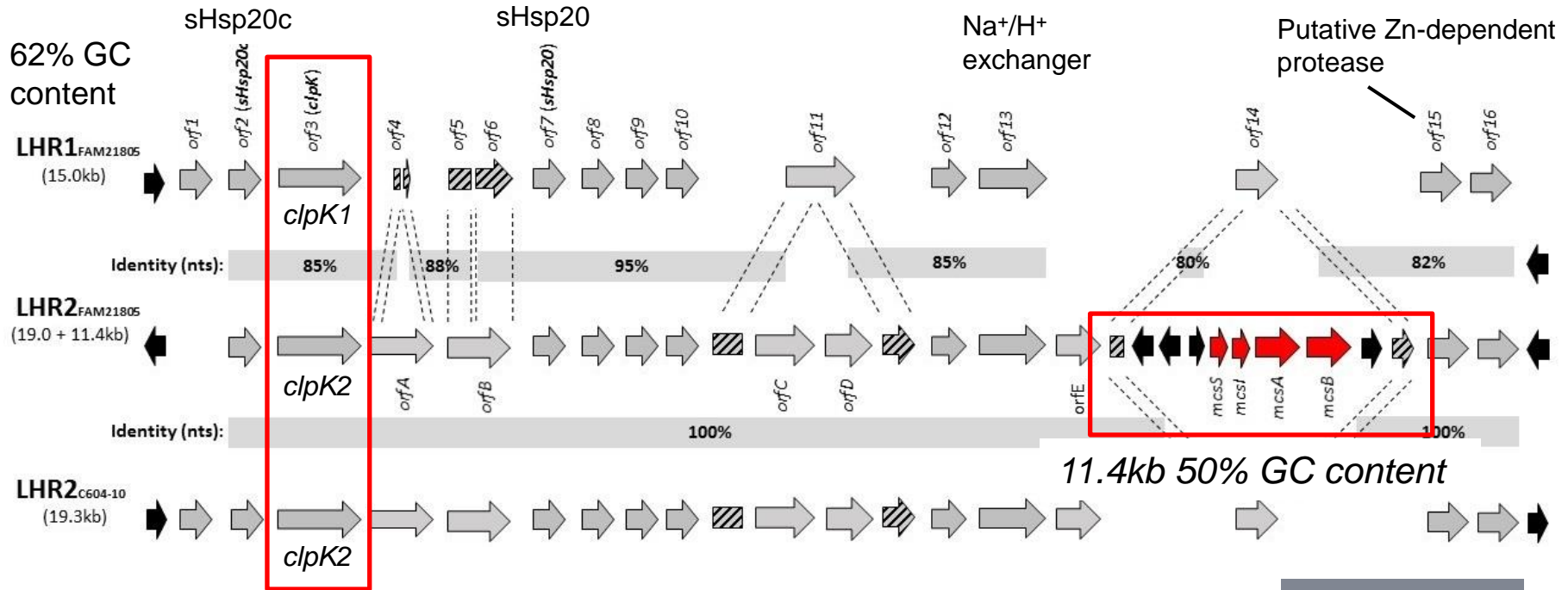
Sequencing of ESBL plasmids

Plasmid (size)	Inc/pST	β -lactamases	Other antimicrobial resistances	plasmid maintenance
pFAM22321 (79.0 kb)	IncFII/-	<i>bla</i> _{CTX-M-14} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>hok/sok</i> , <i>pemIK</i>
pFAM22871_2 (97.8 kb)	IncI1/ pST37	<i>bla</i> _{CTX-M-15} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>parAB</i> , <i>pndAC</i>
pFAM23292_2 (96.1 kb)	IncI1/ pST37	<i>bla</i> _{CTX-M-15} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>parAB</i> , <i>pndAC</i>
pFAM23293_1 (95.2 kb)	IncI1/ pST37	<i>bla</i> _{CTX-M-15} <i>bla</i> _{TEM-1}	<i>aac3-III</i> , tunicamycin resistance	<i>parAB</i> , <i>pndAC</i>

(Marti, Muniesa et al. 2016)

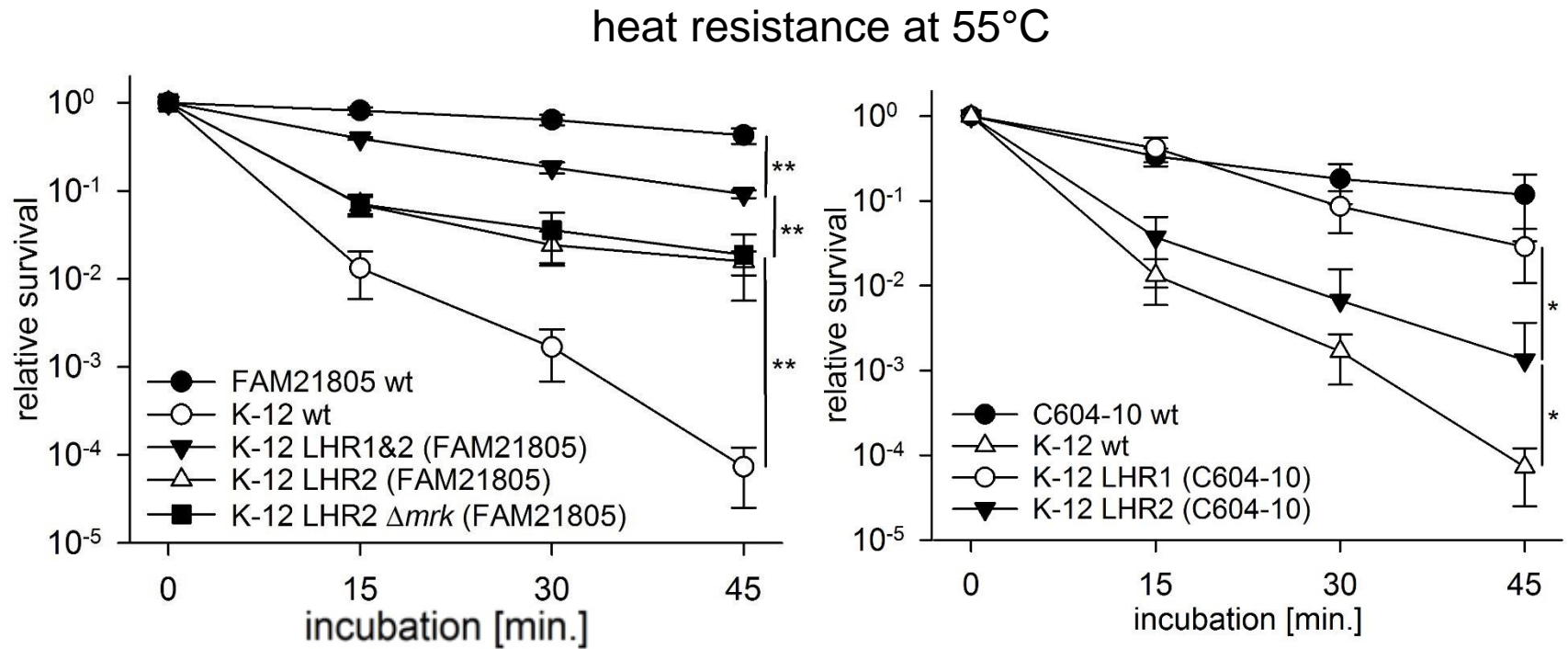
The loci of heat-resistance

- FAM21805 and C604-10 (ESBL) both feature **two** LHR
- LHR1_{FAM21805} extremely similar to AW1.7 (98-99%, Mercer, Zheng et al. 2015)



(Boll, Frimodt-Møller et al. 2016), (Boll, Marti et al. 2017)

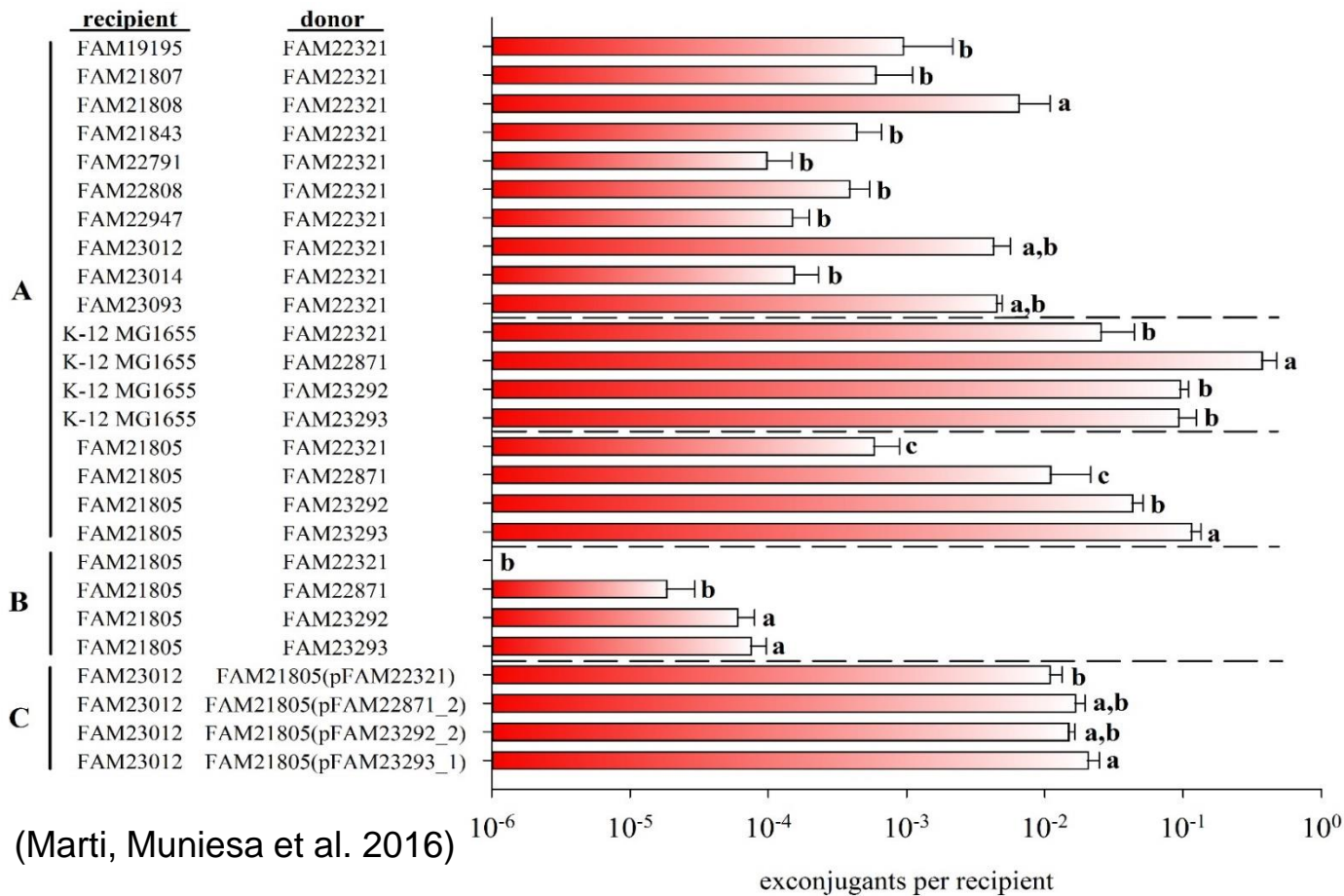
LHRs are transferrable and confer heat resistance



Horizontal gene transfer:

- LHR2_{FAM21805} ($7.56 \times 10^{-01} \pm 2.00 \times 10^{-01}$ transconjugants per recipient)
- LHR1 with very low frequencies

Horizontal gene transfer experiments with sequenced ESBL plasmids (CTX-M-14 & -15)

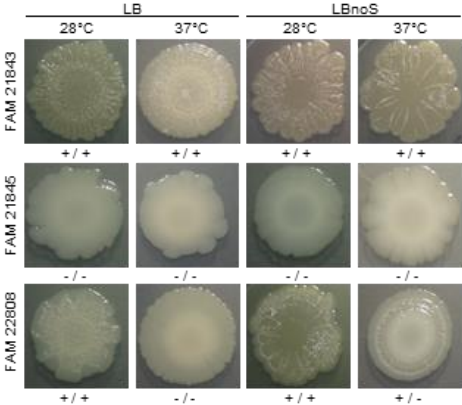
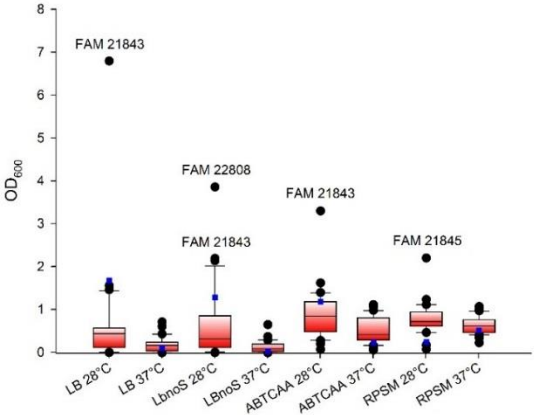


(Marti, Muniesa et al. 2016)

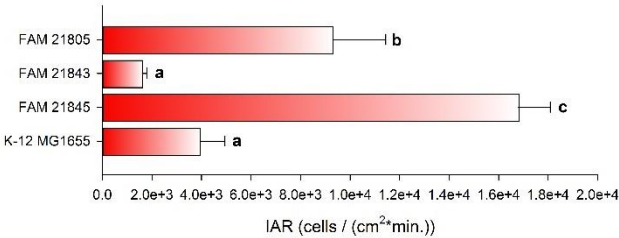
A: wt ESBL strains as donors in plate matings
B: wt ESBL strains as donors in bulk milk

C: FAM 21805 exconjugants as donors in plate matings

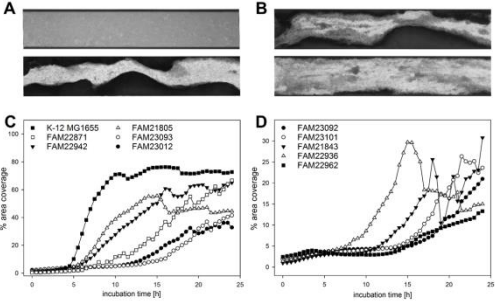
Variability in biofilm formation of *E. coli*



CV assays



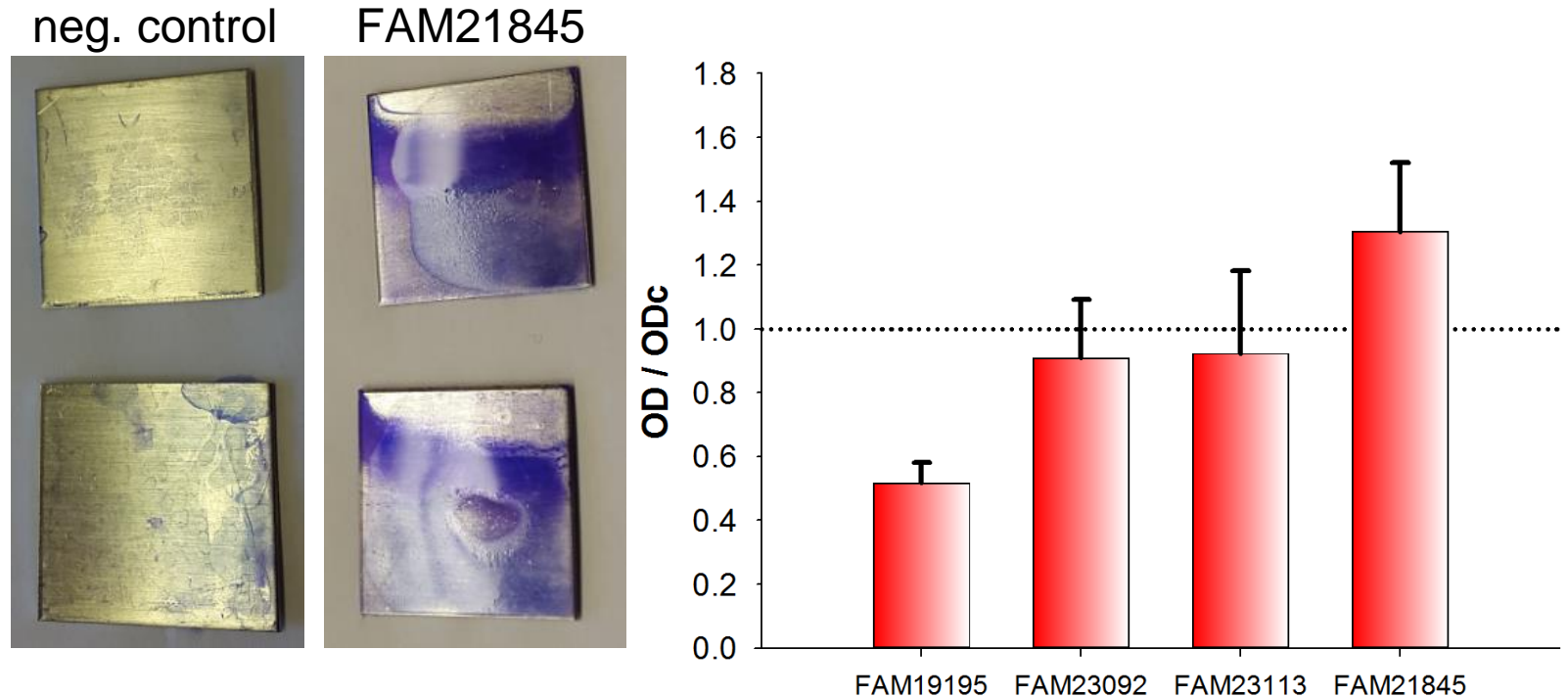
Macrocolony assays



Adhesion assays

Flow cell assays

Biofilm formation on stainless steel



- 48h, 12°C, reconstituted powdered skim milk (10.5%, wt/vol)
- Only FAM21845 exceeds average OD / ODc ratio of one
 - Significantly higher BF formation to all other strains tested together
 - Pair-wise comparisons: significantly higher than all strains except FAM23113 and FAM23092

Biofilm formation under dynamic conditions

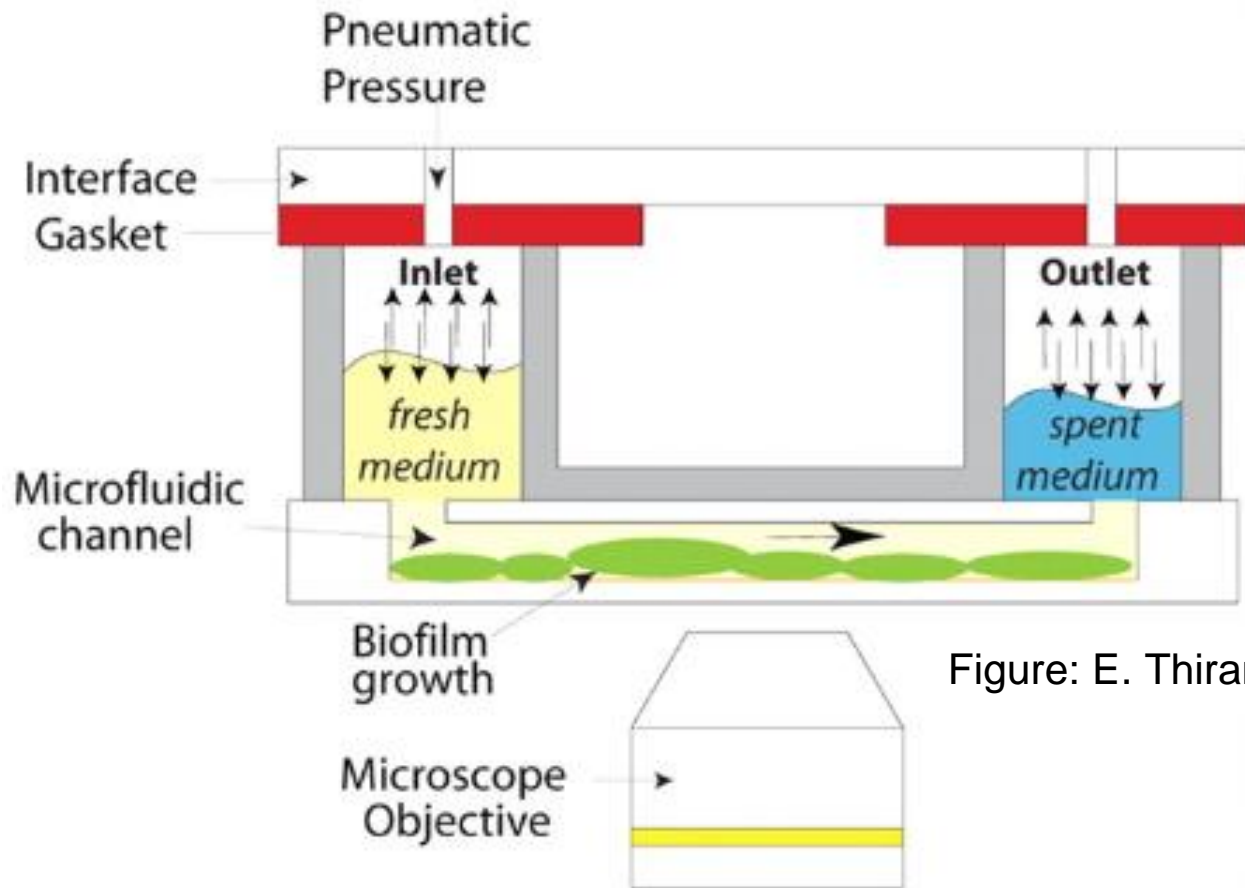
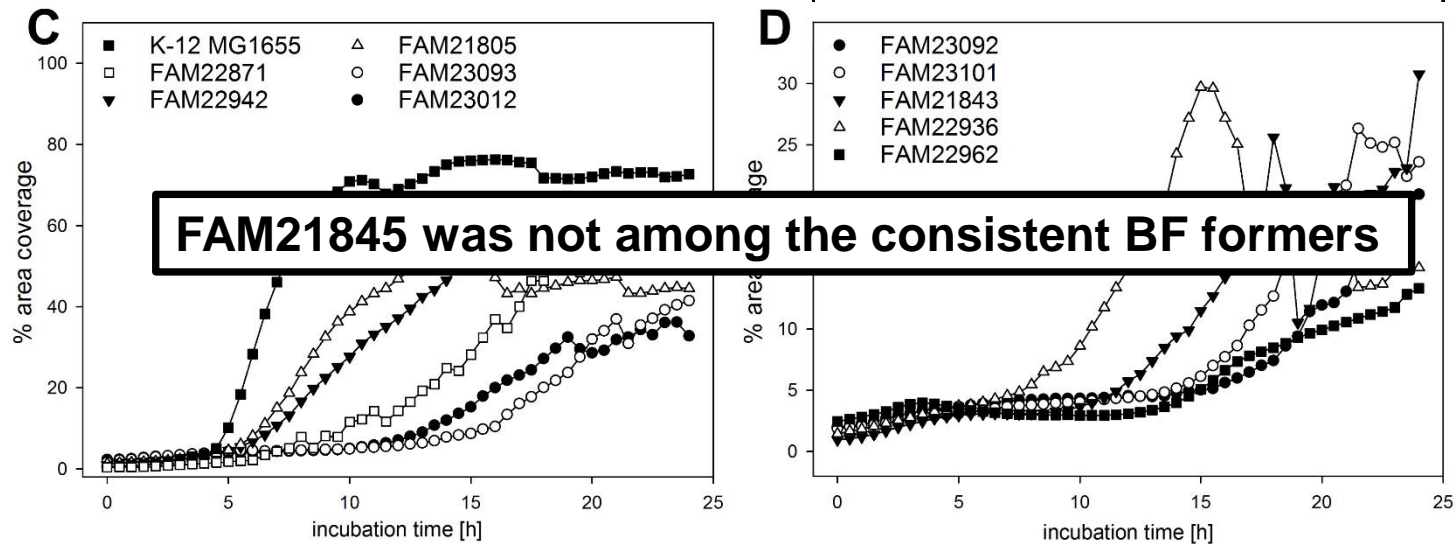
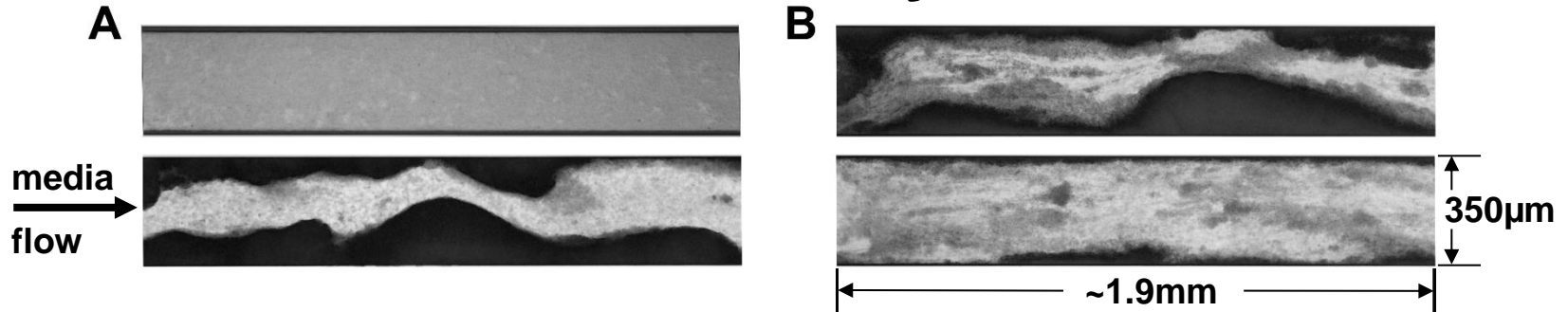


Figure: E. Thiran

Channel cross-sections: 350 × 70 μm

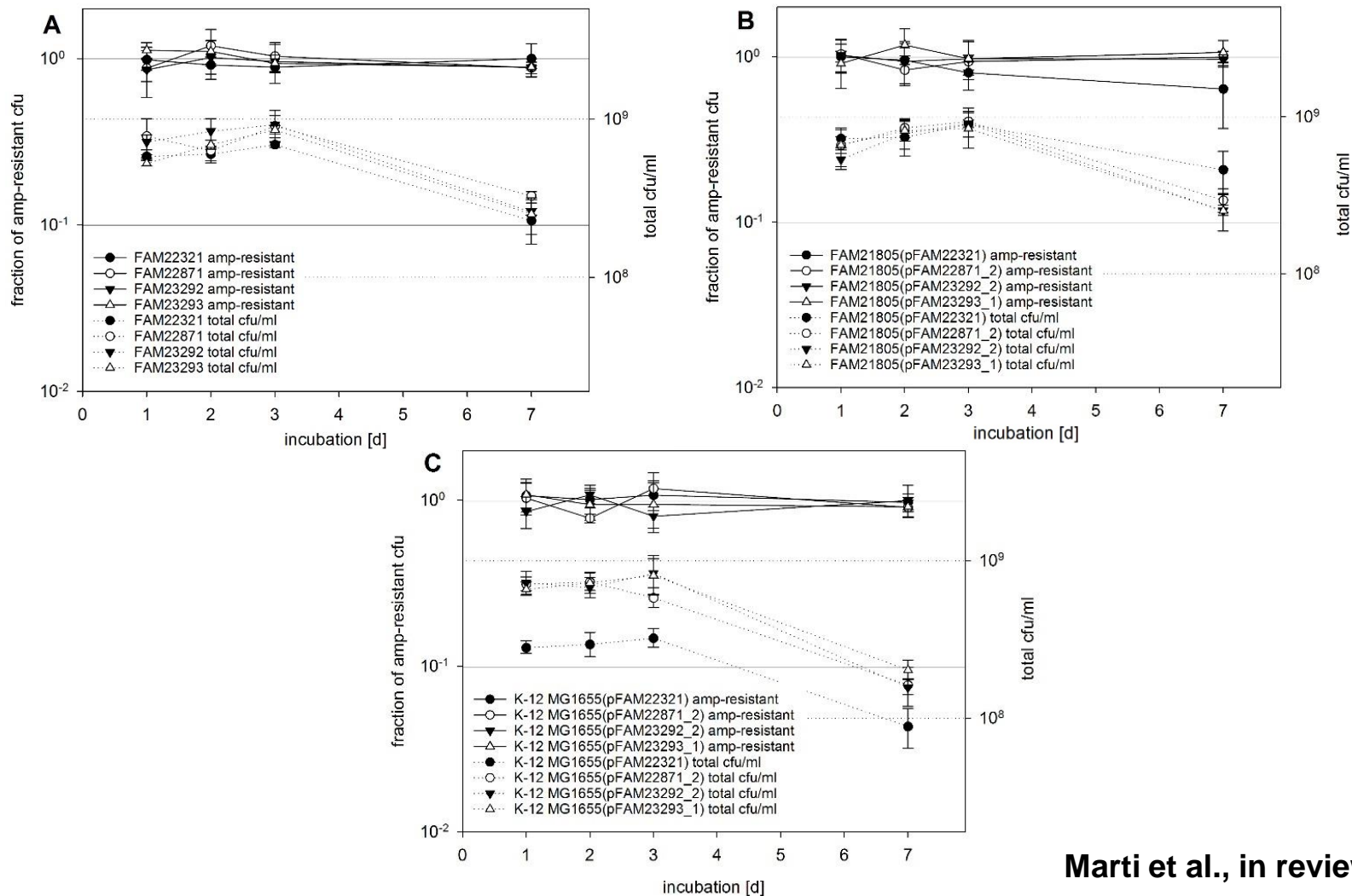
Biofilm formation under dynamic conditions



Marti, Schmid et al., in review

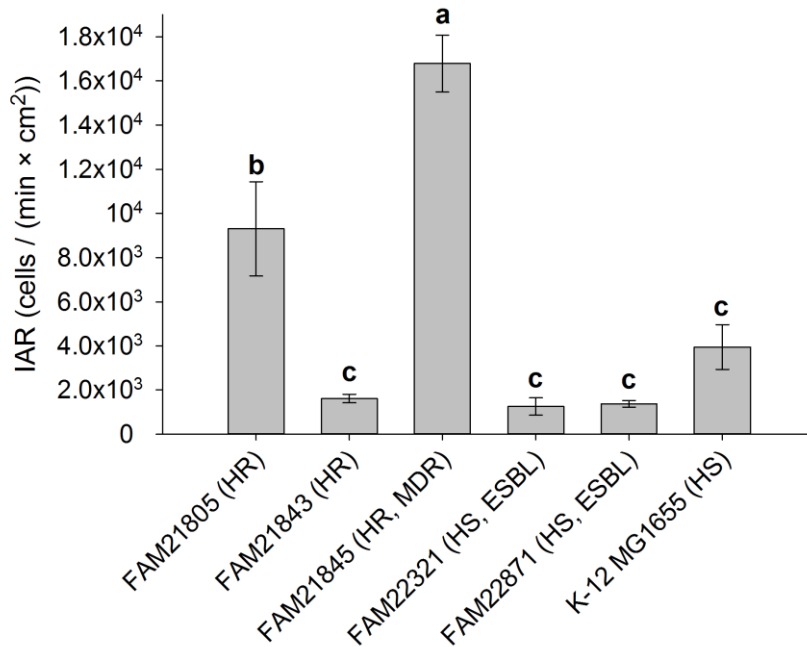
- 24h, 37°C, ABTCAA, 0.15 dyne/cm², cross-sections: 350 × 70µm
- **A**: lawn vs. actual BF; **B**: sloughing event (FAM21843, 18 vs. 18.5h)
- **C&D**: average area coverage over time of consistent BF formers

Plasmid maintenance in ESBL wt strains, FAM21805, K-12 MG1655

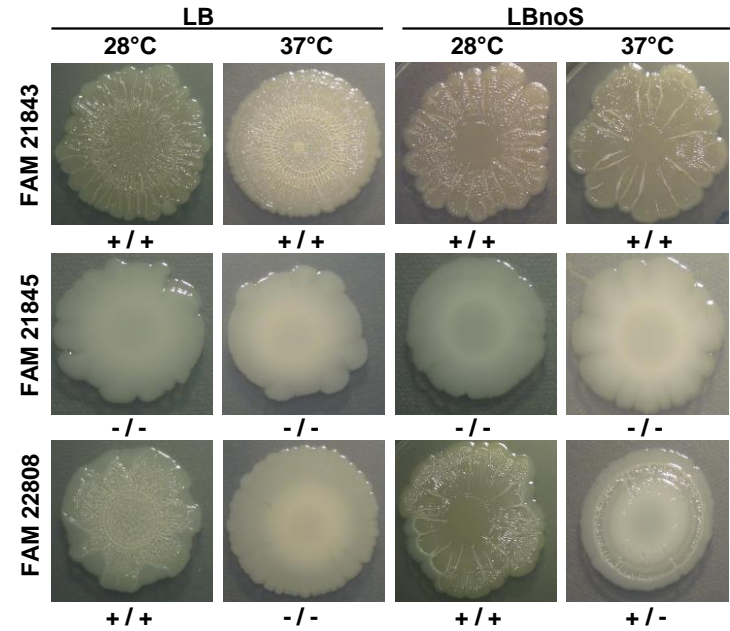


Marti et al., in review

Initial adhesion rates and macrocolony assays



Initial adhesion rates (IAR) were determined in a CoverWell perfusion chamber on PVC slides (Na₂HPO₄ buffer (pH 6.6), 0.0505 Pa; One-Way-ANOVA, all pairwise multiple comparison, Holm-Sidak, $\alpha=0.05$).



Examples of macrocolony assays under different conditions. Production of curli / cellulose based on colony morphology is indicated below each image.

Marti et al., in review

Experiments with FAM21805 loci of heat-resistance

- Single gene (***clpK1&2***) and full **LHR1&2 deletions** have been created and evaluated for reduced heat-resistance by SSI
- **LHR1&2** tagged with antibiotic resistance cassettes have been successfully transferred in **horizontal gene transfer experiments** (plate matings: 24h, 37°C).
 - LHR1 → K-12 MG1655 ($\sim 10^{-8}$ per recipient)
 - LHR2 → K-12 MG1655 & FAM21805 Δ LHR1&2 ($> 10^{-3}$ per recipient)
- Current hypothesis:
 - **LHR2 of FAM21805 might be plasmid-borne.** Frequencies of HGT are high, and K-12 MG1655 x LHR2(FAM21805) is PCR positive for IncFII replicon.