Carbon source influences population heterogeneity in *Pseudomonas putida* KT2440 biofilms

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Pseudomonas putida is well known for its large metabolic capacity, which makes it a potential cell factory for many different biochemicals. The ability of the organism to live in the biofilm state can be advantageous for the production of potentially toxic products due to increased chemical tolerance and general robustness of biofilm associated cell populations. However, biofilm cells frequently differentiate, e.g. by lower yields, and this population heterogeneity may challenge the benefits of biofilm-based production, and therefore knowledge about factors driving the differentiation is of importance in an industrial setting.

We have investigated the influence of different carbon sources on the heterogeneity of *P. putida* KT2440 biofilms growing in a dynamic flow chamber system for 7 days with subsequent phenotypic and genetic analysis of variants. In all cases minimal medium supplied with a single carbon and energy source was used as substrate for the biofilm populations in the flow cells.

Biofilm structure and cell differentiation in *P. putida* KT2440 were highly dependent on the type of carbon source utilised. Low glucose concentrations ranging from 0.3 mM – 15 mM did not alter biofilm structure and showed low or no variation in swimming motility, biofilm capability or growth rate after several days of incubation compared to a one day old planktonic *P. putida* KT2440 culture. However, increasing the glucose concentration to 30 mM introduced filamentous cell structures in the biofilm, which easily detached from the biofilm resulting in lower biomass, while colony morphology and cell motility remained unchanged.
Filamentous and loose biofilm structures were also observed when using citrate in 1 mM – 50 mM range, and small, irregular or wrinkled colony morphotypes appeared. Isolated cells from these colony morphotypes showed a large variation in swimming motility, biofilm formation and growth rate. Whole genome sequencing revealed alterations in cyclic-di-GMP related genes, suggesting selection for mutations in global

regulatory genes involved in biofilm development. No such genetic alterations were observed when changing the glucose concentration. Succinate and glycerol resulted in filaments but no altered colony morphotypes, and no filaments but small colony variants, respectively. This might suggests that filamentous structure is not necessarily an indicator of population heterogeneity, but further investigations need to clarify this.

From these data we conclude that in minimal medium with low concentrations of glucose as carbon source, *P. putida* KT2440 biofilms appear homogeneous. In contrast, citrate as a carbon source induces cell differentiation independent of concentration in KT2440 biofilms. Differentiation in the biofilm population was associated with an apparent selection for mutations of genes involved in cyclic-di-GMP mediated global regulation.