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Screening of a bursa aurealis transposon library identifies major protein families involved in biofilm formation by Staphylococcus aureus USA300

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Background: Methicillin-resistant *Staphylococcus aureus* (MRSA)-USA300 is notorious for its ability to cause community- and healthcare-acquired infections, which are even more difficult to treat when associated with a biofilm phenotype. We utilized a *mariner*-based transposon, *bursa aurealis*, to generate a random library of mutants in a clinical isolate (USA300-UAS391) to query the USA300 genome for nonessential genetic pathways involved in biofilm formation.
Material/methods: The plasmid-cured derivative of *S. aureus* USA300-UAS391 (USA300-UAS391 Ery^S) was sequentially transformed with pFA545 and pBursa. Prospective *bursa aurealis* mutants were selected on solid erythromycin-supplemented medium at 43°C and phenotypically analysed for changes in biofilm formation, growth rates and antibiotic susceptibility. Static biofilms in microtitre plates were stained with crystal violet (2%) after 24 hours and quantified (OD_{492}) (Multiskan™FC photometer; Thermo Scientific). Bacterial growth was analysed by repetitive kinetic turbidometric measurements at 600nm (Multiskan™GO spectrophotometer, Thermo Scientific). Antibiotic susceptibility was determined by disk diffusion (EUCAST recommendations) for ciprofloxacin, gentamicin, sulfamethoxazole-trimethoprim, cefoxitin, chloramphenicol, clindamycin, erythromycin, oxacillin, penicillin and tetracycline. Genomic DNA of knockout mutants showing ≤70% biofilm formation, change in antibiotic susceptibilities or altered growth rates, as compared to parental strains USA300-UAS391 and USA300-UAS391 Ery^S, was isolated (Masterpure™DNA & RNA purification kit,

Epicentre®, an Illumina®company). Transposon insertion sites were identified by inverse PCR and Sanger sequencing. Briefly, DNA was digested with *Aci*I restriction enzyme, ligated with T4 ligase, and PCR-amplified using outward-facing primers ((Buster) 5'-GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTACC-3'; (Martin-ermR) 5'-AAACTGATTTTTAGTAAACAGTTGACGATATTC-3') that anneal to two different regions on the transposon. Sequenced amplicons underwent local BLAST analyses against the *S. aureus* FPR3757 reference genome (NC_007793.1).

Results: A library of 1920 transposon mutants was generated. Of these mutants, 47 showed altered susceptibility to ciprofloxacin (S→I), chloramphenicol (S→I) or penicillin (R→S); 46 isolates showed altered growth rates in the slope of the exponential (logarithmic) portion of the growth curve (R^2=0.84-0.94); and 258 isolates showed a decrease in biofilm formation capacity (OD_{492}<70% of USA300-UAS391 Ery^S), as compared to the parental strains (OD_{492}=0.511; R^2=0.95) (Figure A). In total, 20 mutants showed an interconnection between biofilm forming capacity and antibiotic susceptibility and/or growth rate. Inverse PCR and sequencing identified 92 mutants showing decreased biofilm formation with transposon insertions in single, unique genes involved in several important functional pathways (Figure B).

Conclusions: In this study, the *bursa aurealis* transposon was used for random mutagenesis and for isolation of 1920 *S. aureus* mutants with random insertion sites. By screening for 258 mutants in a static microtiter plate biofilm assay, phage capsid, ATPase associated with various cellular activities, bacterial toxin 50, transposase DDE and NAD/NADP octopine/nopaline dehydrogenase were amongst the identified protein families with major impact on decreased biofilm forming capacity in *S. aureus* USA300. The linkage between the *bursa aurealis* insertion and the mutant phenotype will be further confirmed.

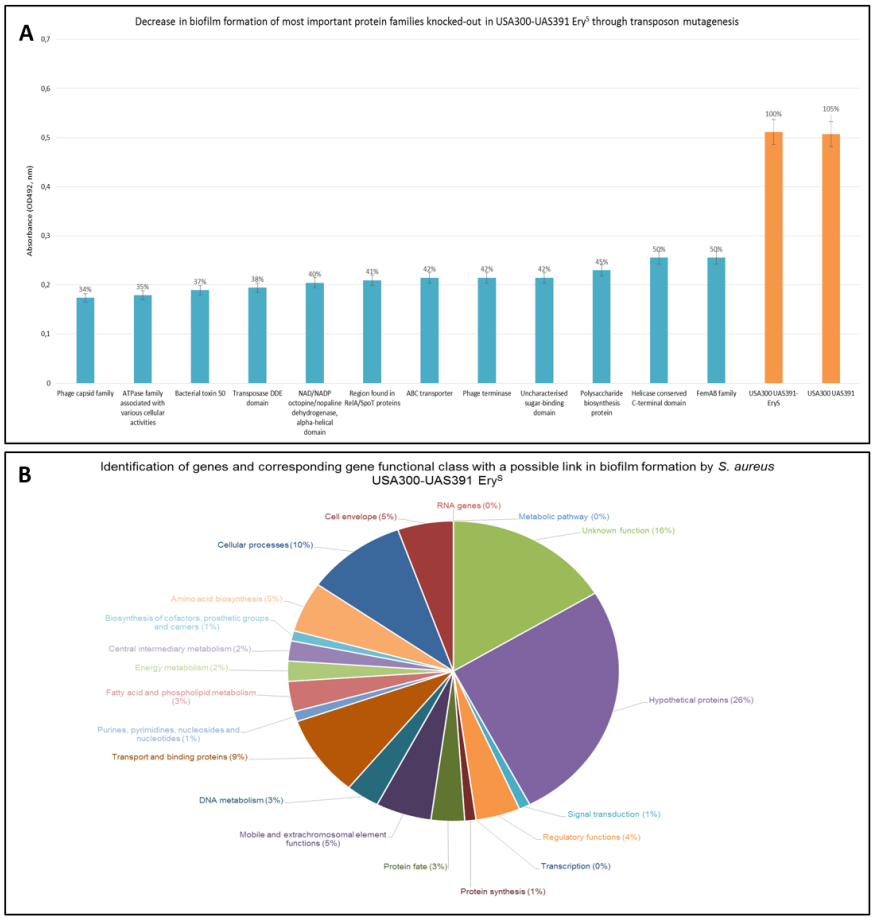

Figure: **(A)** Knocked-out genes per family showing ≤ 50% decrease in biofilm formation by USA300-UAS391 Ery^S. The amount of biofilm formed by each particular strain was measured through quantitative measurement of optical density. The amount of crystal violet bound in each well is proportional to the amount of biofilm formed and can be directly quantified by measuring light absorption. Blue samples are *bursa aurealis* knockout mutants, while orange samples display the parental strains. **(B)** Identification of *bursa aurealis* insertion sites of transposon mutants showing ≤ 70% decrease in biofilm formation as compared to USA300-UAS391 Ery^S. 100-200 nucleotides downstream of the transposon end site CCTGTTA were selected and subjected to Nucleotide BLAST. Annotations of corresponding genes are based on the reference genome of the most closely related strain FPR3757. Classification within the gene functional class (TIGRFam Main Role) as described on AureoWiki, the respository of the *Staphylococcus aureus* research & annotation community (http://aureowiki.med.uni-greifswald.de/Main_Page) is based on metafunction, envelope, cellular processes, metabolism, genetic info processing, regulation, unknown function or RNAs.