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Genetic Analysis of Acetone Carboxylase in Azotobacter vinelandii

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GENETIC ANALYSIS OF ACETONE CARBOXYLASE IN *AZOTOBACTER VINELANDII*

by

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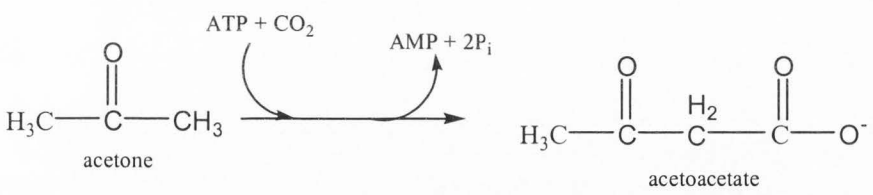
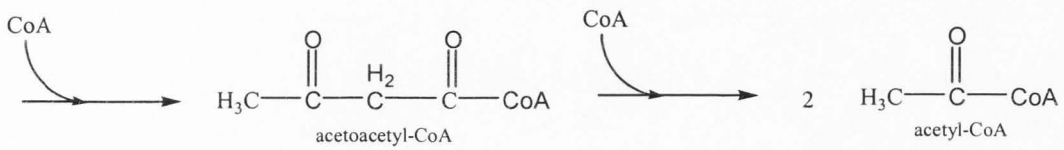
Acetone carboxylase, an inducible multimeric enzyme found in several bacteria, catalyzes the carboxylation of acetone to produce acetoacetate. This is an important reaction for the bacteria because, upon addition of coenzyme A, the acetoacetate can be converted to acetyl-CoA, a common metabolic intermediate. *Azotobacter vinelandii*, one of the bacteria that produces acetone carboxylase, has two different genes encoding this enzyme. We knocked out the first of these by disrupting it with a kanamycin cassette to help us understand the function of the second gene. Knocking out the gene will also make possible the use of site-directed mutagenesis to further study the enzyme.

Introduction

Acetone is a toxic molecule that is synthesized both industrially for use as a solvent, and biologically during fermentation in bacteria or under conditions of starvation or diabetes in mammals. Several bacteria, including *Azotobacter vinelandii*, are able to utilize acetone as a source of carbon and energy. Acetone can be broken down in these bacteria to produce acetyl-CoA, which is a common intermediate in several metabolic pathways. This is shown in the following reactions.





Acetone carboxylase, which catalyzes the carboxylation of acetone to form acetoacetate in the first step of the reaction above, is the multimeric ($\alpha_2\beta_2\gamma_2$) bacterial enzyme that is the focus of this paper. The genome of *A. vinelandii* contains two *acx*A genes that encode the β-subunit of acetone carboxylase. It has previously been shown that the two genes have different DNA sequences, but, because of the redundancy of the genetic code, the two proteins resulting from these genes are very similar. Because the cells need only one acetone carboxylase enzyme, we were interested in learning about the function of the second *acx* gene. To do this we decided to knock out the first gene by disrupting it with a kanamycin cassette. After we have been able to successfully knock out the gene, we will be able to follow similar procedures to perform site-directed mutagenesis. This will give us more information about the enzyme, specifically the role of its metal-binding site. The results from this study will also hopefully give us more insight into the acetone carboxylase genes and enzymes in other bacteria.

Results

PCR amplification of acx*A* – Using the available gene sequence for *A. vinelandii*, we designed primers complimentary to each end of the *acx*A gene and used the polymerase chain reaction (PCR) to amplify the *acx*A gene. We then used gel electrophoresis to run the PCR product through an agarose gel. We observed a single major band of DNA in our gel. We determined that the DNA in this band was the right size to be *acx*A by comparing the band position to a DNA ladder. These results suggested that we had amplified just the *acx*A gene like we wanted to.

Cloning of acx*A in a pGMT-easy plasmid* – We then cloned our PCR product by inserting it into a pGMT-easy plasmid. This plasmid is available already open and ready for insertion of a

DNA sample. There is a thymine at each open end of the plasmid that facilitates insertion of the DNA from the PCR reaction since PCR always places an adenine, which can base pair with thymine, at each end of the DNA segments amplified. After the *acx*A gene was ligated into pGMT-easy, we plated cells containing this plasmid on LB media containing ampicillin. The pGMT-easy plasmid has a gene for ampicillin resistance so cells with this plasmid are able to grow in this media. After incubating the plates to allow growth, we observed blue and white colonies. The blue colonies resulted when the plasmid was just ligated to itself instead of accepting the DNA segment. The opening in pGMT-easy disrupts a *lac*Z gene that codes for the enzyme β-galactosidase. When the plasmid is ligated to itself the gene is restored and the gene product, β-galactosidase, catalyzes the hydrolysis of the modified sugar X-gal that is in the media. This results in the formation of an insoluble blue substance that gives the bacterial colonies their blue color. Alternatively, the plasmid can be ligated to our sample DNA segment. This disrupts the *lac*Z gene so no β-galactosidase is produced and the colonies remain white. We selected the white colonies because the cells in them contained the *acx*A gene. Cloning the gene in this plasmid resulted in the purification and amplification of the gene of interest.

Insertion of a kanamycin cassette – After a thorough search of the DNA sequences of *acx*A and the pGMT-easy plasmid, we found an ApaI restriction site within the *acx*A gene that was not repeated anywhere else in the gene or plasmid. We cut the gene here with the appropriate restriction enzyme and inserted a kanamycin resistance cassette. This cassette disrupts the *acx*A gene, effectively knocking it out. It also allows us to select the cells that contain a successfully knocked out gene because the plasmid and the gene without the cassette lack resistance to kanamycin. Only cells whose DNA has incorporated the kanamycin cassette grew when plated on media containing kanamycin.

Production of knockout A. vinelandii *cells* – Now that the *acx*A gene was knocked out, we needed to put the DNA back in *A. vinelandii* cells. We first excised the DNA and kanamycin cassette from the pGMT-easy plasmid. The PCR primers we designed had terminal regions complementary to the SacI restriction site. As a result, our PCR products had SacI restriction sites flanking the *acx*A gene. The pGMT-easy plasmid and the *acx*A gene do not have any other SacI restriction sites so digestion with SacI restriction enzymes separated the gene and kanamycin cassette from the plasmid. We then ligated the *acx*A with kanamycin cassette into a PMW91 suicide plasmid. This can be taken up by *A. vinelandii* competent cells. The suicide plasmid lacks an origin of replication so it cannot replicate on its own, but the *acx*A gene within the plasmid can associate with the homologous region of DNA in the *A. vinelandii* genome. This region is the first *acx*A gene. Recombination can occur to replace the original *acx*A gene with the disrupted gene. Because the two *acx*A genes of *A. vinelandii* have different DNA sequences and the disrupted gene is only homologous to the first gene, the disrupted gene will only replace this first gene. This leaves the second gene intact for further studies.

Conclusions

We knocked out one of the two genes that encode the enzyme acetone carboxylase in *Azotobacter vinelandii* by disrupting tit with a kanamycin resistance cassette. Reintroduction of this disrupted gene into *A. vinelandii* competent cells can result in recombination to replace the original, intact *acx*A gene with the disrupted gene. Not only will this allow us to learn about the purpose of having a second *acx*A gene, but it also opens up several other related areas of study. For example, acetone carboxylase contains a metal-binding site, but the purpose of binding a metal is not understood. It will be possible to perform site-directed mutagenesis by changing a

single amino acid in the *acx*A gene instead of disrupting it with the kanamycin cassette before reintroducing it into the *A. vinelandii* cells. Causing several different mutations can lead to important information about which amino acid residues are important for metal binding and why metal binding is necessary.

Our results will also let us gain information about enzymes in other bacteria. Several other bacteria have similar genes encoding acetone carboxylase. *Helicobacter pylori* is an interesting example because it has the *acx* gene but doesn't express it. At this time we do not understand this observation. It is possible that the *acx* gene in *H. pylori* is defective in some way that makes it unable to be expressed. Or maybe the cells simply lack the protein machinery necessary to express this gene. To investigate this, we could follow procedures similar to those described here to amplify the *acx* gene from *H. pylori* and insert it into the *A. vinelandii* genome in place of the first *acx* gene. *A. vinelandii* has all the machinery necessary to express the *acx* gene, so we could determine if the *H. pylori* gene can be expressed when provided with the machinery or if the gene is defective and still not expressed. Further work to more fully understand acetone carboxylase, both in *A. vinelandii* and in other bacteria, is currently underway.

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