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Poster Presentation

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Development of an antibiotic-free plasmid selection system based on glycine auxotrophy for recombinant protein overproduction in *Escherichia coli*

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Background

Antibiotics and antibiotics resistance genes have been traditionally used for the selection and maintenance of recombinant plasmids in hosts such as *E. coli*. Although a powerful selection tool, their use has been considered unacceptable in many areas of biotechnology by regulatory authorities. Indeed, there is much international scientific and regulatory focus on this issue [1]. For instance, the use of selection markers that confer resistance to antibiotics in vaccine plasmids may introduce the risk of transforming the patient's microflora and spread resistance genes. Moreover, in recombinant protein production for therapeutic use, the antibiotic must be eliminated from the final product. Another problem arises from the potential loss of selective pressure as a result of antibiotic degradation i.e. ampicillin can be degraded by β-lactamases in less than 30 minutes in high cell density cultures [2].

In this work, an alternative approach to prevent plasmid loss based on an amino acid auxotrophy complementation has been developed. A glycine-auxotrophic strain of *E. coli* M15 has been created using PCR products [3]. This strain contains an internal deletion of the *glyA* gene, which encodes for serine hydroxymethyl transferase (SHMT), an enzyme involved in the main glycine biosynthesis pathway in *E. coli* [4]. As a result, the glyA- strain can not synthesize glycine and, therefore, needs an external source of glycine or a genetic source of SHMT for growing on a defined medium. The construction of a plasmid selection system derived from the commercial vector pQE40 (QIAGEN) with *glyA* under the control of the constitutive weak promoter P3 [5], as well as its evaluation in batch and fed-batch cultures for expressing ramnulose 1-phophate aldolase (RhuA) as a model for recombinant protein production has been evaluated.

Results

Disruption of the *glyA* gene in *E. coli* M15 was accomplished using a directed knockout gene method employing PCR products through homologous recombination [3]. The phenotype of the new strain M5ΔglyA was tested on defined medium cultures with and without glycine, confirming the auxotrophy for glycine.

By using SOE-PCR [6], the *glyA* gene (referred here and after as β fragment) from *E. coli* K-12 was fused to the weak constitutive promoter P3 (referred here and after as α fragment). The fusion αβ product was cloned into pQErham, a pQE40-derived vector for RhuA overexpression [7]. The resulting complementation vector, pQEα βrham (figure 1), was used to transform the *E. coli* M15 ΔglyA host.

Erlenmeyers cultures with glycine-auxotrophic strain M15 ΔglyA transformed with the complementation expression plasmid can grow in defined medium adding neither glycine nor ampicillin. This indicated that the plasmid is

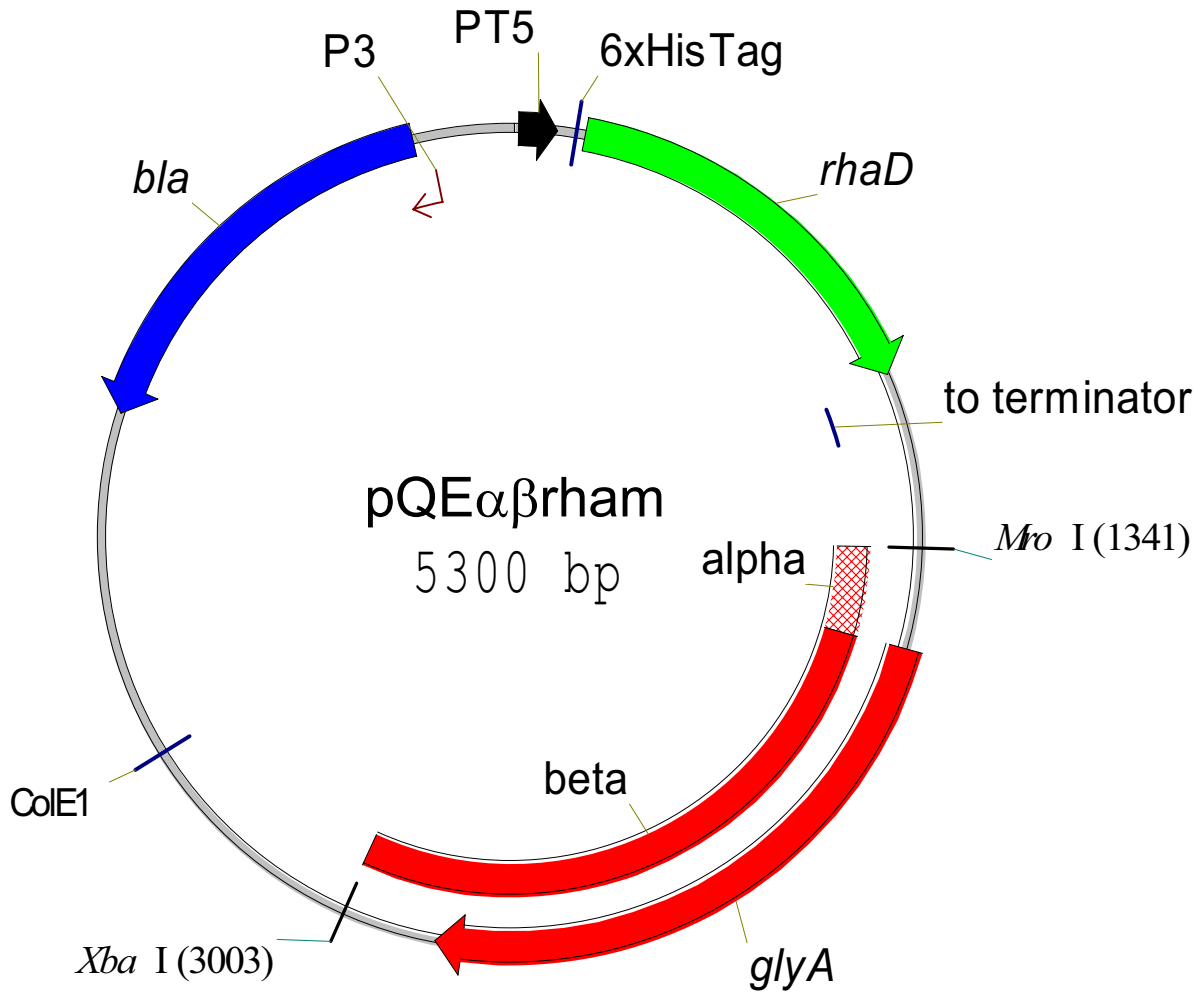

Figure 1
Diagram of the complementation vector pQEαβrham.

maintained and SHMT is being expressed, thereby generating an endogenous source of glycine. SDS-PAGE of soluble cytosolic proteins under non-inducing conditions indicated that SMHT was not being overexpressed.

The capacity of the new complementation system for producing the recombinant RhuA was compared to that of the conventional selection system based on antibiotic resistance (*E. coli* M15 pQErham). Induction with IPTG resulted in high overexpression of recombinant RhuA levels in *E. coli* M15ΔglyA/pQEαβrham. However, such levels were slightly lower than those obtained in the conventional system. SDS-PAGE analyses of cell extracts revealed significantly higher expression levels (compared with non-inducing conditions) of a protein of about 45 kDa, corresponding to SHMT. This suggested that the downstream orientation of αβ fragment in relation to the strong T5 promoter could cause a read-through of RhuA gene in the presence of IPTG, i.e. resulting in higher SHMT expression levels. This phenomenon could exert a metabolic burden on the host cell, resulting in lower specific RhuA activity levels.

High cell density fed-batch cultures using the novel complementation system were performed using an exponential feeding profile strategy analogous to that previously developed for the conventional system [7]. In particular, a fed-batch cultivation with a controlled specific growth rate of 0.2 h^{-1} (final $DO_{600\ nm}$ = 185) was performed without ampicillin. Induction with IPTG allowed obtaining high RhuA production (28483 U l^{-1}) and productivity (1636 U l^{-1} h^{-1}) levels.

Conclusion

The new selection maker based on a glycine-auxotrophy strain plus a plasmid harboring the glyA gene under the P3 weak promoter is a promising tool, not only for recombinant protein production, but also for vaccine plasmids production processes where antibiotics can not be present in the medium formulation. Besides, the use of a chemically defined medium avoids the risk of employing components of animal origin that may contain viruses or prions [1], increasing the safety level of the system for industrial processes.

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