Problem: involves iptg induced production of the protein

Problem: The BL21(DE3) cells harboring the pT7-B0034-glucosidase plasmid and in the presence ofinducer are growing at a slow rate as compared to the untransformed cells. Hypothesis: This system involves IPTG induced production of the protein beta-glucosidase. Theconstitutively active promoter (pLaclq) used for production of the Lac Repressor protein is a mutatedversion of the LacI promoter and results in 10-fold higher expression of LacI protein (Ref. 1). It seemsunlikely that there would be any leaky expression of beta-glucosidase in the absence of inducer, and theproblem states that only " in the presence of inducer" is growth significantly decreased.

This informationleads to the hypothesis that, upon IPTG induction, beta-glucosidase is expressed in these cells at a toxiclevel which subsequently inhibits growth. The best way to resolve this problem would therefore be toreduce the expression level of beta-glucosidase. The choice of Origin of Replication here (RSF1030) could be contributing to this problem. This ORI resultsin high plasmid copy number (Ref. 2) which is not ideal for the expression of toxic proteins.

The T7promoter is a strong promoter which causes high protein expression, so even if beta-glucosidase is onlymildly toxic, using this ORI could result in high beta-glucosidase levels and this could account for the factthat the cells are growing slowly. Using another strain with the capability of tunable expression ofproteins regulated by the T7 promoter such as " NEB Turbo Competent E. coli" (Ref. 3) or " T7 ExpresslysY Competent E. coli" (Ref.

4) or “ BL21(DE3) pLysS” (Ref. 5) could potentially resolve this issue but thequestion says to use the E. coli strain BL21(DE3) so my solution will not involve the use of these cell linesbut will instead involve inserting the beta-glucosidase gene into a plasmid from the available databasewith a different Origin of Replication. 2) Describe in words which plasmids you would construct to test your hypothesis and engineer asolutionTo test my hypothesis that the beta-glucosidase is being expressed at a level which is leading to toxicityand reduced cell growth, I want to remove the beta-glucosidase gene from the original pT7-B0034-glucosidase plasmid and insert it into a plasmid from the available database (which contains pJ23101-B0034-GFP and pJ23101-B0064-GFP plasmids (Ref. 6) with various ORI’s and antibiotic resistance genes, expressed in several E. coli strains). I will use the Golden Gate assembly method to place this gene insertinto the plasmid from genetic construct designation p04: E. coli NEB10b which uses the pSC101 ORI andthe KanR resistance marker.

This plasmid was chosen because it will be present at low copy number (~5copies, Ref. 2) in cells, and if my hypothesis is correct, will result in reduced expression of betaglucosidase, reduced toxicity to the cells, and the growth rate of the cells transformed with this modifiedplasmid will not be diminished as it was with the pT7-B0034-glucosidase plasmid. 3) Describe how you would physically construct these plasmids using any cloning method of yourchoosing. You do not need to write out any DNA sequences – simply naming the genetic parts issufficient.

The plasmid depicted in this problem has BsaI restriction sites flanking the beta-glucosidase gene (aswell as the associated RBS, lac operator, T7

promoter, and T7 terminator). BsaI is a type IIS RestrictionEnzyme and cleaves outside its recognition sequence, therefore to construct this plasmid I want to use Asimov Beta-Glucosidase ProblemDouglas Tait January 19, 2018Page 2the Golden Gate assembly method which makes use of this property to assemble DNA fragmentswithout leaving a scar. This method of plasmid construction will involve performing the following steps: 1) Design and obtain PCR primers to amplify the beta-glucosidase region of the pT7-B0034-glucosidase plasmid making sure to include the two BsaI sites in the PCR product. 2) Design and obtain PCR primers to add BsaI restriction sites to the pJ23101-B0034-GFP plasmidfrom genetic construct designation p04 (Ref.

7). These should be designed such that theresulting PCR product does not contain the GFP gene but does contain the rest of the plasmidbackbone (including the pSC101 Origin and KanR Resistance Marker) they should also bedesigned such that there will be appropriate complementarity, after cutting with BsaI, betweenthe sticky ends of the backbone and the sticky ends of the beta-glucosidase gene insert. 3) Obtain genetic construct plasmid p04 either from previously purified stock or by culturing thep04 NEB10b cells, then performing a miniprep to obtain purified plasmid. 4) Obtain pT7-B0034-glucosidase plasmid. 5) Perform PCR reaction to amplify gene insert from pT7-B0034-glucosidase plasmid using PCRprimers designed in step 1. 6) Perform PCR reaction to amplify plasmid backbone from pJ23101-B0034-GFP plasmid using PCRprimers designed in step 2.

7) Purify amplicons, set up assembly reaction by addition of insert gene and destination vector toGolden Gate assembly mix, incubate at 37 degrees

Celsius for 1 hour, then 55 degrees Celsiusfor 5 minutes. 8) Transform into a suitable E. coli strain for plasmid cloning, such as NEB10b, plate on agarcontaining the antibiotic Kanamycin, incubate overnight. 9) Select several colonies and perform colony PCR. 10) If results of colony PCR look good, grow successful colonies in broth overnight, performminiprep, send purified plasmid away for sequencing, save the rest of the plasmid, andcryopreserve the cells for later use, verify sequencing results when they become available. 11) Use the successfully prepared plasmid to transform the expression strain BL21(DE3). 12) Observe bacterial growth rate and beta-glucosidase protein production levels in transformedBL21(DE3) cells to evaluate accuracy of original hypothesis. 13) Perform further modifications to the expression system as necessary, for instance the methoddescribed here to test my hypothesis used only two fragments and did not incorporate theconstitutively expressed LacI gene and pLacIq promoter from the original pT7-B0034-glucosidaseplasmid into the final construct, these could be added in the Golden Gate assembly as well.