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FINAL REPORT

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Title: Development of an Expression System for Eukarytoic Proteins in Methylotrophic Bacteria

ABSTRACT

The objective of this project was to develop an expression vector for methylotrophic bacteria for use in the production of ^{13}C- and ^{2}H-labelled eukaryotic proteins by growing methylotrophic bacteria on labelled methanol or methylamine. The eukaryotic proteins calmodulin and troponin C were chosen as test cases. Genes encoding both proteins were cloned into different constructions and tested for expression. Moderate amounts of troponin C were found with one of the constructions.

OBJECTIVES

1. Construction of a Eukaryotic Expression Vector for Methylotrophs

A cloning vector that replicates in a variety of methylotrophic bacteria, the broad-host-range vector pECT5, was used for the initial constructions of the expression vectors. It contained an IncQ replicon, a streptomycin-resistance gene for selection, a polylinker to facilitate cloning, and a *tac* promoter for expression. A second vector was also generated, containing the *mau* promoter, for methylamine-regulated expression.

2. Demonstration of the Expression System

Clones containing the gene for either chicken calmodulin or avian troponin C were provided by Cliff Unkefer's group, and appropriate restriction fragments each containing the entire gene were subcloned into both types of expression vectors. These clones were confirmed to be correct, and then were transferred into three different methylotrophic bacteria, *Methylophilus methylotrophus* AS1, *Methylobacterium flagellatum* KT, and *Methylobacterium extorquens* AM1. Each strain was grown under various conditions in attempts to optimize expression, and cells were tested for production of either calmodulin or troponin C. Production of these proteins was assessed by screening the cells for appearance of new polypeptides of the correct size on SDS-PAGE gels. No evidence was obtained for expressin of troponin C, but it appeared that calmodulin was expressed in two strains, *Methylophilus methylotrophus* AS1 and *Methylobacterium flagellatum* KT

DISCLAIMER

containing the *tac* expression vector (plasmid pGak35). The level appeared to be about 3-fold higher in the latter strain, and was estimated at 0.5 - 1% of the total protein.

In order to further pursue this, antibody blots using antibody to calmodulin were used for screening. This work confirmed production of calmodulin by *Methylophilus methylotrophus* W3A1. Attempts were made to increase the level of expression, by growing cells on different substrates and harvesting at different stages of growth. However, no greater expression was achieved in any of these experiments.

A computer analysis of the sequences of vector and insert was undertaken to analyze expression sequences, and it was discovered that the troponin C ribosome binding site was a very weak one for *Methylophilus methylotrophus* W3A1. Therefore, it seemed likely that changing the ribosome binding site sequence to one more optimal for *Methylophilus methylotrophus* W3A1 would enhance expression. A strategy was worked out for making this change.

PUBLICATIONS: none

DELIVERABLES

1. The successful expression system for calmodulin has been provided to the Unkefer lab at Los Alamos National Laboratories, as well as documentation of the expression (antibody blots).
2. All instructions for maintaining the expression vector, subcloning into this vector, and mobilizing this vector into methylotrophic bacteria have been provided to the Unkefer lab at Los Alamos National Laboratories. In addition, the information on how to make changes to this vector to enhance expression has been provided.