Pseudomonas 2.0

Robust fermentation production of tacrolimus and related immunosuppressors: Molecular genetics and metabolic engineering to construct a by-product free superproducer

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The potential of non-pathogenic Pseudomonas as a platform microorganism for the industrial production of chemicals, pharmaceuticals and fuels has been discussed for decades in Europe, mainly inspired by its metabolic versatility, ease of genetic programming and high solvent tolerance. These properties enable growth in the presence of a second phase of toxic solvents, such as styrene or octanol, or high concentrations of inherently toxic compounds, such as furaldehydes, originating from cheap and renewable feedstocks (for example, biomass hydrolysates). Furthermore, Pseudomonas displays an extensive enzymatic inventory (for example, hydrocarbon degradation pathways) and the potential to efficiently regenerate the redox cofactors necessary for productive syntheses. However, Pseudomonas strains currently play only a minor role as production strains for the bio-industry (with Bacillus, Corynebacterium glutamicum, Escherichia coli and Saccharomyces cerevisiae as the most important ones). Novel biocatalytic processes must successfully overcome economic barriers before greater realization is possible. The desirable properties of microbes therefore include high solvent tolerance, cofactor regeneration capacity, carbon efficiency and operational stability. Furthermore, the consideration of process conditions and scaling is central. In this project, we have tackled and overcome some of the remaining molecular bottlenecks that still hamper Pseudomonas applications, and have exploited some of their unique characteristics to provide major benefits for application. To this end, various genetic and analytic tools, as well as stable production hosts and cultivation methods, have been established, as described below.

'With this project, ERA-IB enabled a very fruitful and close international collaboration, which was key to success and prone to inspire future collaborative projects. Beside the successful scientific and technical output, the project enabled academic-industrial networking not only among project partners but also beyond. Several contacts for possible industrial implementations have been established.'

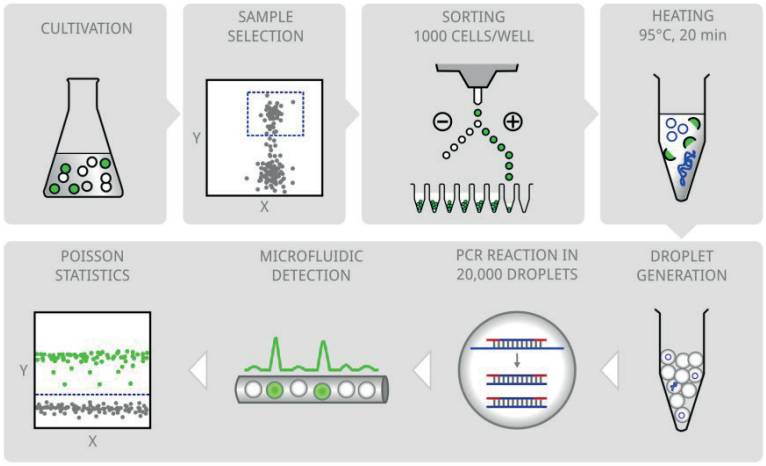
Development of genetic tools

Using the Standard European Vector Architecture (SEVA, nearly one hundred vectors), a set of calibrated synthetic promoters (to control recobinant protein synthesis) and pBAMDs (a series of novel broad host-range mini-Tn5 vectors to insert DNA into the genome), it was possible to develop a core collection of genetic tools. These tools can now be used as bricks to construct engineered Pseudomonas strains. Bricks of this kind were intensively exchanged within the consortium.

Physiology and variability

Metabolic engineering experiments showed that the Entner-Doudoroff pathway plays a central role in counteracting oxidative stress, which is an important feature

for productive application. For efficient aromatics production, the availability of the metabolite PEP was identified as a critical factor, especially on lignocellulosic C5-substrates, such as xylose. As far as variability/reproducibility issues are concerned, indications were found that genetically homogeneous populations of P. putida mt-2 employ a strategy of phenotypic variation (metabolic bet-hedging) when confronted with nutrient mixtures. Based on the green fluorescent protein eGFP, a fusion construct with the synthetically applied enzyme styrene monooxygenase was generated to evaluate variability in its intracellular level in different Pseudomonas strains under different cultivation conditions. The results showed, for example, that some strains formed two subpopulations in one culture, with one population synthesizing high levels and the other no or low levels of the fusion protein (bimodal expression pattern). Such expression variability was found to depend on the combination of the strain and the regulatory system, and could be avoided using suitable combinations.Subpopulation proteomics was established for the first time and subpopulations with differing expression levels were successfully sorted and subjected to proteome analysis, giving interesting insights into subpopulation-based differences in the proteomic cell inventory. For physiology analysis under stress conditions, a continuous cultivation platform was established. The population heterogeneity was found to be growth-rate dependent and P. putida KT2440 was found to recover quickly after phases of oxygen depletion. Flow cytometry in combination with modeling and simulation showed that stress in terms of iron limitation, oxygen limitation and organic solvent exposure significantly increases the replication speed above the maximum level observed under non-stress conditions.



Work flow for the quantification of plasmid copy numbers in individual separated subpopulations within a single culture.

Production strains and conditions

Ethanol-producing E. coli and P. putida strains were constructed and compared. P. putida consistently outperformed E. coli with regard to tolerance towards ethanol, whether produced in the cells or added exogenously. This highlights the value of this bacterium as a microbial cell factory for the production of biofuels, owing to its naturally pre-evolved ability to withstand different kinds of chemical stress. The solvent tolerance characteristics of Pseudomonas taiwanensis VLB120 were investigated and a constitutively solvent-tolerant strain was generated, overcoming the necessity of tedious and unpredictable adaptation to the presence of a toxic solvent. Importantly, this strain not only showed an improved stability but also a significantly improved production performance under process conditions. Growing cells of this mutant strain showed the highest specific styrene epoxidation activity reported so far, which augurs well for process implementation. Another approach to construct a robust Pseudomonas-based microbial cell factory consisted of the deletion of 11 non-adjacent genomic elements within P. putida, thereby enhancing desirable traits and eliminating attributes that are detrimental in an expression host. A suite of functions that drain high-energy phosphate from the cells and/or consume NAD(P)H were targeted; in particular, the whole flagellar machinery. Furthermore, elements potentially causing genetic instability (4 prophages, 2 transposons and 3 components of DNA restriction-modification systems) were eliminated. The resulting strain P. putida EM383 displayed improved growth properties (lag times, biomass yield and specific growth rates) and a recombinant protein synthesis performance clearly superior to the precursor wild-type strain KT2440.

Conclusion

Overall, several molecular and technical bottlenecks in respect of the practical application of Pseudomonas have been resolved successfully and interesting production strains have been discovered. This will now enable the development of ecologically and economically more efficient processes for the production of diverse goods, including chemicals, pharmaceuticals and fuels from renewable resources. Relevant issues were continuously discussed with the industrial consultants, with the aim of placing the European bio-industry in a prime position within the global biotechnology market and supporting a bio-refinery approach in the chemical industry on the basis of the European Lead Market Initiative.