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Search for the transcription factors involved in the regulation of biofilm formation in *Escherichia coli* K-12

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Motivation and Aim: Bacterial biofilms are one of the main reasons for the worldwide expanding antibiotic resistance. It takes 1,000 times as much antibiotic to treat an infection if bacteria "decide" to form a biofilm. Lots of studies are being conducted for *Pseudomonas* and *Staphylococcus*. However, the regulation of biofilm formation in *Escherichia coli*, which can also be pathogenic, is still poorly understood. Uropathogenic *E. coli* (UPEC), forming extensive biofilms, leads to chronic urinary tract infections, while the biofilms formed by EPEC can be the reason for chronic colitis or gastroenteritis. The laboratory opportunistic *E. coli* K-12 MG1655 strain is also characterized by biofilm formation. A variety of candidate regulators of this process have been proposed but the master regulator is not yet known. The main aim of our research is to find the key factors promoting changes of the *E. coli* lifestyle, and to study the possible connection between biofilm formation and metabolic features.

Methods and Algorithms: RNA was extracted from the free-living *E. coli* cells and biofilms, and RNA-seq was performed in triplicates. After QC, reads were mapped on the reference genome using Bowtie2, and differential expression was analysed with FeatureCounts and DESeq2. Biofilm intensity was measured in 96 well plates after staining with 0.1 % crystal violet. Bacterial adhesion efficiency was tested on the CaCo-2 monolayer. Target gene expression was evaluated using qRT-PCR. Calculations were performed using $2^{-\Delta\Delta Ct}$.

Results: Based on the RNA-seq data, regulators of hexuronate metabolism, UxuR and YjjM, could serve as repressors of the *E. coli* motility and activators of biofilm formation. To form a biofilm, bacteria first need to attach to a surface. As such, we measured the adhesion ability of the wild type *E. coli* K-12 MG1655 and its *ΔuxuR, ΔexuR, ΔyjjM,* and *Δcrp* derivatives to the intestinal epithelial cells CaCo-2 (Fig. 1). cAMP-CRP was earlier shown to be involved in regulation of these processes, and ExuR is the UxuR paralog [1].

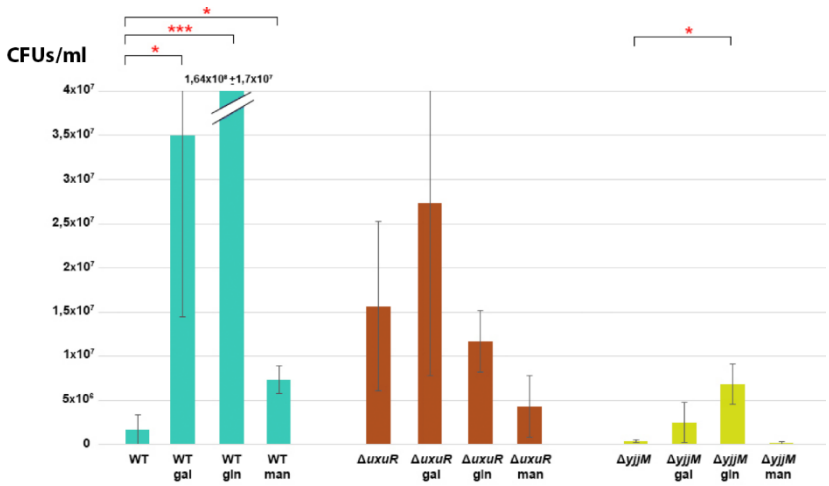

Fig. 1. Adhesion of *E. coli* K-12 and its deletion mutants for the *uxuR*, and *yjjM* genes in the presence of 0.2 % galacturonate (gal), glucuronate (gln), or mannose (man) in the medium to CaCo-2 cells. The standard deviation for three biological replicates is presented. Statistical significance was calculated using the t-test: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) relative to K-12 MG1655

YjjM indeed affected adhesion efficiency, while *uxuR* deletion led to the decrease of hexuronate-mediated adhesion activation (Fig. 1). Deletion of *crp* also decreased the number of attached cells, while deletion of *exuR* caused no significant effects (not shown). These results confirm the importance of hexuronate metabolism for successful surface attachment of bacteria to host tissues and the key role of UxuR and YjjM in this process. The next step was to evaluate the effect on the biofilm formation (Fig. 2). As expected, deletion of *yjjM* and *crp* resulted in decreased biofilm formation, either in the absence or presence of glucose, but the effect of the *uxuR* deletion was unstable. At the same time, deletion of *exuR* did not cause any effect, that was rather unexpected.
According to the transcriptomic data, the absence of uxuR resulted in dramatic activation of all fli – genes (responsible for flagella formation and motility), but only in two out of three cases. This explains the data on the proteomic maps of the K-12 and ΔuxuR in our earlier study (Fig. 4A) [2]. As such, the next part of our research was to verify or refute the assumption that the deletion of uxuR resulted in an imbalance in the transcription level of genes responsible for biofilm formation thus leading to different results on the plates that we had observed (Fig. 2). For this purpose, we studied biofilm formation and expression of genes encoding key regulators, in the individual colonies of E. coli K-12 MG1655, and its ΔuxuR, ΔexuR, Δcrp, and ΔyjjM derivatives. Since within uxuR small non-coding RNAs can be synthesized that are involved in the regulatory processes [3], two versions of the mutant were taken: full gene deletion and the strain where UxuR is not translated but all RNAs can be synthesized (ΔuxuR-tr). We measured expression of four genes: fliA coding for the “flagellar” sigma-factor σ28 [1]; csgD coding for the the pili responsible for the cell-surface adhesion [1]; csrC, small RNA that regulates csrA expression and was earlier shown to regulate biofilm formation [1]; and ssrS, 5S rRNA that was dramatically overexpressed in the biofilm-forming cells independent on the carbon source (Fig. 3).

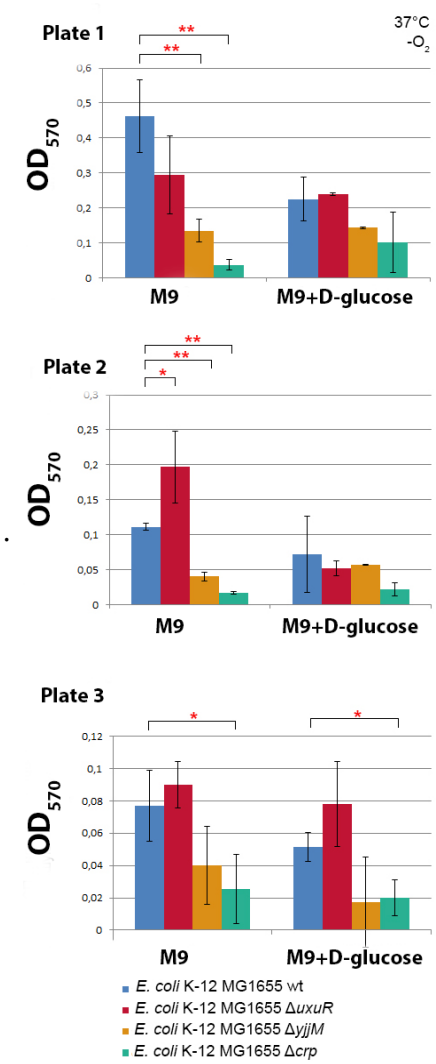

Fig. 2. Biofilm formation in 96-well plates. Data were normalized to the optical density. Data are presented for three identical plates grown simultaneously (biological repeats), and each sample was grown in three wells (three technical repeats). Cells were cultured for 48 hours in M9 + 5% LB + 0.2% D-glucose at 37 °C, microaerobic conditions. Spreads are presented as the standard deviation of three technical repeats. Statistical significance was calculated using t-test: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) relative to K-12 MG1655

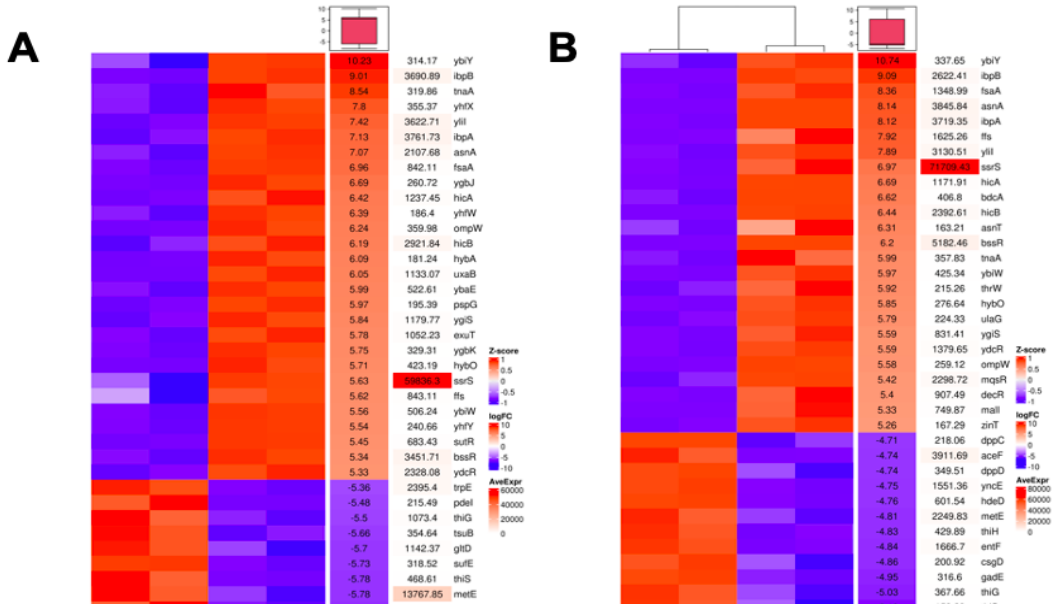

Fig. 3. Differential expression of genes for the free-living *E. coli* K-12 MG1655 and its biofilm during growth on D-glucose (A) and D-glucuronate (B)

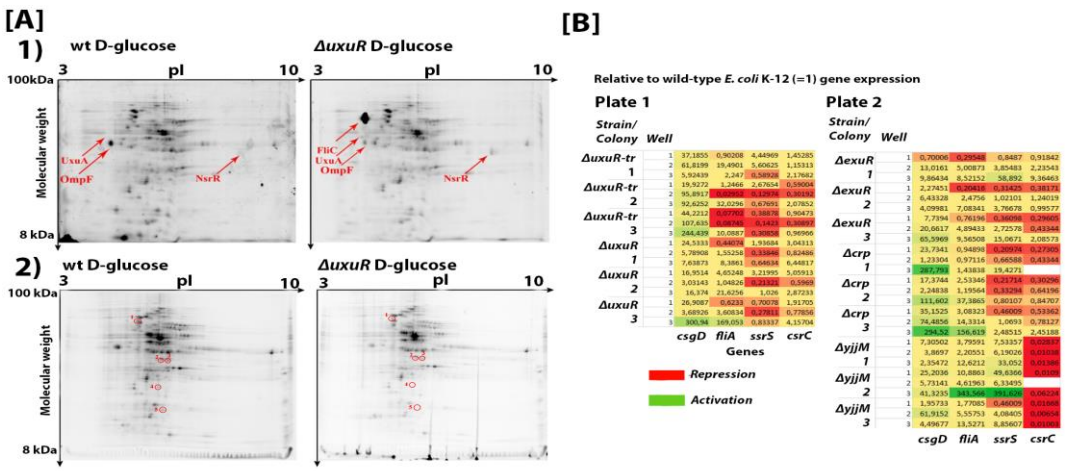

Fig. 4. [A] Proteomic maps of the wild type *E. coli* K-12 MG1655 and *E. coli* K-12 MG1655 *ΔuxuR* during growth with D-glucose: first experiment (1) and second experiment (2) [2]. [B] Gene expression of mutant strains presented as a heat map relative to *E. coli* K-12 (=1)

qRT-PCR (Fig. 4B) confirmed that transcription of fliA in ΔuxuR indeed appeared to be "floating", and a burst was observed only in several wells. In the ΔuxuR-tr mutant, fliA was repressed reflecting the impact of RNAs synthesized from within uxuR. The csgD expression was activated in both strains that corresponded to the efficiency of biofilm formation in the respective wells in this experiment. YjjM appeared to directly activate CsrC. Deletion of crp activated csgD and inhibited csrC, but the effects were less prominent than for deletion of yjjM. This is consistent with the previously shown CRP-mediated effect on csgD.

Conclusion: Hexuronates metabolism and its regulators, UxuR and YjjM, play the key roles in successful bacterial attachment and biofilm formation via modulating these processes, while cAMP-CRP probably act indirectly. Also, our data suggest that small non-coding RNAs are deeply involved in regulation of biofilm formation in *E. coli* K-12 and might serve as possible signaling molecules.

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