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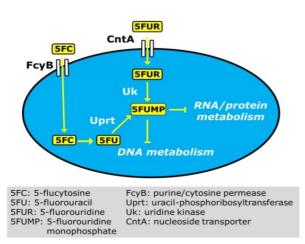


Fungal multiplex genetic engineering exploiting the pyrimidine salvage pathway

Reference Number TO 42-00019

Challenge

Functional manipulation is a perquisite for approaches involving production of recombinant proteins or compounds of biotechnological/ medical importance. Genetic engineering is typically based on genomic integration of genes requiring selectable marker genes to distinguish between transformed and non-transformed cells employing either dominant antibiotic resistance genes or auxotrophy-curing genes that require specific recipient strains. Thereby, the restricted availability of dominant selection marker genes represents a limitation in genetic manipulation. To circumvent the problem of selection marker gene shortage, different recycling strategies have been developed based on the excision of the marker gene after successful integration into the genome. However, the remnants in the chromosome after each transformation step (e.g. FRT or *loxP* sites), the laborious efforts to remove the inserted markers as well as difficulties in maintaining purity of such isolates due to the absence of the selection marker after recycling represent major drawbacks of these systems. In addition, with the application of current marker systems, targeted integrations remain a challenging task and transformations are often accompanied with ectopic integrations only with unpredictable side effects.



Metabolic conversion of 5FC, 5FU and 5FUR into the cell toxic metabolite 5FUMP by enzymes of the pyrimidine salvage pathway

Commercial Opportunity

Technology

A solution to these disadvantages is provided by researchers from the Medical University of Innsbruck offering a new versatile counter-selectable marker system using endogenous counterselectable markers that are non-auxotrophic but still (should) allow highly efficient selection in suitable organisms. The technology is based on homologous recombination-driven replacement of these negative selection markers with DNAs of interest (DOIs) giving resistance to 5-fluorocytosine (5FC), 5fluorouracil (5FU) or 5-fluorouridine (5FUR). Proof of principle experiments in Aspergillus fumigatus using reporter cassettes uncovered several loci suitable for transformation selection: fycB (5FC permease), fycA (5FC deaminase), uprt (5FU phosphoribosyltransferase) and uk (5FUR uridine kinase) or cntA (5FUR nucleoside permease). Loss of individual activities resulted in differential 5FC/5FU/5FUR resistance, allowing the simultaneous use of markers for the insertion of multiple DOIs.

Homologous, site-directed integration of any gene or sequence of interest into the genetic loci of *fcyB*, *fycA*, *uprt*, *cntA* or *uk* without the need for additional selectable marker genes becomes possible in several organism groups including bacteria, fungi and plants because of an evolutionary conservation of the pyrimidine salvage pathway. This versatile applicability further highlights the potential of the technology for genetic and metabolic engineering of strains used for heterologous protein expression and the production of any biotechnological relevant substance (e.g. carbohydrates, lipids, antibiotics, vitamins, amino acids, cosmetic ingredients, pharmaceutical active proteins/peptides). In the case of integration of homologous sequences, this approach allows self-cloning and avoidance of the GMO status. The technology is open for licensing, further co-development is highly welcomed.

Developmental Status

Various reporter cassettes (BFP, GFP, RFP, luciferase, lacZ) have been integrated into the respective genomic loci of *A. fumigatus* via 5FC, 5FU or 5FUR selection to validate the suitability of the described strategy. Proof-of-principle applications



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Patent Situation

Patents are pending in EP and US (priority June 2018).

Further Reading

Birštonas et al. (2020), mBio 11:e00230-20



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