Video Article

Functional Complementation Analysis (FCA): A Laboratory Exercise Designed and Implemented to Supplement the Teaching of Biochemical Pathways

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Abstract

Functional complementation assay (FCA) is an *in vivo* assay that is widely used to elucidate the function/role of genes/enzymes. This technique is very common in biochemistry, genetics and many other disciplines. A comprehensive overview of the technique to supplement the teaching of biochemical pathways pertaining to amino acids, peptidoglycan and the bacterial stringent response is reported in this manuscript. Two cDNAs from the model plant organism *Arabidopsis thaliana* that are involved in the metabolism of lysine (L,L-diaminopimelate aminotransferase (dapL) and tyrosine aminotransferase (tyrB) involved in the metabolism of tyrosine and phenylalanine are highlighted. In addition, the bacterial peptidoglycan anabolic pathway is highlighted through the analysis of the UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-*meso*-2,6-diaminopimelate ligase (murE) gene from the bacterium *Verrucomicrobium spinosum* involved in the cross-linking of peptidoglycan. The bacterial stringent response is also reported through the analysis of the rsh (**r**elA/**s**poT **h**omolog) bifunctional gene responsible for a hyper-mucoid phenotype in the bacterium *Novosphingobium sp.* Four examples of FCA are presented. The video will focus on three of them, namely lysine, peptidoglycan and the stringent response.

Video Link

The video component of this article can be found at https://www.jove.com/video/53850/

Introduction

Functional complementation in the context of elucidating the function(s)/role(s) of a gene is defined as the ability of a particular homologous or orthologous gene to restore a particular mutant with an observable phenotype to the wild-type state when the homologous or orthologous gene is introduced in *cis* or *trans* into the mutant background. This technique has been widely used to isolate and identify the function(s)/role(s) of many genes. One particular example is the isolation and identification of orotidine-5-phosphate decarboxylase from *Candida albicans* using the *ura3* mutant of *S. cerevisiae* and the *pyrF* mutant of *E. coli*.[1] The authors have used this technique to elucidate the function of genes that are involved in the metabolism of amino acids, peptidoglycan and the stringent response in their research programs and have incorporated this technique into their teaching programs in the Biotechnology and Molecular Bioscience (BMB) program at the Rochester Institute of Technology (RIT).

The authors teach Fundamentals of Plant Biochemistry/Pathology (FPBP) (Hudson) and Bioseparations: Principles and Practices (BPP) (Hudson/Savka), two upper division elective laboratory based courses in the BMB Program at the RIT. Since some of the topics that are discussed in the courses are affiliated with their research interests, the authors have incorporated many of the techniques and experimental tools that are used in their respective research programs into these two laboratory-based courses. One such example is functional complementation as a laboratory exercise to reinforce the lecture materials pertaining to amino acid metabolism from plants, peptidoglycan and the stringent response metabolism from bacteria.

Three of the amino acid pathways from plants that are discussed in the FPBB course are that of lysine (lys), tyrosine (tyr) and phenylalanine (phe). The lys pathway is highlighted in the course because of the importance of the amino acid as an essential amino acid for all animals particularly humans since animals lack the genetic machinery to synthesize lys *de novo*. In addition, it was recently discovered that plants employ a pathway for the synthesis of lys that is significantly different from that of bacteria. This discovery was partially facilitated by functional complementation of the *E. coli* diaminopimelate (*dap*) mutants using a gene that encodes the enzyme L,L-diaminopimelate aminotransferase (DapL) from the model plant *Arabidopsis thaliana*.[2] The variant pathways for the synthesis of lys through the intermediate diaminopimelate are shown in **Figure 1**. In addition, the synthesis of lys facilitates through the aspartate derived family of amino acids which is highly regulated.[3] In addition to their importance in protein synthesis, the pathways for tyr and phe are highlighted given their importance in serving as precursor compounds for the anabolism of phenylpropanoid compounds involved the synthesis of plant defense compounds such as: alkaloids, lignins, flavonoids, isoflavonoids, hydroxycinnamic acid among others.[4] The tyr and phe pathways are also highlighted to show the difference between the plant and bacterial anabolic pathways. In bacteria, the enzyme tyrosine aminotransferase (TyrB) is involved in the anabolism of both amino

acids, whereas in plants, the enzyme is primarily involved in the catabolism of tyr and phe and is not involved in the anabolism of these amino acids. (**Figure 2**).[4]

The differences between Gram positive and Gram negative bacteria regarding the structure of peptidoglycan (PG) are highlighted in the FPBP course. The PG of Gram negative bacteria is of interest regarding plant pathology based on to the fact that most plant pathogens are Gram negative. A recent review regarding the top 10 bacterial phyto-pathogens revealed that all are Gram negative. The bacteria were from the genera: *Pseudomonas*, *Ralstonia*, *Agrobacterium*, *Xanthomonas*, *Erwinia*, *Xylella*, *Dickeya* and *Pectobacterium*.[5] One of the chemical differences when comparing the PG stem of Gram negative and Gram positive bacteria is the difference between the cross-linking amino acids of both types. The initial step for that different cross-linking of PG occurs in the cytoplasmic step of PG anabolism and is facilitated by the enzyme UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-*meso*-2,6-diaminopimelate ligase (MurE) (**Figure 3A**). MurE catalyzes the addition of a particular diamine compound at third position of the peptide stem.[6] In most Gram negative bacteria, the penultimate lys precursor, *meso*-diaminopimelate (*m*-DAP) serves as the cross-linking amino acid and lys serves the same role in the PG of most Gram positive bacteria (**Figure 3B**).[7] This is due to the fact that both *m*-DAP and lys possess two amine groups and are capable of forming two peptide bonds for peptide stem cross-linking.

In the Bioseparations: Principles and Practices (BPP) course, the differences between open and closed systems for the cultivation of bacteria and how nutrient levels will change significantly in both systems due to environmental changes are discussed. These events are linked to regulatory changes called a "shift down" or "shift up" triggered by starvation or an adequate supply of amino acids or energy. The "shift down" response can occur when a bacterial culture is transferred from a rich and complex medium to a chemically defined medium with a single carbon source. This change in environment leads to the rapid cessation of tRNA and rRNA synthesis. This cessation results in the lack of ribosomes, protein and DNA synthesis even though the biosynthesis of amino acids are upregulated.

Following the "shift down" response, the existing ribosomes are used to produce new enzymes to synthesize the amino acids no longer available in the medium or environment. After a period of time, rRNA synthesis and new ribosomes are assembled and the population of bacterial cells begins to grow although at a reduced rate. The course of events is termed the "*stringent response"* or "*stringent control"* and is an example of global cellular regulation and can be thought of as a mechanism for adjusting the cell's biosynthetic machinery to compensate for the availability of the required substrates and energy needs.[8] The stringent response thus enables bacteria to respond rapidly to fluxes of nutrients in the environment and contributes and enhances the ability of bacteria to compete in environments that can change rapidly with regards to nutrient and or substrate availability.[8-9]

The stringent response has an integral role in gene expression when the availability of amino acids, carbon, nitrogen, phosphate, and fatty acids are limited.[8,10-14] This stringent response is coordinated by two nucleotides, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) commonly referred together as the *alarmone* (p)ppGpp. For example, when amino acids are limited-which can lead to a bottleneck in protein synthesis-the alarmone, guanosine 3,5-(bis) pyrophosphate (ppGpp), derived from anabolism of guanosine 3-diphosphate 5-triphosphate (pppGpp) accumulates in the cell. The change in (p)ppGpp level is involved in expression of genes that regulate the response to overcome the lack of substrates in the environment that are directly involved in cell growth and development. Two of the genes that are involved in this process are called relA and spoT. RelA is a ribosome-associated (p)ppGpp synthetase that is involved in the response to the accumulation of uncharged tRNAs that is the result of amino acid limitation. SpoT functions as a bifunctional (p)ppGpp synthetase and hydrolase. The synthetase activity of SpoT is involved in the response to the lack of carbon and fatty acid starvation.[8] The RelA/SpoT homologs are widespread in plants and bacteria and are referred to as Rsh for **R**elA/**S**poT **h**omologs.[8,10-12,16] A recent manuscript showed that there is a specific Rsh protein involved in the synthesis of these alarmones from the bacterium *Novosphingobium* sp Rr 2-17.[17]

Here we present four biochemical pathways tethered to the functional complementation assays. The complementation assays outlined in this manuscript provides an avenue to explore employing this *in vivo* assay as a means of identifying and or characterizing enzymes that are predicted to have unknown/putative function(s) or as teaching tools to supplement the teaching of biochemical pathways.

Protocol

NOTE: The authors are willing to provide bacterial strains and recombinant plasmids to facilitate the incorporation of functional complementation analysis for teaching purposes for individuals who are interested. The plasmids that were used to facilitate functional complementation experiments are listed in **Table 1**.

1. Construction of Plasmids for Functional Complementation

1. Cloning of Diaminopimelate Aminotransferase (dapL) for Functional Complementation.
    1. Amplify the dapL open reading frame (ORF) from the *V. spinosum* and cDNAs from *A. thaliana* and *C. reinhardtii* by PCR. Use 1 cycle at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 2 min. Include 12 pmoles of each primer, 1 mM $MgSO_4$, 0.5 mM each of the 4 deoxynucleotide triphosphates, and 0.5 ng of template DNA and 1 unit of *Pfx* DNA polymerase.
    NOTE: The complete description pertaining to the recombinant cloning of three dapL orthologs from the plant *A. thaliana* (*At*-dapL), the bacterium *Verrucomicrobium spinosum* (*Vs*-dapL) and the alga *Chlamydomonas reinhardtii (Cr*-dapL) has been previously published.[2,18-20]
    NOTE: The primers used for the cloning of the dapL orthologs are as follows:
    *Vs*dapL F- 5'-CCCCGAATTCATGGCCCTCATCAACGAAAACTTCCTCAAG-3'
    *Vs*dapL R 5'- CCCCGTCGACCTACTTCAGCGCGGCGATACGGCGGCAGAC-3'
    *At*dapL F- 5'-GGGGCATTGGAAGGAGATATAACCATGGCAGTCAATACTTGCAAATGT-3'
    *At*dapL R-5'- GGGGGTCGACTCATTTGTAAAGCTGCTTGAATCTTCG-3'
    *Cr*dapL F-5'-*CCCCCGAATTCATGCAGCTCAACGTGCGGTCCACCGCCAGC-3'*
    *Cr*dapL R-*5'*- CCCCCAAGCTTCTAGTTACGCTTGCCGTAGGCCTCCTTAAA-3'
    2. Clone the PCR fragments into the plasmid pET30A to produce the recombinant plasmids, pET30A::*Vs*dapL, pET30A::*At*dapL and pET30A::*Cr*dapL using the primers, restriction enzymes and T4 DNA ligase.

1. Briefly, incubate 50 ng of insert and 20 ng of vector in 1x ligase buffer and 1 unit of T4 DNA ligase overnight at 17 °C. Transform ligation into *E. coli* Dh5α cells and screen for colonies on LB plates supplemented with 50 μg/ml^{-1} kanamycin by incubating at 37 °C for 24 hr.[18-20]

3. To create the plasmid for functional complementation, digest pET30A::*Vs*dapL and pET30A::*At*dapL plasmids with the restriction enzymes XbaI and SalI and XbaI and HindIII for the pET30A::*Cr*dapL. Ligate the inserts into the plasmid pBAD33 using T4 DNA ligase to produce the plasmids pBAD33::*Vs*dapL; pBAD33::*At*dapL and pBAD33::*Cr*dapL.
    1. Incubate 50 ng of insert and 20 ng of vector in 1x ligase buffer and 1 unit of T4 DNA ligase overnight at 17 °C. Transform ligation into *E. coli* Dh5α cells and screen for colonies on LB plates supplemented with 34 μg/ml^{-1} kanamycin by incubating 37 °C for 24 hr.[20]

2. Cloning of Tyrosine Aminotransferase (tyrB) from *A. thaliana* for Functional Complementation.
NOTE: The complete details regarding the cloning of the cDNA from *A. thaliana* annotated by the locus tags At5g36160 has been described previously.[4]
    1. Amplify the cDNA by PCR. Use 1 cycle at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 2 min. Include 12 pmoles of each primer, 1 mM $MgSO_4$, 0.5 mM each of the four deoxynucleotide triphosphates, and 0.5 ng of template DNA and 1 unit of *Pfx* DNA polymerase.
    NOTE: The primers used for the cloning of the At5g36160 are as follows:
    *At*tyrB F- 5'-CCCCGAATTCATGGGAGAAAACGGAGCCAAGCGAT-3'
    AttyrB R- 5'- CCCCGAATTCATGGGAGAAAACGGAGCCAAGCGAT-3'
    2. Clone the fragment into the plasmid pET30A to produce the recombinant plasmids pET30A::At5g36160 by digesting the PCR fragment with EcoR1 and Sal1; ligate in the fragment into pET30A using T4 DNA ligase as described below.[4]
    3. To create the functional complementation plasmid, digest the pET30A::At5g36160 with the restriction enzymes XbaI and HindIII, and ligate the insert into pBAD33 using the same restriction enzyme sites to produce the recombinant plasmid pBAD33::At5g3160 using T4 DNA ligase.
        1. Incubate 50 ng of insert and 20 ng of vector in 1x ligase buffer and 1 unit of T4 DNA ligase overnight at 17 °C. Transform the ligation into *E. coli* Dh5α cells and screen for colonies on LB plates supplemented with 34 μg/ml^{-1} chloramphenicol by incubating 37 °C for 24 hr.[4]

3. Cloning of UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-*meso*-2,6-diaminopimelate Ligase (murE) from *V. spinosum* for Functional Complementation.
NOTE: The cloning of the murE ORF from *V. spinosum* has been described previously.[18]
    1. Amplify the open reading frame by PCR. Use 1 cycle at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 2 min. Include 12 pmoles of each primer, 1 mM $MgSO_4$, 0.5 mM each of the four deoxynucleotide triphosphates, 0.5 ng of template DNA and 1 unit of *Pfx* DNA polymerase. Then clone into the plasmid pET100D to produce the plasmid pET100D::*Vs*murE.
    NOTE: The primers used for the cloning of the *Vs*murE are as follows:
    *Vs*MurE F- 5'- CACCATGACCATTTTGCGCGATCTTATCGAGGGT -3'
    VsMurE R- 5'- GTCGACTCACTGACGGTCATCCCTCCTTTGGCGTGC-3'
    2. To produce the plasmid for functional complementation, digest the pET100D::*Vs*murE
    With the restriction enzymes XbaI and Sal1 and ligate the insert into pBAD33 to produce the recombinant plasmid pBAD33::*Vs*murE using T4 DNA ligase.
        1. Incubate 50 ng of insert and 20 ng of vector in 1x ligase buffer, along with 1 unit of T4 DNA ligase, overnight at 17 °C. Transform ligation into *E. coli* Dh5α cells and screen for colonies on LB plates supplemented with 34 μg/ml^{-1} chloramphenicol by incubating 37 °C for 24 hr.[8]

4. Cloning of relA/spoT (rsh) from *Novosphingobium sp* for Functional Complementation.
NOTE: The cloning of the rsh from *Novosphingobium sp.* has been described previously.[17]
    1. Amplify the rsh ORF in addition to 599 nucleotides upstream of the initiation start site and 46 nucleotides downstream at the termination site by PCR. Use 1 cycle at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 2 min. Include 12 pmoles of each primer, 1 mM $MgSO_4$, 0.5 mM each of the four deoxynucleotide triphosphates, 0.5 ng of template DNA and 1 unit of Taq DNA polymerase. Then clone into the plasmid pCR2.1 to produce pCR2.1::*Nsp*rsh.
        1. Incubate 1 μl of amplified PCR fragment, 1 μl of pCR2.1 vector, 1 μl of salt solution and 1 μl of water. Incubate at 25 °C for 5 min and 2 μl of ligation into *E. coli* cells, and screen for colonies on LB plates supplemented with 50 μg/ml^{-1} kanamycin by incubating 37 °C for 24 hr.
        NOTE: The primers used for the cloning of the rsh are as follows:
        rsh F- 5'- GTTGAAAAACGCCGAATAGC -3'
        rsh R- 5'- GAGACCTGTGCGTAGGTGGT -3'
        2. For functional complementation, digest the plasmid pCR2.1::*Nsp*rsh with EcoR1 and ligate the insert into the broad host range pRK290 to produce the recombinant plasmid pRK290::*Nsp*rsh using T4 DNA ligase.
            1. Incubate 50 ng of insert and 20 ng of vector in 1x ligase buffer and 1 unit of T4 DNA ligase overnight at 17 °C. Transform ligation into *E. coli* Dh5α cells and screen for colonies on LB plates supplemented with 10 μg/ml^{-1} tetracycline by incubating 37 °C for 24 hr.[17]

2. Preparation of Electro-competent Bacterial Cells to Facilitate Transformation

NOTE: The preparation of electro-competent cells is based on the protocol 26 for 1.0 L of culture that can be scaled down to a smaller volume (*i.e.,* 250 ml). Please note that this protocol can be used to make all the strains competent that are described in this manuscript to facilitate FCA.[22]

1. Inoculate 50 ml of liquid medium with a single colony into a flask and grow over night, making sure to check the genotype of the mutant before commencing this step (**Table 2**).
2. On day two, inoculate 1.0 L of the appropriate medium (LB for *E. coli* mutants and PD for *Novosphingobium sp*) with the 50 ml of the overnight culture, and grow to an OD_{600} to 0.4-0.6 (log phase) at 30 °C.
3. Harvest cells by centrifuging at 5,000 x g for 15 min at 4 °C, decant the supernatant, and resuspend the pellet in 500 ml of sterile ice-cold pure H_2O.
4. Centrifuge the cells at 5,000 x g for 20 min at 4 °C, decant the supernatant and resuspend the pellet in 250 ml of sterile ice-cold 10% glycerol.
5. Centrifuge the cells at 5,000 x g for 20 min at 4 °C, decant the supernatant, and resuspend the pellet in 2.0 ml of 10% glycerol.
6. Aliquot the cells by transferring 50 µl into micro-centrifuge tubes and immediately freeze by placing the tubes in a dry-ice-ethanol bath. Store the competent cells at -80 °C for electroporation.

3. Electroporation of Bacterial Cells with Complementation Plasmids

1. For electroporation, add 1.0 µl (10-50 ng) of recombinant plasmid to an aliquot (50 µl) of competent cells and place on ice for 5 min.
2. Transfer the mixture via pipetting to an electroporation cuvette and set the electroporation apparatus to the following setting: 25 µF capacitance, 2.5 kV, and 200 ohm resistance and deliver a pulse.
3. Add 1.0 ml of recovery media (LB) to the electroporation cuvette and transfer using a pipette to a 15 ml conical tube. Recover with gentle rotation for 60 min in a shaking incubator.
4. Plate 100 µl of the culture from the recovery step onto the agar plates to select for transformants by pipetting and spreading using a sterile spreader.
   NOTE: Make sure to check **Table 1** and **Table 2** before commencing this step to check the antibiotics and genotypes to make the proper agar plates.

4. Transformation of *AOH1* to Facilitate Functional Complementation Using L,L-diaminopimelate Aminotransferase (dapL)

1. For complementation analysis, transform *AOH1* with the empty vector (pBAD33), and with the DapL expression vectors in separate transformation events using the electroporation protocol outlined in section 3.0.
2. Select transformants by plating on LB agar medium supplemented with 50 $\mu g/ml^{-1}$ DAP and 34 $\mu g/ml^{-1}$ chloramphenicol and 50 $\mu g/ml^{-1}$ kanamycin.
3. Test for functional complementation by replica-plating colonies by streaking onto LB medium with 0.2% (w/v) arabinose with and without 50 $\mu g/ml^{-1}$ DAP and 34 $\mu g/ml^{-1}$ kanamycin.
4. Incubate plates at 30 °C for 24 hr and observe results.
   NOTE: The result should show that the mutant is only able to grow on DAP free media only when the dapL gene is expressed in the mutant background when compared to the vector only control.

5. Transformation of TKL-11 to Facilitate Functional Complementation Using UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2,6-*meso*-diaminopimelate Ligase (murE)

1. Transform the mutant with the empty vector (pBAD33) and with the MurE expressing vector (pBAD33::*VsmurE*) using the protocol outlined in section 3.0.
2. Plate and select transformants on LB agar medium supplemented with 50 $\mu g/ml^{-1}$ thymine and 34 $\mu g/ml^{-1}$ chloramphenicol and incubate the plates at 30 °C for 24 hr.
3. Test for complementation by streaking or plating colonies from both the control and the experimental transformations onto two LB medium plus 0.2% (w/v) arabinose and 50 $\mu g/ml^{-1}$ thymine.
4. Incubate one plate at 30 °C and the other at 42 °C for 24 hr to visually assess the growth phenotype.
   NOTE: The results should show that the mutant is able to grow at 42 °C only when the murE gene is expressed in the mutant background compared to the vector only control.

6. Transformation of DL39 to Facilitate Functional Complementation Using Tyrosine Aminotransferase (tyrB)

1. Transform *DL39* with either pBAD33 or pBAD33::At5g36160, and select transformants on LB agar plates supplemented with 50 $\mu g/ml^{-1}$ tyrosine, 50 $\mu g/ml^{-1}$ phenylalanine, and 34 $\mu g/ml^{-1}$ chloramphenicol using the protocol outlined in section 3.0.
2. Replica-plate colonies on minimal (M9) media with 50 $\mu g/ml^{-1}$ phenylalanine and 50 $\mu g/ml^{-1}$ tyrosine, 50 $\mu g/ml^{-1}$ aspartate, 50 $\mu g/ml^{-1}$ leucine, 50 $\mu g/ml^{-1}$valine, 50 $\mu g/ml^{-1}$ isoleucine, 10 $\mu g/ml^{-1}$ uracil 0.5% (w/v) glycerol, 0.2% (w/v) arabinose. Also replica-plate colonies on plates lacking phenylalanine and tyrosine by streaking the same colony of both plates.
3. Incubate plates at 30 °C for 48 hr to observe growth phenotype.

NOTE: The result should show that the mutant is only able to grow on phenylalanine and tyrosine free media, only when the tyrB gene is expressed in the mutant background when compared to the vector only control.

7. Transformation of *Hx699* to Facilitate Functional Complementation of the Hypomucoid Phenotype of *Novosphingobium sp*. Strain *Hx699*

1. Transform wild-type strain *Novosphingobium sp.* (Rr2-17) and the mutant *Hx699* with pRK290 and pRK290::rsh_{Nsp} in 2 separate transformation events using the transformation protocol outlined in section 3.0.
2. Plate the transformations on potato dextrose (PD) agar supplemented with 10 µg/ml^{-1} tetracycline, and incubate for at least 24 hr or until transformants appear.
3. Streak both transformations in an "X" pattern on fresh PD agar plates and incubate at 30 °C for at least 4 days. Observe the phenotypes of the vector only, and experiment by visually examining the growth phenotype of both plates.
   NOTE: The result should show that the hypo-mucoid phenotype is complemented when rsh is expressed in the mutant background when compared to the vector only control.

Representative Results

The bacterial strains that are employed in the various functional complementation analyses are listed in **Table 2**.

Functional complementation analysis: L,L-diaminopimelate aminotransferase (dapL)

The *E. coli* double mutant *AOH1* (Δ*dapD::Kan2, dapE6*) harbors a mutation in the dapE gene and a complete deletion of the dapD gene (**Figure 1**). As such, the mutant is unable to grow unless DAP is provided. Due to these mutations, *AOH1* is deemed auxotrophic. *AOH1* is suitable for functional complementation analysis of L,L-diaminopimelate aminotransferase (DapL) orthologs as DapL catalyzes the synthesis of L,L-diaminopimelate directly from THDP to facilitate PG and lys synthesis (**Figure 1**).

The results from the experiment should show that only *AOH1* mutant expressing DapLs are able to grow on L,L-DAP-free media demonstrating that the recombinant enzymes are able to convert THDP to L,L-DAP circumventing the DapD and DapE enzymatic steps in the DAP/lys pathway (**Figure 5**).

Functional complementation analysis: UDP˙*N*-acetylmuramoyl-L-alanyl-D-glutamate-*meso*-2,6-diaminopimelate ligase (murE)

meso-diaminopimelate (*m*-DAP) is the cross-linking amino acid in the PG of Gram-negative bacteria. The enzyme UDP-*N*-acetylmuramoylalanyl-*D*-glutamyl-2,6-*meso*-diaminopimelate ligase (MurE) facilitates the addition of *m*-DAP in this process (**Figure 3A**-**B**). The *E. coli* mutant *TKL-11* harbors a mutation in the *murE* gene which results in the ability of the mutant to grow at 42 °C due to the fact that the mutated enzyme is not able to fold properly at this temperature.

The results will demonstrate that there is growth at the permissive temperature of 30 °C by both the control and the experiment. However, only the cells expressing MurE (experiment) will be able to grow at the non-permissive temperature of 42 °C (**Figure 6**).

Functional complementation analysis: Tyrosine aminotransferase (tyrB)

Arabidopsis thaliana encodes a tyrosine aminotransferase that is able to interconvert tyrosine and 4-hydroxyphenylpyruvate, and phenylalanine and phenylpyruvate annotated by the At5g36160 (**Figure 2**).[4] The *E. coli* mutant (*DL39*) harbors a mutation in the tyrB gene which makes auxotrophic for phenylalanine and tyrosine.[24-26] It should be noted that the mutant is auxotrophic for the amino acids tyrosine, phenylalanine, aspartate, leucine, isoleucine and valine, as well due to other mutations. The strain is suitable to assess the function of tyrB orthologs since the TyrB enzyme ortholog from *E. coli* is directly involved in the synthesis of tyrosine and phenylalanine to facilitate protein synthesis.

The result should show that while the *DL39* mutant is able to grow on M9 only when tyrosine and phenylalanine are provided. However, the strain expressing the *A. thaliana* (At5g36160) is able to grow on M9 media lacking tyrosine and phenylalanine demonstrating that the enzyme is able to synthesize tyrosine from 4-hydroxyphenylpyruvate, and phenylalanine from phenylpyruvate t (**Figure 7**).[4]

Functional complementation analysis: Rsh (RelA and *S*poT homolog)

The Rsh protein from *Novosphingobium sp*. (Rr2-17) contains both the N-terminal phosphohydrolase and the (p)ppGpp synthase domains, and thus has the proposed bifunctional role like SpoT of *E. coli.* This has been confirmed using complementation analysis of an *E. coli* double mutant, *CF1693* which harbors mutation in relA and spoT using the rsh gene from *Novosphingobium sp*. The phenotype of the Rr2-17 *rsh* mutant, named *Hx699* (*rsh::EZ-Tn5, Kan*^{R}), results in a hypo-mucoid and this is due to a non-functional rsh. The hypo-mucoid phenotype of *Hx699* was assessed by complementation facilitated by the native rsh_{Nsp} gene that was cloned into the broad-host range vector pRK290. Consistent with the hypo-mucoid phenotype, the *Hx699* harboring empty vector pRK290 show less soluble polysaccharide of that produced by the trans-complemented *Hx699* (pRK290::rsh_{Nsp}) and Rr2-17 containing pRK290 or pRK290::rsh_{Nsp} (**Figure 8**).

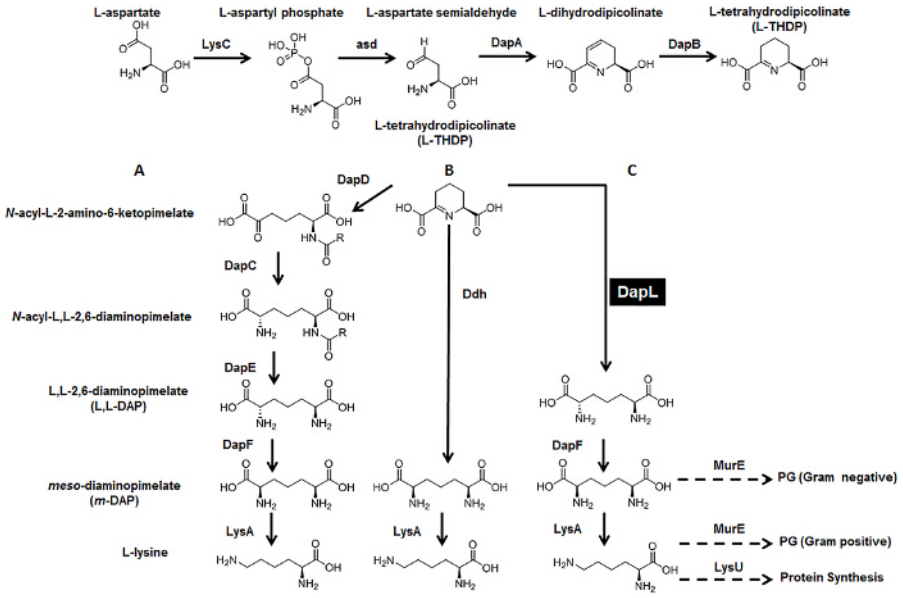

Figure 1: The variants of the lysine anabolic pathways from aspartate that proceed through the intermediate diaminopimelate (DAP). The pathways are annotated by (**A**) the acyl pathways, (**B**) the diaminopimelate dehydrogenase (Ddh) pathway and (**C**) the L,L-diaminopimelate aminotransferase (DapL) pathway. The abbreviation definitions for the enzymes are as follows: aspartate kinase (LysC), aspartate semialdehyde dehydrogenase (asd), tetrahydrodipicolinate synthase (DapA), tetrahydrodipicolinate reductase (DapB), tetrahydrodipicolinate acylase (DapD), *N*-acyl-2-amino-6-ketopimelate aminotransferase (DapC), *N*-acyl-L,L-2,6-diaminopimelate deacylase (DapE), diaminopimelate epimerase (DapF), *meso*-diaminopimelate dehydrogenase (Ddh), *meso*-diaminopimelate decarboxylase (LysA), L,L-diaminopimelate aminotransferase (DapL), UDP-*N*-acetylmuramoyl-L-alanyl-d-glutamate:*meso*-2,6-diaminopimelate ligase (MurE) and lysyl-tRNA synthetase (LysU). Please click here to view a larger version of this figure.

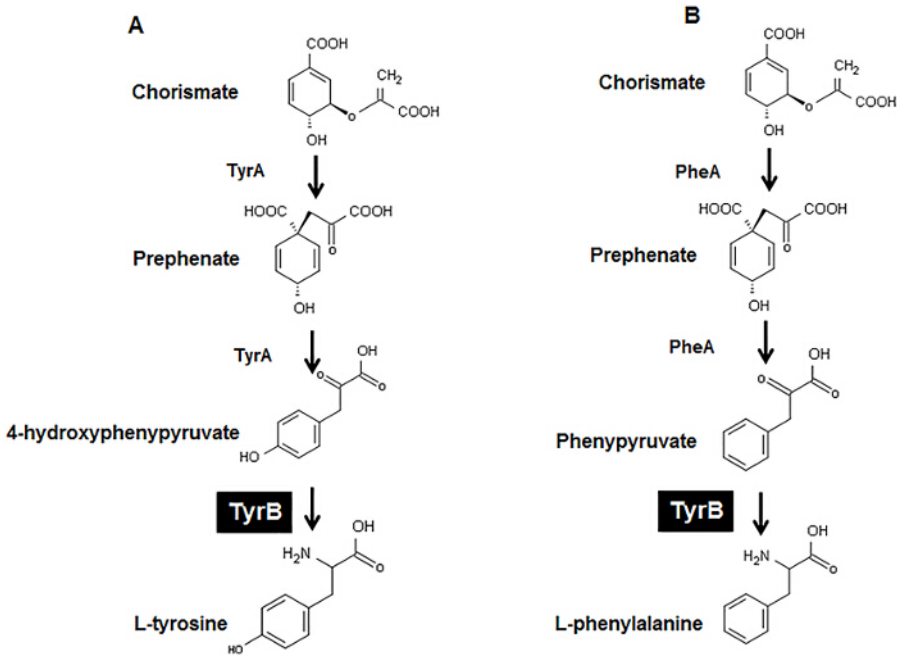

Figure 2: Anabolic pathways for the amino acids tyrosine and phenylalanine. (**A**) The bacterial (*E. coli*) pathway through the intermediate chorismate and (**B**) the plant pathway through the intermediate arogenate. The abbreviations for the enzymes are as follows: chorismate mutase/prephenate dehydratase (PheA), chorismate mutase, prephenate dehydrogenase (TyrA), and tyrosine aminotransferase (TyrB). The diagram was adopted and modified form Prabhu and Hudson, 2010.[4] Please click here to view a larger version of this figure.

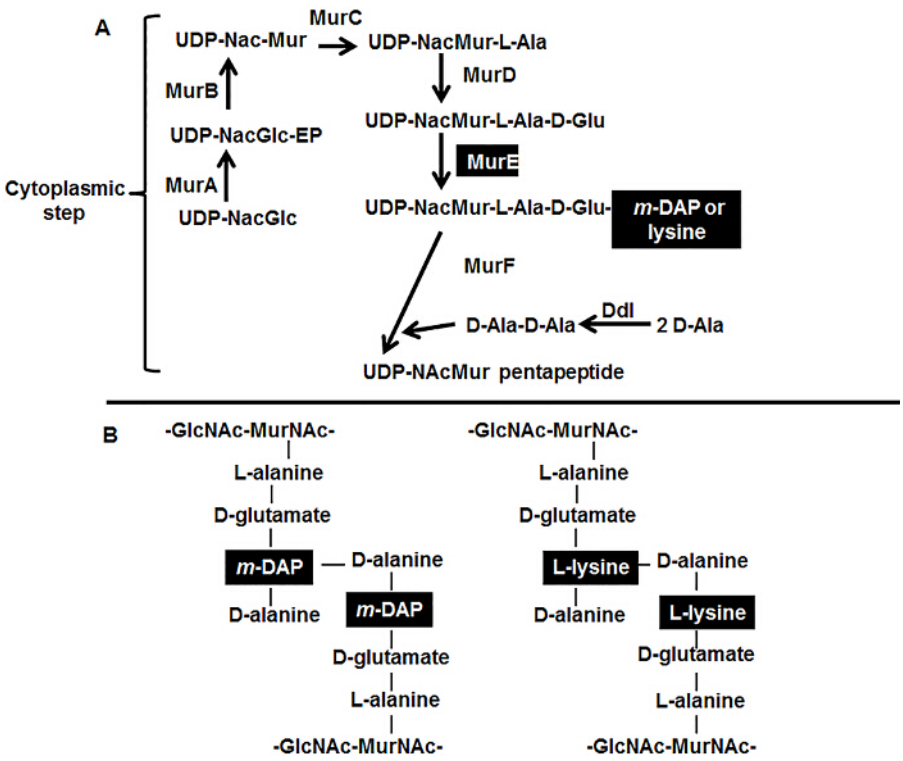

Figure 3: The cytoplasmic step of peptidoglycan synthesis. (**A**) Schematic representation of the cytoplasmic step of peptidoglycan synthesis. The abbreviations of the enzymes are as follows: UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), UDP-*N*-acetylenolpyruvoylglucosaminereductase (MurB), UDP-*N*-acetylmuramate-L-alanine ligase (MurC), UDP-*N*-acetyl-muramoylalanine-D-glutamate ligase (MurD), UDP-*N*-acetylmuramoyl-L-alanyl-d-glutamate 2,6-*meso*-diaminopimelate ligase (MurE), UDP-*N*-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase (MurF), D-alanine-D-alanine ligase (Ddl). (**B**) Schematic representation of a monomeric unit of peptidoglycan showing the disaccharide *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic (MurNAc) linked via a β-1,4 glycosidic bond. The amino acid at position 3 of the stem peptide is involved in cross-linking denote by *meso*-diaminopimelate (*m*-DAP) in most Gram-negative bacteria and lys in most Gram-positive bacteria. Please click here to view a larger version of this figure.

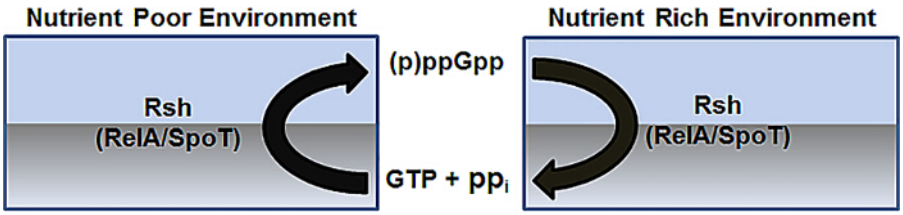

Figure 4: Schematic model representation of the stringent response in bacteria. Exposure to a nutrient poor environment induces Rsh (RelA or SpoT) to anabolize the alarmone (p)ppGpp (guanosine pentaphosphate) by adding a phosphate group to GTP (guanosine triphosphate). Exposure to a nutrient rich environment induces the expression of Rsh to hydrolyze (p)ppGpp which is a growth inhibitor. The diagram was adopted and modified from Raskin *et al.,* 2007.[27] Please click here to view a larger version of this figure.

Figure 5: Functional complementation using DapL. Functional complementation of the *AOH1E. coli* mutant with L,L-diaminopimelate aminotransferase (dapL) gene from the bacteria *V. spinosum* (*Vs*dapL), the plant *A. thaliana* (*At*dapL) and the alga, *C. reinhardtii* (*Cr*dapL). The mutant harboring the plasmids, pBAD33, pBAD33::*Vs*DapL, pBAD33::*At*dapL and pBAD33::*Cr*dapL were replica-plated on LB agar plates supplemented with 0.2% (w/v) arabinose with or without 50 µg/ml L,L-diaminopimelate and were grown at 30 °C for 24 hr. The diagram was adopted and modified from Nachar *et al.,* 2012.[18] Please click here to view a larger version of this figure.

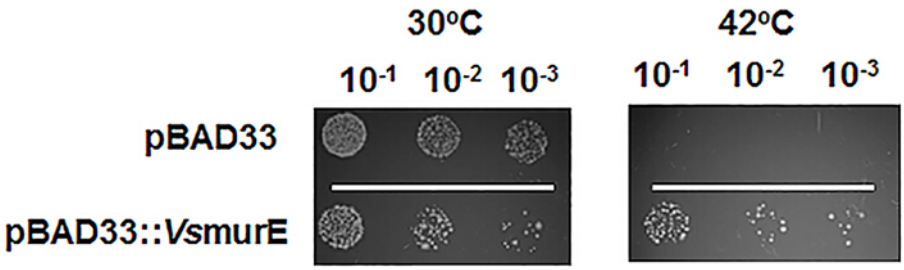

Figure 6: Functional complementation using MurE. Functional complementation of the *TKL-11 E. coli* mutant with the UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-*meso*-2,6-diaminopimelate ligase (murE) gene from *V. spinosum*. The mutant harboring the plasmids BAD33 or pBAD33::*VsmurE* were grown in LB medium to an OD_{600} of 0.1 and were serially diluted to 10^{-1}, 10^{-2}, and 10^{-3} using 0.85% (w/v) saline. 5 µl of various dilutions were replica-plated on LB medium supplemented with 0.2% (w/v) arabinose and were grown assessed at 30 °C and 42 °C for 24 hr. Please click here to view a larger version of this figure.

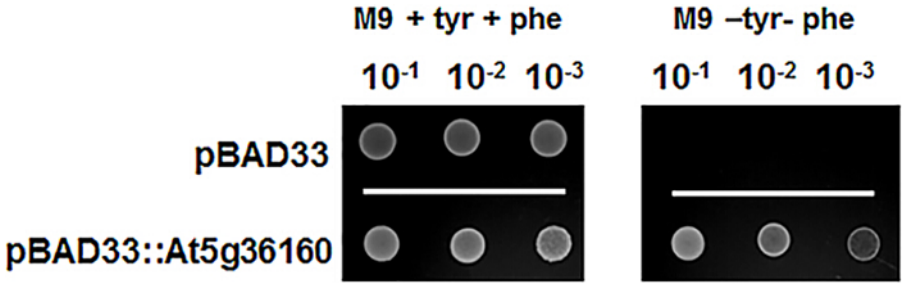

Figure 7: Functional complementation of the *DL39E. coli* with a tyrosine aminotransferase gene annotated by the locus tag At5g36160 from *A. thaliana*. The mutant harboring plasmids pBAD33 or pBAD33::At5g36160 were selected on LB agar plates supplemented with 50 µg/ml^{-1} tyrosine, 50 µg/ml^{-1} phenylalanine, and 34 µg/ml^{-1} chloramphenicol. The strain was grown in LB broth to an OD OD_{600} of 1.0 and were serially diluted to 10^{-1}, 10^{-2}, and 10^{-3} using 0.85% (w/v) saline. 5 µl of the various dilutions were then replica-plated onto M9 agar plates supplemented with 50 µg/ml^{-1} phenylalanine and 50 µg/ml^{-1} tyrosine, 0.5% (w/v) glycerol, 0.2% (w/v) arabinose, 50 µg/ml^{-1} aspartate, 50 µg/ml^{-1} leucine, 50 µg/ml^{-1} valine, 50 µg/ml^{-1} isoleucine, 10 µg/ml^{-1} uracil, and also on plates lacking phenylalanine and tyrosine and were incubated at 30 °C for 48 hr. The diagram was adopted and modified form Prabhu and Hudson, 2010.[4] Please click here to view a larger version of this figure.

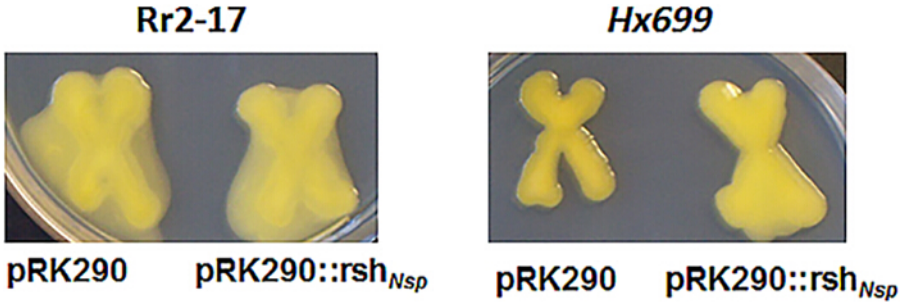

Figure 8: Functional complementation of the hypomucoid phenotype of the *Novosphingobium sp.* mutant strain *Hx699*. The wild-type strain *Novosphingobium sp.* (Rr2-17) and the mutant strain *Hx699* were transformed with pRK290 or pRK290::*rsh*$_{Nsp.}$ The strains were streaked in an "X" and were further grown on PD agar at 30 °C for at least 4 days to observe the phenotypes. Please click here to view a larger version of this figure.

Complementation plasmids	Antibiotic resistance markers	Citation
pBAD33	Cm^r	21
pBAD33::*At*dapL	Cm^r	2
pBAD33::*Cr*dapL	Cm^r	20
pBAD33::*Vs*dapL	Cm^r	18
pBAD33::At5g36160	Cm^r	4
pBAD33::*Vs*murE	Cm^r	18
pRK290	Tet^r	28
pRK290::rsh_{Nsp}	Tet^r	17

Table 1: Functional complementation plasmid used in this study. List of plasmids used for functional complementation analysis. Cm^R and Tet^R denote chloramphenicol and tetracycline resistance respectively.

Strain name	Organism	Genotype	Phenotype	Source
AOH1	*E. coli*	*ΔdapD::Kan2, dapE6*	Auxotrophic for diaminopimelate	Hudson Laboratory
*TKL-11**	*E. coli*	*thr-1*, *leuB6* (Am), *murE1*, *fhuA21*, *codA1*, *lacY1*, *tsx-95*, *glnV44* (AS), λ^-, *pyrF101*, *his-108*, *thyA6*, *argG66*, *ilvA634*, *thi-1*, *deoC1*	Growth sensitive phenotype were the mutant grows at 30 °C but not a 42 °C	CGSC (#5989)
*DL39**	*E. coli*	*LAM-, aspC13, fnr-25, rph-1 ilvE12, tyrB507*	Auxotrophic for the amino acids; tyrosine, phenylalanine, leucine, isoleucine and valine	CGSC (#6913)
Rr2-17	*Novosphingobium sp.* (wild-type)	-	Hyper-mucoid	Savka Laboratory
Hx699	*Novosphingobium sp.*	*rsh::EZ-Tn5, Kan^R*	Hypo-mucoid	Savka Laboratory

Table 2: Bacterial strains used in this study. List of bacterial strains along with respective genotypes and phenotypes used in this study. Please note that strains (*TKL-11* and *DL39*) denoted by the asterisk can be obtained directly from the Coli Genetic Stock Center (CGSC) (http://cgsc.biology.yale.edu/).

Discussion

Many of the courses that are integral to the Biotechnology and Molecular Bioscience curriculum at the Rochester Institute of Technology have a laboratory component in addition to the lecture portion of the course. The curriculum for the academic year 2014-2015 contains a total of 48 courses, 29 of which contain a laboratory component which represent approximately 60%. One such course is Fundamentals of Plant Biochemistry and Pathology (FPBP), a blended lecture/ laboratory course and Bioseparations: Principles and Practices (BPP), a laboratory based course.

The laboratory component of each course is designed to reinforce the lecture materials. For example, biochemical pathways and metabolism are heavily stressed in FPBP and BPP. Some of the topics include amino acid metabolism, peptidoglycan biosynthesis, plant secondary metabolism, bacterial response to environmental niches, among others. The authors have integrated functional complementation as laboratory exercises in both courses to reinforce the understanding of metabolic pathways that are discussed in the lecture and or pre-laboratory presentations. Four examples of using functional complementation experiment to analyze the function of genes involved in biochemical pathways of lys, PG, tyr, phe and the stringent response of bacteria are discussed.

There are several reasons why the authors have integrated this experimental module into their courses. Firstly, exposure to functional complementation analyses is an excellent tool to reinforce or introduce topics that are related to genetics, evolution, genomics, bioinformatics and biochemistry. Secondly, the experiment is facile and can be accomplished in a course laboratory environment. All of the reagents that are used are safe and the bacteria that are used are denoted as biosafety level 1 and are not pathogenic. As such, the authors are willing to make reagents such as plasmids and bacterial strains available to anyone who is in interested in incorporating functional complementation as a part of their teaching experience. It should be noted that two of the bacterial strains (TKL-11 and DL39) were obtained directly from the Coli Genetic Stock Center (CGSC) (http://cgsc.biology.yale.edu/).

It is important to note that there are several critical steps to the functional complementation protocol. The first step is to make sure that the mutant(s) are able to be cultured before commencing any experiments. The reason is that as per **Table 2**, there are several genotypes associated with each mutant. In order to grow the mutant to prepare competent cells before the experiment, try growing the mutant with or

without the required chemicals and or temperature requirements regarding the PG murE mutant. This is also a teaching moment because it will prove that the mutant is authentic before any experiments.

It should also be noted that although this is an excellent tool to test the function(s) of genes, it does not always work due to several factors such as codon usage where gene(s) of interest cannot be properly translated due to the lack of the appropriate tRNAs in the mutant organism. However, this can be circumvented by codon optimization of the open reading frame or cDNA during the cloning step which is normally done by synthesizing the gene of interest by changing the nucleotides to match the codon usage of the mutant(s). Another issue is that some eukaryotic proteins require post-translational modifications for function. Post translational modification is not a feature of bacteria and as such one might not be able to use this technique to test the function(s) of genes using bacterial mutants.

The significance of the technique that we emphasize in the courses is that FCA is an excellent way to test to function of genes *in vivo*. Characterization of enzymes are mostly based on *in vitro* studies where the enzyme is purified and traditional enzymatic assays are performed.[29] Also traditional enzymatic assays are great to measure or detect enzymatic activity, one can often "force feed" the enzyme with commercially available substrates that are not pure or natural for the enzyme.[30] An *in vivo* system such as FCA provides a more authentic proof regarding the function of genes given the fact that it is operating under physiological conditions as appose to *in vitro* which is normally not under physiological conditions.[2] Given lack of information regarding the functions of many genes and the fact that most annotations are based on prediction,[31] mastering this technique will facilitate experiments to elucidate the functions of many genes that remain nebulous or those that are currently deemed to have putative functions in the various public databases.

One of the issues regarding the technique that is often experienced is in the preparation of the competent cells. One should make sure that the cells are prepared correctly to ensure that there is enough cells to be transformed in addition to making sure the cells are washed properly with water and glycerol to prevent arching during the electroporation process. One should also be cognizant of the phenotype of the mutant by making sure that the cells are grown with the appropriate chemical or at the proper temperature to facilitate both the initial growth of the mutant and also for the functional complementation analysis.

Once this technique is mastered, researchers can use functional complementation assay to assess the function of genes as long as there is an appropriate mutation in an organism available to facilitate this analysis. Please note that the protocol in this manuscript describes the use of bacteria. However, other organisms such as plants and mammalian cells, among others, can be used to assess the function of genes.[32-33] One would just have to make sure that the proper vectors are used in addition to optimizing the transforming and or transfection of the cells to facilitate the experiment.

Disclosures

The authors declare that they have no competing financial interests.

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