Engineering *Corynebacterium glutamicum* with a comprehensive cosmid library and phage-based vectors

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Background

The club-shaped actinobacteria *Corynebacterium glutamicum* sustain the global production of amino acids, notably glutamate and lysine. Generally recognized as safe, this microorganism has been extensively engineered for the commercial production of metabolites such as organic acids, from sustainable carbon sources [1]. Through the heterologous expression of biosynthetic pathways, *C. glutamicum* has become a new source of added value compounds for the pharmaceutical and food industry [2].

Genetic engineering in *C. glutamicum* has been carried out using non-replicative vectors with small homologous recombination platforms for targeted gene editing and delivery [3]. Recently, programable endonucleases were also developed for *C. glutamicum* (reviewed in [4]). Though powerful, the current technologies for gene editing of *C. glutamicum* often require long preparation time, result in low editing frequencies, and, thus, involve laborious mutant screening. This work [5] addresses the current issues of gene editing and delivery using a novel platform for gene editing and phage-based integrative systems for gene delivery into *C. glutamicum.*

Methods

A cosmid library was generated by transfection of *E. coli* with ~35 kbp fragments of the genome of *C. glutamicum* and mapped by sequencing. Existing excisable markers [6] were co-opted for cosmid modification by recombineering in *E. coli* and standard methods were used for allelic exchange in *C. glutamicum*. Marker excision was performed as described elsewhere [6]. The mutant strain was characterized by genome re-sequencing. The pigments produced by derivative strains harboring biosynthetic genes for the polyketide flaviolin or the non-ribosomal peptide indigoidine were quantified by spectrophotometry [7, 8].

Results

We generated a cosmid library of the genome of *C. glutamicum* covering more than 94% of the chromosome, with average 5.1x coverage. Non-represented genomic

regions were consistent with other libraries of *C. glutamicum.* We replaced the 7.6-kbp *crt* locus encoding the yellow pigment of *C. glutamicum* by an attachment site for the ΦBT1 actinophage. For that, we modified one cosmid from the generated library by recombineering in *E. coli* with an excisable marker obtained by PCR. After transformation and selection of the cosmid in *C. glutamicum*, we obtained albino mutants with up to 94% efficiency. Importantly, our cosmid-based allelic exchange attained higher first recombination frequency, higher second recombination frequency, and less reversion to wild-type events than the conventional plasmid-based system. We obtained a marker-less deletion strain after transient expression of the marker excision system. Overall, we performed the procedure of gene editing in *C. glutamicum* using a modified cosmid and marker excision in 14 days, with no associated off-target effects.

We named the generated mutant as the BCA strain, after the encoded attachment sites of ΦBT1 and ΦC31, and its albino phenotype. We showed the BCA strain was readily transformable with ΦC31- and ΦBT1-based vectors, in a site-specific manner. We calculated integration efficiencies of up to 600 and 10 000 colony-forming units per µg of ΦC31- and ΦBT1-based plasmids, respectively. To demonstrate the utility of the *C. glutamicum* BCA strain, we generated derivative strains to produce specialized metabolites. A BCA derivative to produce the naphthoquinone flaviolin was generated by integrating a genetic device constituted of the Ptac promoter and the polyketide synthase gene *rppA* using the ΦC31-based vector pTES [9]. This strain produced up to 40 mg L^{-1} of flaviolin in 72 hours of culture. The backbone of the ΦC31-based vector, featuring two *loxP*-sites, was excised by transient expression of the Cre recombinase [9] rendering the strain marker-free and maintaining flaviolin production. We also generated a BCA derivative to produce the pyridone indigoidine by integrating the non-ribosomal peptide synthetase gene *bpsA*, under a strong synthetic promoter [10], using the ΦBT1-based vector pFM69SD. The expression of the high-GC, codon-biased *bpsA* gene required prior optimization of 5'-UTR and first codons. This strain produced up to 7.3 g L^{-1} of indigoidine, in 72 hours of culture, without the need for heterologous 4'-phosphopantetheinyl transferase co-expression.

Conclusion

A comprehensive cosmid library of the genome of *C. glutamicum* is now available for the community. Combined with standard techniques of recombineering (in *E. coli*) and allelic exchange, this library allows seamless and efficient gene editing of medium-sized regions (20-30 kbp) of the chromosome of *C. glutamicum*. Thanks to the developed methodology, an engineered *C. glutamicum* strain with expanded capacities for phage-based integration and reduced metabolic footprint is also available for the community. Vectors based on ΦC31 and ΦBT1 phages can integrate into the BCA strain with moderate to high frequency at the cognate attachment sites, assisted by the correspondent phage integrases. By analogy to other actinobacteria, the delivery and inheritance of large heterologous biosynthetic pathways (>100 kbp) shall now be possible in the *C. glutamicum* BCA strain. Proof-of-principle heterologous expression in *C. glutamicum* BCA using phage-based vectors allowed the production of the small molecules flaviolin and indigoidine with applications for the pharmaceutical and

chemical industries. The ability to express highly biased heterologous genes encoding for enzymes requiring post-translational modification in *C. glutamicum* supports the relevance of this actinobacteria as heterologous host.

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