Site-directed mutagenesis of cell division proteins for contraction generation *in vitro*

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28th May 2013
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Bacterial cell division

Figures reproduced from Ghigo et al. [2] and Vicente and Lowe [12]
This project focuses on three proteins known to be involved in the formation of the Z-ring [3, 6–8, 11].
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Three Z-ring proteins — ZipA

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The aim of this project is to tag FtsZ, ZipA and ZapA.

This is with a view to observing these proteins interact in an “artificial cell”, enabling us to determine their role in the mechanism of Z-ring contraction.
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Why (site-directed) mutagenesis?

- We could tag the protein by expressing it with a fluorescent tag attached (e.g. GFP) ... 

- ... but these proteins are bulky.

<table>
<thead>
<tr>
<th>Protein</th>
<th>GFP</th>
<th>FtsZ</th>
<th>ZipA</th>
<th>ZapA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of residues</td>
<td>238</td>
<td>383</td>
<td>328</td>
<td>109</td>
</tr>
</tbody>
</table>
Why (site-directed) mutagenesis?

- An alternative approach — tag the cysteine residues using their thiol groups (see Kim et al. [4] for example).

![Chemical structure](image.png)

- If we cannot find an appropriate side-chain in a suitable place, instigate a mutation to provide one.
Mutation positions

- No mutations in ZapA.
- Four mutations in each of FtsZ and ZipA chosen.
- 7 out of 8 of these are Gly or Ala to Cys.

\[
\begin{align*}
\text{Glycine} & : & \text{GGA} & \text{GGC} & \text{GGG} & \text{GGT} \\
\text{Alanine} & : & \text{GGA} & \text{GGC} & \text{GGG} & \text{GGT} \\
\text{Cysteine} & : & \text{TGC} & \text{TGT} \\
\end{align*}
\]

- Other mutation — phenylalanine residue coded for upstream of the ZipA gene in pET-52b plasmid.
**Primer Design**

*QuikChange*\(^1\) primer calculator used to generate primers (protocol used by others [1, 10]). Basic principles behind primer building:

- Need the primer size to be just right.

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\(^1\)Trademark of Stratagene / Agilent Technologies.
Primer Design

QuikChange\textsuperscript{2} primer calculator used to generate primers (protocol used by others [1, 10]). Basic principles behind primer building:

- Online tool to see if primers will self-anneal because of repeated subsequences.

\textsuperscript{2}Trademark of Stratagene / Agilent Technologies.
Protocol summary I

- Methylated DNA plasmids
- Forward primer with mutation
- Reverse primer with mutation
- plus free nucleotides, DNA polymerase and buffer

**Performing the mutagenesis**

1. PCR
2. Mixture of plasmids with and without mutation
3. Dpn1 Digest at 37°C for 1 hour
4. Original methylated DNA plasmids digested by Dpn1
Protocol summary II

Transform Top10 E. coli cells

Grow cells

Top10 cells replicate and produce many copies of the mutated plasmid

Mini-prep

Harvest the mutation-containing plasmids
Protocol summary III

Transform over-expression strain *E. coli* cells

Grow cells

Clones of cells and plasmids produced

Add IPTG

Incubate at 37°C for 4 hours

Induce cells

Cells contain protein with desired mutation.
Results ...?

Mutagenesis PCR is tricky!

1. TRY
2. FAIL
3. COLLAPSE
4. SOB
Results — DNA gels

DNA gels were carried out to indicate the presence of PCR product.

FtsZ - Gly to Cys at residue 21
Phusion DNA polymerase
GeneRuler Ladder

Annealing Temp / °C
61.7 66 68.5 70

GeneRuler Ladder

Annealing Temp / °C
Results — DNA gels

Most gels looked like this: ...

ZipA - Ala to Cys at residue 193
Phusion DNA polymerase

GeneRuler Ladder
Annealing Temp / °C
50.0 51.7 52.8 54.3 56.0 57.4 58.5 60.0

ZipA - Ala to Cys at residue 328
Phusion DNA polymerase
Results — DNA gels

...which meant a lot of this:

![Image of 4. SOB]

We did the transformations anyway. Result — lots of colonies!
Results — DNA Sequence Validation

Sequencing results of transformations that produced colonies were varied:

- Some colonies had no mutation.
- Others had multiple copies of the primer found at the point of mutation.
Results – Success!!

We obtained plasmids for three mutations.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue</th>
<th>Residue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>FtsZ</td>
<td>Gly</td>
<td>21</td>
</tr>
<tr>
<td>ZipA</td>
<td>Ala</td>
<td>193</td>
</tr>
<tr>
<td>ZipA</td>
<td>Phe</td>
<td>Upstream in plasmid</td>
</tr>
</tbody>
</table>
Improving the yield

How do we avoid inserting multiple copies of the primer?

One way is **Single Primer Reactions IN Parallel (SPRINP)**, proposed by Edelheit et al. [1].
**“If I had more time . . .”**

- SPRINP improvement.
- Protein verification.
  - MALDI — amino acid sequence check.
  - Circular dichroism — secondary structure check.
  - Linear dichroism — mutated FtsZ polymerisation check.
- Attach tags.
- Construct “artificial cells” containing tagged proteins (SMALPs [5]).
- Observe using confocal microscopy.
“Artificial Cells” and SMALPs

- Membrane bound FtsZ — Osawa et al. [9].

- **Styrene Maleic Acid Lipid Particles (SMALP)** — Knowles et al. [5].
Conclusions

- Site directed mutagenesis can be used as an alternative approach to tagging proteins to study their kinetics ... but there are limitations.

- Protein verification – it remains to be seen whether the mutated proteins expressed in this project retain key properties of the wild-type form.

- Beyond this, the next steps are to tag the proteins (the effect of the tag will need to be considered), construct an artificial cell containing these proteins and observe the resulting interactions and Z-ring contraction.
References


Acknowledgements

- Claire Dow & David Roper
- All of the Structural Biology Lab (C10) at Gibbet Hill
- MOAC DTC staff and fellow students
- The University of Warwick, EPSRC and BBSRC

Thank you for listening! :-) Any questions?