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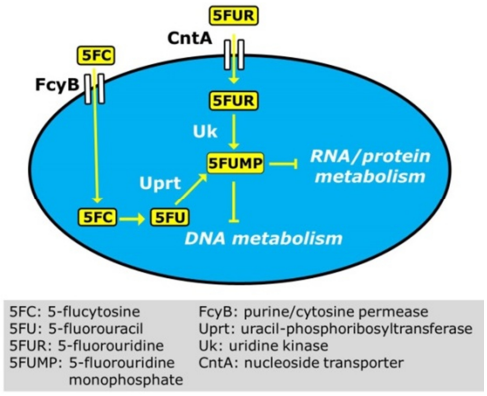
Novel marker-free selection strategy for site-directed homologous recombination targeting the pyrimidine salvage pathway

Reference Number TO 42-00019

Challenge

Functional manipulation is a perquisite for approaches involving production of recombinant proteins or compounds of medical/ biotechnological importance. Genetic engineering is typically based on genomic integration of genes requiring selection marker genes to distinguish between genetically 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 possibilities. 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) represent a major drawback of these systems as they may cause problems via recombination and chromosome instability. Another disadvantage of this approach is the time-consuming selection for positive clones that have lost the resistance feature. Integration is either locus targeted based on homologous recombination or ectopically with unpredictable side effects.



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

Technology

A solution to these disadvantages is provided by a new versatile counter-selectable marker system using markers, which are non-auxotrophic and thus allow for an efficient selection marker-free transformation of suitable organisms. Researchers at the Medical University of Innsbruck present a method allowing site-directed integration of sequences of interest into independent genetic loci exploiting the pyrimidine salvage pathway in a host cell. This novel selection strategy is based on loss of transport/enzymatic activities involved in the metabolism of toxic nucleobase as well as nucleoside analogs: 5-flucytosine (5FC) selects for loss of cytosine permease (FycB) or uracil-phosphoribosyl-transferase (Uprt), i.e. integration in the encoding gene (*fcyB* or *uprt*). 5-fluorouracil (5-FU) selects for loss of Uprt, 5-fluorouridine (5-FUR) selects for loss of uridine kinase (Uk) or nucleoside uptake (CntA).

Commercial Opportunity

On the basis of these markers, homologous site-directed integration of any gene or sequence of interest into the genetic loci of *fcyB*, *uprt*, *cntA* or *uk* without the need for additional selection marker genes becomes possible in several organism groups including bacteria, fungi and plants. Modified strains can be used in 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 GMO status.
The technology is open for licensing, further co-development is highly welcomed.

Developmental Status

The selection method has been successfully tested for *fcyB* and *uprt* loci in the pathogenic model organism *Aspergillus fumigatus*. Various reporter cassettes (eGFP, RFP, luciferase, lacZ) have been integrated into the respective genomic loci via 5FC or 5FU selection to validate the suitability of the described strategy. In addition, the feasibility of the approach for the *uprt* locus in the biotechnological relevant *Penicillium chrysogenum* and the plant pathogen *Fusarium oxysporum* has been validated, and the use of the *cntA* and *uk* locus in *A. fumigatus* are explored regarding their suitability. Simultaneously, investigations on the use of the system in further biotechnological important species such as *Saccharomyces cerevisiae* and *Escherichia coli* are ongoing.

Patent Situation

A priority claiming GB patent application has been filed in June 2018.

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