

Multiplex genetic engineering exploiting pyrimidine salvage pathway-based endogenous counterselectable markers.

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Abstract

Selectable markers are indispensable for genetic engineering, yet their number and variety are limited. Most selection procedures for prototrophic cells rely on the introduction of antibiotic resistance genes. New minimally invasive tools are needed to facilitate sophisticated genetic manipulations. Here, we characterized three endogenous genes in the human fungal pathogen *Aspergillus fumigatus* for their potential as markers for targeted genomic insertions of DNAs of interest (DOIs). Since these genes are involved in uptake and metabolism of pyrimidines, resistance to the toxic effects of prodrugs 5-fluorocytosine and 5-fluorouracil can be used to select successfully integrated DOIs. We show that DOI integration, resulting in the inactivation of these

genes, caused no adverse effects with respect to nutrient requirements, stress resistance, or virulence. Beside the individual use of markers for site-directed integration of reporter cassettes, including the 17-kb penicillin biosynthetic cluster, we demonstrate their sequential use by inserting three genes encoding fluorescent proteins into a single strain for simultaneous multicolor localization microscopy. In addition to *A. fumigatus*, we validated the applicability of this novel toolbox in *Penicillium chrysogenum* and *Fusarium oxysporum*. Enabling multiple targeted insertions of DOIs without the necessity for exogenous markers, this technology has the potential to significantly advance genetic engineering. **IMPORTANCE** This work reports the discovery of a novel genetic toolbox comprising multiple, endogenous selectable markers for targeted genomic insertions of DNAs of interest (DOIs). Marker genes encode proteins involved in 5-fluorocytosine uptake and pyrimidine salvage activities mediating 5-fluorocytosine deamination as well as 5-fluorouracil phosphoribosylation. The requirement for their genomic replacement by DOIs to confer 5-fluorocytosine or 5-fluorouracil resistance for transformation selection enforces site-specific integrations. Due to the fact that the described markers are endogenously encoded, there is no necessity for the exogenous introduction of commonly employed markers such as auxotrophy-complementing genes or antibiotic resistance cassettes. Importantly, inactivation of the described marker genes had no adverse effects on nutrient requirements, growth, or virulence of the human pathogen *Aspergillus fumigatus*. Given the limited number and distinct types of selectable markers available for the genetic manipulation of prototrophic strains such as wild-type strains, we anticipate that the proposed methodology will significantly advance genetic as well as metabolic engineering of fungal species.

Beteiligte Forschungseinheiten

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