Genetic approaches to uncover gene products involved in iron-sulfur protein maturation.

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Abstract

Iron-sulfur (FeS) clusters are one of the most ubiquitous and versatile prosthetic groups exploited by nature. FeS clusters aid in conducting redox reactions, carbon activation, and environmental sensing. This chapter presents an overview of the genetic approaches that have been useful for identifying and characterizing bacterial factors involved in FeS protein assembly. Traditional genetic screens that assess viability or conditional auxotrophies, and bioinformatic approaches have identified the majority of the described genes utilized for FeS protein assembly. Herein, we expand upon this list of genetic methods by detailing the use of transposon-sequencing (TnSeq) to identify gene products that are necessary for the proper function of metabolic pathways that require FeS enzymes. TnSeq utilizes the power of genomics and massively parallel DNA sequencing to allow researchers to quantify the necessity of individual gene products for a specific growth condition. This allows for the identification of gene products or gene networks that have a role in a given metabolic process but are not essential for the process. An advantage of this approach is that it allows researchers to identify mutants that have partial phenotypes that are often missed using traditional plate-based selections. Applying TnSeq to address questions of FeS protein maturation will result in a more comprehensive understanding of genetic interactions and factors utilized in FeS biogenesis and FeS protein assembly.

1. Introduction

FeS clusters are cofactors found in almost all living organisms. The synthesis of FeS clusters and the maturation of FeS proteins are essential processes [1,2]. FeS proteins are utilized for a wide range of physiological processes. In bacteria, there are three primary systems that have evolved to build FeS clusters. The ISC (iron-sulfur cluster), SUF (sulfur mobilization) and NIF (nitrogen fixation) biosynthetic systems are phylogenetically and biochemically distinct, but they all share a similar strategy to build the FeS clusters (reviewed in [3]). In general, FeS cluster biogenesis occurs in three steps. First, sulfur (S^0) is acquired from L-cysteine by a cysteine desulfurase and combined with iron (Fe^{2+}) and electrons (e^-) on a proteinaceous scaffold [4]. Once assembled, the FeS cluster can be transferred directly to an apo-protein or via an intermediate, FeS cluster carrier protein. This results in the activation of the target protein [5,6].

Despite the progress that has been made in understanding FeS cluster assembly and maturation, a number of important questions remain. For instance, in most species, the Fe or electron donors are yet to been identified and we still do not have a complete picture on how FeS cluster synthesis is regulated. It is known that many organisms utilize multiple FeS cluster carrier proteins. It has not yet been established if there is an underlying specificity of carriers for target proteins or if cells prioritize the maturation of certain FeS proteins over others. Moreover, a number of factors have been identified that are utilized in FeS protein maturation, but their biochemical functions are unknown [7,8].

Microorganisms have been exploited to characterize gene products involved in maturating FeS proteins because of their genetic tractability, high growth rate, and metabolic diversity. The first genes utilized for FeS protein maturation were discovered by scoring mutant strains of the diazotroph *Azotobacter vinelandii* for growth in the presence and absence of a reduced nitrogen source. *A. vinelandii* strains lacking the *nifSU* genes, which were later shown to encode a cysteine desulfurase and a FeS cluster synthesis scaffold, respectively, were not able to grow in the absence of a reduced nitrogen source [9-11]. The requirement for FeS clusters for nitrogenase function, and the sensitivity of the cofactors to oxygen, places high demand on the FeS cluster assembly

machinery when reduced nitrogen is not provided in the medium. The process of nitrogen fixation requires a dedicated maturation system (Nif) for maturation of simple and complex FeS clusters [12]. Scoring for diazotrophic growth has also been successfully applied to study the redundancy between maturation factors [13].

Escherichia coli can synthesize nicotinic acid, which is required for survival. NadA is utilized in nicotinic acid synthesis and an *E. coli* mutant strain lacking the *isc* operon is auxotrophic for nicotinic acid [14]. Using an Δ*isc* mutant, researchers isolated strains that contained a second site suppressor mutation providing nicotinic acid prototrophy. The suppressor strains also showed increased growth compared to the parent when alternate vitamins or amino acids that require an FeS enzyme for synthesis were absent from the media. The mutations mapped to the promoter region of the *suf* operon and ultimately resulted in the discovery of the third system for FeS cluster synthesis.

Salmonella enterica synthesizes thiamine using the SAM-radical enzymes ThiH and ThiC, which have solvent exposed and oxygen labile FeS clusters [15,16]. Mutations that decrease carbon flux through the purine synthesis pathway, which provides precursors for thiamine synthesis, has allowed researchers to identify a number of genes that effect thiamine synthesis. Many of these mutations are in genes that encode proteins that are not dedicated members of the core thiamine biosynthetic machinery and have provided hints as to how metabolic pathways are tethered together (reviewed in [17]). Some of thiamine auxotrophic mutants had a general defect in maturating FeS proteins [17] and at least one was biochemically demonstrated to be a FeS cluster carrier [18]. Thiamine-dependent growth phenotypes have been used to analyze heterologous expression of homologues [19] and site-directed variants [20] of FeS cluster assembly factors.

S. enterica also has the ability to grow using tricarballylate as a carbon and energy source. Tricarballylate is transported into the cells where it is reduced to *cis*-aconitate by the flavoprotein TcuA [21]. The FeS protein TcuB is thought to provide electrons for the reduction of TcuA [22]. Strains lacking specific FeS maturation factors did not grow on medium containing tricarballylate as a sole carbon source, but were proficient for growth on medium containing glucose. It has been hypothesized that the high demand for FeS assembly machinery is the result of poor TcuB maturation resulting in sustained

tricarballylate import and decreased reduction. This results in cytosolic accumulation, which inhibits isocitrate dehydrogenase [23]. Tricarballylate growth has been used to conduct suppressor analysis and study functional redundancy between FeS cluster assembly systems.

Other phenotypes utilized to identify and characterize FeS cluster biosynthesis systems include growth in the presence of elevated oxygen [24] or Fe limitation [25]. It was recently demonstrated [26] that FeS cluster synthesis is essential because it is required to maturate the FeS enzymes IspG and IspH, which are utilized isoprenoid synthesis in *E. coli* and *Bacillus subtilis* [27,28]. The need for IspH and IspG can be bypassed by the heterologous expression of a mevalonate-dependent pathway for isoprenoid synthesis, which contains no FeS proteins, and supplementation of the growth media with mevalonate. Removing mevalonate from the growth medium provides a strong selective pressure. Researchers have used these genetic systems to isolate strains with second site mutations that provided information about ISC- and SUF-dependent FeS cluster synthesis.

The examples provided above and others discussed in [29] (typically) allowed researchers to answer questions by scoring for a conditional auxotrophy or viability. Although powerful, especially when applied to study second-site mutations, they do not allow researchers to identify genes that may contribute to a process but do not have a strong solid medium phenotype. Transposon-sequencing (TnSeq) utilizes the power of genomics and massively parallel DNA sequencing to quantify the necessity of individual gene products for cellular fitness under specific growth conditions [30]. By comparing the fitness needs of loci under two growth conditions, such as with and without thiamine supplementation, we can gain a systems level understanding of the genes that contribute to a given metabolic process, which allows us to build models of gene interaction. There are a couple of potential drawbacks to using Tn-seq. First, the network analyses do not allow for the incorporation of essential genes. Second, the genes of interest require mutant construction or acquisition for further study.

Herein, we outline the use of Tn-Seq to determine the importance of specific gene products for the fitness of the Gram-positive bacterium *Bacillus subtilis*. Applying TnSeq to address questions of FeS protein maturation will result in a more comprehensive

understanding of genetic interactions and factors utilized in FeS cluster biogenesis and FeS protein assembly.

2. Materials

2.1. *Genomic DNA preparation*.

a. DNeasy Blood and Tissue kit (Qiagen);

b. Enzymatic Lysis Buffer: 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100. Tris-HCl and EDTA stock solutions should be autoclaved prior to addition. A stock solution of Triton-X should be filter sterilized.

c. RNase A (100 mg/ml)

d. Lysozyme

2. 2. *Transposon library generation.*

a. Plasmid pCJ41 (see Note 1)

b. MarC9 transposase (see Note 2)

c. 3M sodium acetate, pH 5.2

d. Ethanol (100% and 70%)

e. 2X Buffer A: 41 mM HEPES, pH 7.9, 20% glycerol, 200 mM NaCl, 20 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin (BSA), 4.0 mM dithiothreitol (DTT). Prepare a small volume from autoclaved or filtered stock solutions fresh before use.

f. 10X Buffer B: 500 mM tris-HCl, pH 7.8, 100 mM $MgCl_2$, 10 mM DTT.

g. Bovine serum albumin (1 mg/ml)

h. dNTP solution mix (New England Biolabs)

i. 3000 U/ml T4 DNA polymerase (New England Biolabs)

j. 10,000 U/ml *E. coli* DNA ligase (New England Biolabs)

k. 2.6 mM nicotine adenine dinucleotide (NAD)

2. 3. *Competent cells.*

a. Competence media. 1X SS base: 6 g/L (44 mM) KH_2PO_4, 14 g/L (80.4 mM) K_2HPO_4, 2 g/L (38 mM) $(NH_4)_2SO_4$, 1 g/L (4 mM) sodium citrate. Autoclave to sterilize. To 500 ml of 1X SS media, add 5 ml of 10% yeast extract (autoclaved twice prior to addition), 5 ml of 2% casein hydrolysate (autoclaved), 5 ml of 50%

glucose (autoclaved), 2.5 ml of 0.5 M $MgCl_2$, and auxotrophic requirements (final concentration 50 µg /ml).

b. 0.5 M $CaCl_2$

c. Luria-Bertani (LB) broth agar and media.

d. 100 mg/ml spectinomycin in dH_2O. Solution should be filtered sterilized before use. When added to plates, use at a final concentration of 100 µg/ml.

e. S7 + metals: 5 mM potassium phosphate, pH 7.0, 10 mM $(NH_4)_2SO_4$, 100 mM MOPS (adjusted to pH 7.0 with KOH), 20 mM sodium glutamate, pH 7.0, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 µM $MnCl_2$, 5 µM $FeCl_2$, 1 µM $ZnCl_2$, 2 µM thiamine, 100 mM glucose + required auxotrophic requirements (50 µg/ml). All salt solutions should be autoclaved. MOPS and thiamine should be filter sterilized. To recover the transposon library, leave out all carbon sources (glucose, glutamate and amino acids).

f. Glycerol

2. 4. *Preparation of DNA for Illumina sequencing*

a. Primers (see Table 2)

b. MmeI restriction endonuclease (New England Biolabs)

c. Phenol:chloroform:isoamyl alcohol (25:24:1)

d. 400,000 U/ml T4 DNA ligase (New England Biolabs)

e. Phusion DNA polymerase (ThermoFisher)

f. Agarose

g. Electrophoresis equipment

h. MinElute Gel Extraction kit (Qiagen)

3. Methods

3.1 *Preparation of genomic DNA*

Genomic DNA can be prepared using any standard method, or kit that produces a large quantity of a highly pure product. The following protocol contains instructions for use with the DNeasy Blood and Tissue kit (Qiagen), using the Gram-positive bacteria protocol.

1. Inoculate 5 ml of liquid LB media with a single colony of the desired *Bacillus* strain and incubate overnight at 37°C with aeration (shaking).
2. Centrifuge 1.5 ml of cells in an Eppendorf tube for 5 min at 11,000 *x g* and remove and discard the resulting supernatant. Repeat this step again (same tube), to collect a total of 3 ml of cells.
3. Resuspend the pellet in 180 µl of Enzymatic Lysis Buffer that has 40 mg/ml lysozyme (final concentration) freshly added. Incubate at 37°C for 30 min.
4. Add 4 µl of RNaseA (stock concentration of 100 mg/mL) and mix well. Incubate for 15 minutes at room temperature.
5. Add 25 µl Proteinase K and 200 µL Buffer AL (provided in the DNeasy kit). Vortex samples to mix. Incubate at 56°C for 30 min.
6. Add 200 µl 100% ethanol, and vortex to mix.
7. Transfer the sample to a DNeasy spin column. Centrifuge 1 min at 6000 *x g*. Discard the flow through, and transfer column to a new collection tube.
8. Wash the column by adding 500 µl of Buffer AW1, and centrifuge for 1 min at 6000 *x g*. Discard the flow through, and transfer column to a clean collection tube.
9. Wash column a second time by adding 500 µl Buffer AW2, and centrifuge for 3 min at maximum speed. Discard the flow through and transfer column to a clean Eppendorf tube for sample elution.
10. Add 200 µl Buffer AE to the spin column. Incubate for 1 minute at room temperature. Centrifuge for one minute at 6000 *x g* to collect the genomic DNA. Samples may be stored at -20°C for future use.

3.2 *Generation of transposon libraries* in vitro

More information on transposon library preparation can be found for *Streptococcus pneumoniae* [30,31]*, Bacillus subtilis* [32] and *Staphylococcus aureus* [33,34]. The *B. subtilis* detailed protocol is presented below. Each reaction can be used for two library transformations. It is recommended to perform at least 5 separate reactions in total.

1. Mix together in an Eppendorf tube, 1.3 µg pCJ41 (*magellan6X* transposon donor, see **Note 1**), 5 µg target gDNA, 10 µl 2x Buffer A and dH_2O to 18 µl. Finally,

add 2 µl MarC9 transposase (Final concentration of 15-20 µg/ml, see **Note 2**). Incubate overnight at 30°C.

2. The following morning, add 2 µl of 3M sodium acetate (pH 5.2), mix, and then add 50 µl ice-cold 100% ethanol. Incubate on ice for at least 20 minutes (but can go longer).

3. Centrifuge samples at 4°C, at maximum speed for 30 minutes.

4. Remove the supernatant (with a pipette) and add 100 µl ice-cold 70% EtOH. Centrifuge samples at 4°C, at maximum speed for 15 min. Remove supernatant with a pipette and allow to dry at 37°C.

5. Resuspend the pellet by adding 2 µl 10x Buffer B, 2 µl of 1 mg/ml BSA and 11 µl H_2O. Incubate at 37°C for 3-4 hours.

6. Add 4 µl of a 2.5 mM dNTP solution. Add 1 µl T4 DNA Polymerase (3000 U/ml, New England Biolabs (NEB)) and incubate for 20 minutes at 12°C (see **Note 3**).

7. Following incubation, heat inactivate the polymerase by incubating the reactions at 75°C for 15 minutes. Cool the reactions on ice for 3 minutes.

8. Add 0.2 µl 2.6 mM NAD and 1 µl *E. coli* DNA ligase (10,000 U/ml, NEB), and incubate overnight at 16°C.

9. The transposed DNA in theory can be stored at -20°C for future use; however, the effects of freeze-thaw cycles are unknown. It is recommended to immediately proceed with the transformation procedure described below.

3.3 *Preparation of the* in vivo *transposon library*

1. Streak out the desired *Bacillus* strain on LB agar plates with appropriate antibiotics (if necessary), and incubate overnight at 30°C.

2. The following morning, inoculate two flasks with 10 ml of competence media with enough bacteria for a starting Klett of 15-20 (If using OD_{600}, start at an OD of ~0.05).

3. Incubate cells at 37°C with aeration for 2.5 hours. Take a starting Klett reading. From this point, take Klett readings every 10 minutes. When the Klett value changes by 2-5 units every 10 minutes, your cells are in exponential phase. Dilute

each culture 1:10 into 10 ml of pre-warmed competence media supplemented with 0.5 mM $CaCl_2$ (final concentration).

4. Incubate cells with aeration for 90 minutes.

5. Centrifuge cells for 10 minutes at 4500 *x g* at 4°C to collect cells. Resuspend each pellet in 1 ml of spent competence media (supernatant).

6. Divide the competent cells into ten 200 µl aliquots (in test tubes, or 15 ml conical tubes), and add 10 µl of the *in vitro* transposed gDNA (2.5 µg each, or ½ of the reaction mixture) to each tube.

7. Incubate the reactions for 90 minutes at 37°C with aeration (shaking or rotation).

8. Add 1 ml of pre-warmed LB to each tube and incubate for an additional 30 minutes.

9. Plate cells on LB agar supplemented with 100 µg/ml spectinomycin (250 µl/plate, to select for the transposon). Incubate plates overnight at 37°C.

10. OPTIONAL: Plate a 10-fold serial dilution series to determine the number of transposants in your library. Do not recover these cells as these clones have more space to grow on the plate and may become over-represented in the library.

11. Recover the library. Add 2 ml of S7 with metals (no carbon source) to each plate, and use a 1 ml pipette to disrupt the colonies and recover the fluid from the plate. Pool all of the recovered cells. This approach yields a library of ~4.0 X 10^5 independent clones.

12. Mix the library with ¼ of the volume with 50% glycerol, then freeze in 100 µl aliquots for storage at -80°C (see **Note 4**).

3.4 *Selection for mutants involved in FeS cluster biosynthesis*

There are numerous biosynthetic pathways that encode FeS cluster proteins in *B. subtilis* (See Table 1). Any individual pathway or multiple pathways, including additional ones not listed, could be chosen for the screen. The base media for all of the following studies is S7 with metals. Alternate selection pressures including culturing in Fe deplete and replete conditions or including a small molecule that catalyzes ROS formation could also be examined. As in all genetic selections, care should be taken to minimalize variables to prevent pleotropic effects caused by the selective pressure.

Before beginning the selection process, it is recommended to perform simple growth experiments to adjust concentrations of added supplements, and to verify that the selection process will be successful. To do this, deletion mutants can be either acquired (i.e., from Bacillus Genetic Stock Center) or constructed. For example, the *leuC* deletion mutant requires leucine for growth, and does not grow in its absence, which was confirmed by growth in different media (Figure 1). When all nutritional requirements are determined, known FeS cluster biosynthetic genes are tested in the supplemented S7 and minimal media (Figure 2). Deletions of known, non-essential assembly factors, such as *nfu* [5], *sufA* [35], or *sufT* [36] were grown in each media, and growth defects of each mutant were apparent. Consequently, these mutants should be under-represented in the population following selection, and thus serve as a positive internal selection control. Once the media conditions are selected, the following steps are done to perform the selection.

1. Thaw a 100 µl aliquot of the transposon library. Inoculate 20 ml of LB media.
2. Incubate at 37°C with shaking until the culture reaches an OD_{600} of 1.0. It will likely take between 2-3.5 hours.
3. Collect two 1 ml samples of cells and centrifuge at 11,000 *x g* for 5 minutes. Wash each pellet three times in either supplemented or minimal S7 media.
4. Resuspend each pellet in supplemented or minimal media, and add to 100 ml of the same media (1:100 dilution). It is recommended to use 1 ml of cells to have a good representation of the mutant clones in the library. Let cells grow overnight (~24 hours) at 37°C with shaking.
5. The next morning, record OD readings. Back dilute the culture 1:100 in the same media, and let grow for another 24 hours at 37°C with shaking.
6. Record OD readings, and collect six, 1.5 ml aliquots of each culture in Eppendorf tubes. Centrifuge at 11,000 *x g* for 10 minutes. The pellets may be frozen at -20°C for future processing. Typically, 2-3 of these tubes will need to be processed to ensure enough gDNA, but collecting extra aliquots of cells is recommended.
7. It is recommended to perform this selection at least three independent times for appropriate statistical analyses.

8. Isolate gDNA from the pellets as described in section 3.1. 6 µg of gDNA are required to proceed.

3.5 *Preparation of DNA for Illumina sequencing*

The following protocol was adapted from Johnson and Grossman [32]. See Table 2 for the primer sequences.

3.5.1 *Precipitate genomic DNA*

a. Add 20 µl of 3M sodium acetate pH 5.2, and 500 µl of ice cold 100% ethanol to 200 µl of gDNA.

b. Incubate at -20°C (or on ice) for at least 30 minutes.

c. Centrifuge at 20,000 *x g* at 4°C for 30 minutes.

d. Discard supernatant, and wash with 500 µl of ice cold 70% ethanol. Centrifuge at 20,000 *x g* at 4°C for 10 minutes.

e. Discard supernatant and dry at 37°C for 10 minutes.

f. Once dry, resuspend in 60 µl of 10 mM Tris-HCl, pH 8.5.

g. Determine the concentration and purity of DNA using a Nanodrop, or other DNA quantification methods. If you have less than 6 µg of total DNA for one condition, prepare more gDNA from the frozen pellets.

3.5.2 *Anneal the adapter*

a. The oligo nucleotides AP-A and AP-B should be prepared as 200 mM solutions in 1 mM Tris, pH 8.5 (see **Note 5**).

b. Mix equal volumes of the oligonucleotides and place in 96°C heat block for 2 minutes.

c. Remove the heat block, and let cool to room temperature for 15 minutes. This will allow the oligonucleotides to anneal.

d. The annealed adapter will be at a 100 µM concentration. The adapter can now be stored at -20°C.

3.5.3 *Digest gDNA with MmeI*

a. Set up the following reaction:

6 µg gDNA

3 µl MmeI (NEB, 2000 U/ml)

0.66 µl SAM (32 mM)

30 µl CutSmart buffer

Adjust volume to 300 µl total volume with dH_2O.

b. Incubate for 2.5 hours at 37°C.

c. Add 0.6 µl of calf intestinal phosphatase (6 units, NEB) and incubate for an additional 1 hour at 37°C.

d. Add 300 µl of phenol:chloroform:isoamyl alcohol (25:24:1), vortex to mix, and centrifuge at max speed for 10 minutes. Recover the top aqueous layer into a fresh Eppendorf tube.

e. Ethanol precipitate the DNA as described in section 3.5.1, with one modification. Resuspend in 27.5 µl of 2 mM tris-HCl, pH 8.5 and incubate for at least 2 hours at 37°C to dissolve the pellet.

3.5.4 *Adapter ligation*

a. Prepare the following reaction:

27.5 µl MmeI-digested gDNA

3.5 µl 10X T4 DNA ligase buffer

2.0 µl 100 µM annealed adapter

1.5 µl T4 DNA ligase (400,000 U/ml, NEB)

b. Incubate overnight at 16°C.

c. The following morning, the reaction products were purified using a PCR purification kit (Qiagen) as per manufacturer's instructions. Elute in 50 µl warm elution buffer (pre-heated to 37°C, 10 mM tris-HCl, pH 8.5).

3.5.5 *PCR amplification of the fragment of interest*

For PCR, any high-fidelity polymerase can be used. This protocol describes use with Phusion High Fidelity DNA Polymerase (ThermoFisher).

a. Set up the following reaction (per condition):

10X buffer	5 µl
Adaptor ligation	2- 5 µl (Can vary template concentration)
25 mM dNTP mix	0.5 µl
10 µMGEX-PCR primer	1.0 µl
10 µM oCJ22-NNNN	1.0 µl
Phusion	0.5 µl
Adjust volume to 50 µl with dH_2O	

b. Set up the PCR conditions as follows. Adjust temperatures and times according to manufacturer's instructions (see **Note 6**).

1) 98°C 30 s (1 cycle)
2) 98°C 10 s
3) 58°C 25 s
4) 72°C 10 s (18 cycles to step 2)
5) 72°C 10 min (1 cycle)
6) 4°C HOLD

c. Load the entire PCR sample (mixed with loading dye) onto a 1.8% agarose gel, with a 100 bp ladder. Excise the appropriate band (size ~130 bp, Figure 3) from the gel using the MinElute gel extraction kit (Qiagen) following manufacturer's instructions. When following the instructions, perform the optional column wash step with buffer QG to ensure removal of residual agarose. Elute with 14 µl of elution buffer (10 mM Tris, pH 8.5).

d. This DNA sample is now ready for Illumina sequencing. Submit the custom sequencing primers Prim-seq (primary sequencing primer to determine the genomic locations) and Bar-seq (sequences barcode). Our sequencing facility utilized the NextSeq 500 and MiSeq instruments.

3.6 Bioinformatic analysis

This protocol is written with the assumption that the genome of the organism of interest has been fully sequenced and annotated with genes, rRNA, tRNA, pseudogenes, etc.

3.6.1 *Demultiplexing and trimming of raw reads*

Because of the way the Tn-Seq libraries are constructed, the raw reads include barcode sequences, sequencing adaptors, and remaining transposon sequence which need to be removed before any downstream analyses.

a. The first 8 bp of each read form a barcode added before the transposon insertion to enable the pooling of different conditions together. With a simple bash /perl/python script that utilizes the barcode sequence, the reads can be split so there is one file per each sample. This can also be achieved with the cutadapt program [37].

b. Remove Illumina sequencing adapters from the 3'end of the reads. There are many open-source programs that deal with sequencing adapter trimming such as cutadapt and Trimmomatic [38].

c. Trim the remaining transposon sequence and filter out any sequence that is longer than 17 bp or shorter than 16 bp. All of the programs mentioned above can be used for this step as well.

3.6.2 *Determine the number of insertions in each TA site*

The trimmed reads are genomic sequences found next to the insertion sites (TA). The goal is to align the trimmed reads to the reference genome and count the number of reads aligned to each TA site. There are many programs that can perform this task but the two most popular programs are bowtie [39] and BWA [40]. The reads are short (16-17 bp), so do not allow any mismatches between the reads and the genome in the alignment process. Each alignment program uses different parameters to control the number of mismatches allowed, consult with the manual of the program in use. From the alignment output, extract the coverage (e.g. number of aligned reads) at each TA site, including the sites with zero read counts. If your experiment includes more than one condition, such a file should be generated for each condition.

3.6.3 *Predicting putative Essential Genes*

The goal here is to identify genes or regions in the genome that are essential or advantageous for growth under the specific selection conditions. The basic idea is that mutants with insertions in conditionally essential genes would not be viable or underrepresented in the population.

The different methods used to identify essential genes can be divided into 2 general categories: 1) Annotation dependent- The total count of disrupted sites per annotation feature (i.e. gene) are counted and compared with the rest of the genes to determine which genes are essential and which are not, and 2) Annotation independent- The total counts of disrupted sites are counted independently of annotation features. One such method uses a Hidden Markov Model to determine the essentiality of each disrupted site, which are then crossed with the gene annotations to identify which genes are essential. There are several programs that can be used for determining essential genes in Tn-Seq experiments, some options are: TRANSIT [41], ESSENTIALS [42], Tn-seq Explorer [43], and TnseqDiff [44]. Some of these programs contain methods for both annotation-dependent and annotation-independent methods such as TRANSIT. The required input files for these programs are usually a gene annotation file and the read alignment file (one for each library). The output in most cases is a tab-delimited file that holds all the genes as rows while the columns represent the results (i.e. essential or non-essential). Some programs add probability values to their results and are able to compare the essential genes between two conditions and quantify the statistical significance.

4. Notes

1. The plasmid pCJ41 serves as the source of the *magellan6X* transposon [30,32]. This plasmid was modified from the original plasmid pR412-*magellan6* [30], to remove the *B. subtilis* BsuM restriction site. The inverted repeats on both sides of the transposon contain a MmeI restriction site. This enzyme cleaves the DNA downstream of the restriction site. Following digestion, approximately 16 bp of genomic DNA will be attached to the ends of the transposon, facilitating mapping of the insertion sites.

2. MarC9 must be purified first, as described [30,45]. The plasmid containing the MarC9 transposase is available upon request [30].

3. The temperature is important for this reaction. The T4 DNA polymerase fills in 4 nucleotide single-stranded gaps on each end of the transposon insertion. This enzyme also has 3' to 5' exonuclease activity that increases at temperatures above 12°C. If a cooling block is not available, a heat block placed in a cold room can be used to achieve this temperature.

4. To regenerate a previously used library: Take a 100 µl aliquot of the transposon library and inoculate 20 ml of LB media. Let the cells grow, with shaking, at 37°C until stationary phase. Measure the OD_{600}. Centrifuge the cells for 10 minutes at 5000 *x g,* at 4°C, and discard the supernatant. Resuspend the cells in 10 ml of S7 media (no carbon source) and centrifuge as before. Resuspend the pellet in S7 media (no carbon source) to achieve an OD_{600} of 10. Add glycerol to a final concentration of 12.5%, and freeze at -80°C in 100 µl aliquots.

5. AP-A contains a random two base overhang (NN). MmeI digestion leaves a random 3 base overhang, so this allows for ligation to occur. AP-B must be phosphorylated at both ends. The 5' phosphorylation allows for ligation to the dephosphorylated genomic DNA fragments. The 3' phosphorylation prevents formation of self-ligated adapter products.

6. Touchdown PCR can be used in this step. Drop the annealing temperature from 66°C to 58°C in successive cycles. Do not change the total number of cycles.

5. References

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Table 1. Select genes encoding FeS enzymes and the consequences of mutation.

Gene	Phenotype	Reference
nadA	Nicotinic acid auxotroph	[23]
leuC	Leucine auxotroph	[7]
ilvD	Isoleucine and valine auxotroph	[5]
lipA	Lipoic acid auxotroph	[46]
bioB	Biotin auxotroph	[47]
thiC	Thiamine auxotroph	[15]
thiH	Thiamine auxotroph	[48]
citB	Glutamate auxotroph	[8]

Table 2. Primer sequences utilized for Tn-Seq

Name	Sequence (5' to 3')	Purification
AP-A	GTTCAGAGTTCTACAGTCCGACGATCACACNN	PAGE
AP-B	5'-Phos/GTGTGATCGTCGGACTGTAGAACTCTGAACCTGTC/3'Phos	PAGE
oCJ22	CAAGCAGAAGACGGCATACGAGATNNNNNNTGTGTGAGACCGGGGACTTATCATCCAACCTGT	PAGE
GEX-PCR	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA	PAGE
Prim-seq	TACACGTTCAGAGTTCTACAGTCCGACGATCACAC	None
Bar-seq	ACAGGTTGGATGATAAGTCCCCGGTCTCACACA	None

Primer oCJ22 is specific for the inverted repeat sequences of the *magellan6* transposon, and attaches a barcode (NNNNNN, where N is any randomly selected nucleotide) and Illumina-specific sequences. The barcode can be any combination of nucleotides, and should be 4-6 nt in length. Ordering multiple different barcoded primers allows for multiplexing when performing Illumina sequencing. Primer GEX-PCR is specific for the ligated adaptor and adds Illumina-specific sequences. The primers oCJ22, GEX-PCR, and Prim-seq are optimized for use with the MiSeq instrumentation. It is recommended to speak with an Illumina specialist when using new or different instrumentation to check the compatibility of the sequences (For use with the HiSeq platform, see [32]).

Figures.

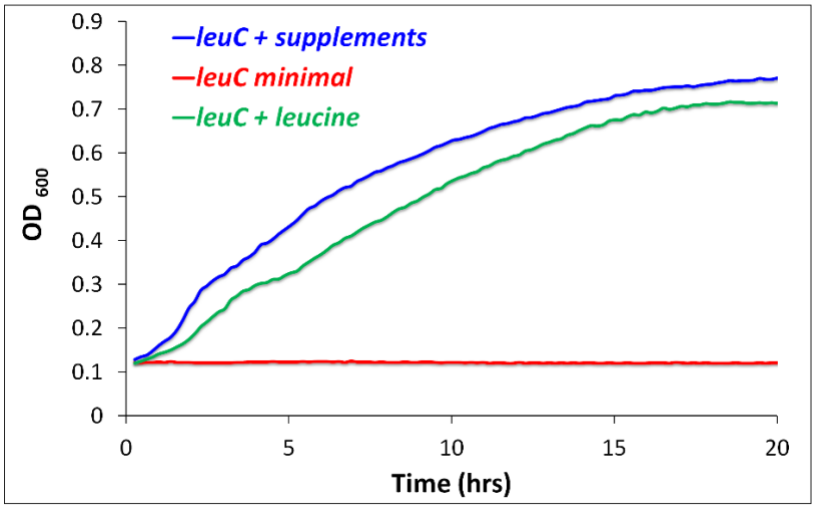

Figure 1: Sample growth curves with the *leuC* deletion mutant. The *leuC* mutant was cultured in S7 media with the addition of all selected supplements listed in Table 1 (blue), S7 media with leucine (green) and minimal S7 media (red). The *leuC* mutant was acquired from the Bacillus Genetic Stock Center. Growth curves were acquired by measuring OD_{600} in an EnVision multimode 96-well plate reader (PerkinElmer). Data are presented as the average of two growth experiments.

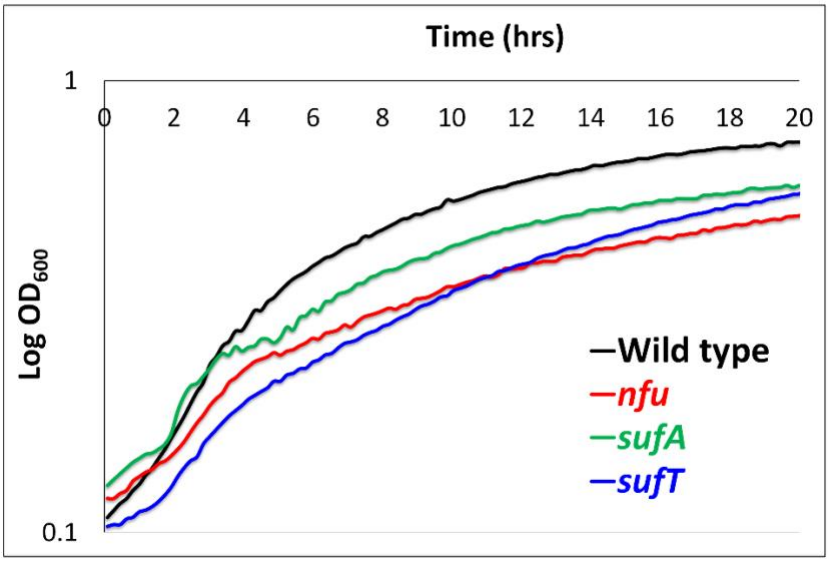

Figure 2: Sample growth curves of wild type (black), *nfu* (red), *sufA* (green) and *sufT* (blue) *B. subtilis* deletion mutants. Strains were obtained from the Bacillus Genetic Stock Center. The strains were grown, in duplicate, in minimal S7 media. Growth curves were acquired by measuring OD_{600} in an EnVision multimode 96-well plate reader (PerkinElmer).

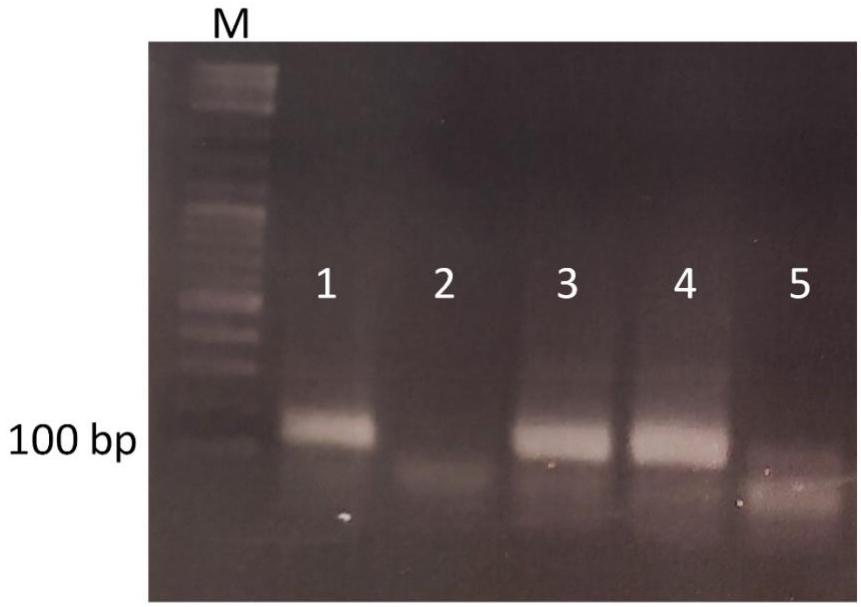

Figure 3: Example of PCR products separated on a 1.8% agarose gel. Lane M: 100 bp ladder (New England Biolabs). The 100 bp band is marked. Lanes 1,3-5 are experimental PCR samples. Lanes 1, 3, and 4 contain the desired product, which runs slightly above the 100 bp band. This is the band that should be extracted. Lane 2 is a negative control (adaptor only). This control is important to include to properly localize the correctly sized band for purification. The bottom two bands are artifacts.