Centre For Bacterial Cell Biology

Investigation of Molecular Mechanism of CRISPR Spacer Acquisition by Using an *in vivo* Experimental System

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Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (cas) comprise adaptive immune systems of many bacteria, and their function is to recognise and destroy invading mobile genetic elements. Immunity is achieved in three steps: spacer acquisition (in which short fragments of foreign DNA are inserted in CRISPR loci), CRISPR transcription (cr-RNA is produced) and cr-RNA guided interference, in which a double-stranded DNA break is made in targeted foreign genetic element. Mechanisms of spacer acquisition process still remain a mystery.

Acquisition in E.coli	Naive	Primed
Proteins required	Cas1 and Cas2	Cas1, Cas2, Cas3 and Cascade
Memory	No prior information about a protospacer	Stored information about a protospacer
Result	Acquisition of one protospacer	Acquisition of multiple protospacers from the same mobile genetic element, adaptive immunity

Experimental in vivo system used to study spacer acquisition in E.coli. Diagram schematically showing cas and CRISPR loci in **KD263 strain** – a derivative of K-12 Escherichia coli, which contains promoters fused with cas genes expressed on chromosomal DNA, that can be induced by IPTG (cas3) and arabinose (Cascade and Cas1 and Cas2) as well as g8 spacer flanked by two repeats. pT7blue-based plasmids were used containing a **g8 protospacer** (derived from M13 bacteriophage) also has a gene for ampicillin resistance. A g8 protospacer with an **escape mutation C1T** was used to see the effects of mutated protospacer on CRISPR/Cas system.

Aims

- To test an *in vivo* experimental system (KD263 strain) and study CRISPR spacer acquisition
- To construct a transposon library in KD263 strain with and without plasmid carrying a g8 protospacer in order to establish random insertions in different genes of E.coli genome to screen for other proteins that may play role in CRISPR/Cas system
- To purify Cas1 and Cas2 separately and in complex and assess their activity *in vitro*

Methods and Materials

- Plasmid purification using QIAprep Spin Miniprep kit
- DNA purification by phenol-chloroform DNA extraction ethanol precipitation and Sigma GenElute Bacterial Genomic DNA kit
- PCR reactions performed using PCR Phusion Hugh-fidelity DNA Polymerase in the GC buffer. Primers complimentary to leader sequence and g8 protospacer used to amplify CRISPR cassette. PCR products analysed by 2% agarose gel.
- E.coli cells transformed using TSB with g8 and g8_C1T carrying plasmids; via electroporation with pRL27 carrying Tn5, recovered by one hour phenotypic expression at 37°C, plated on large LB+KAN (to select for Tn5, which has aph gene that confers kanamycin resistance) and LB+AMP+KAN (to select for g8) Petri dishes (25-30 plates, each containing approx. 3000 colonies) and grown ON at 37°C.
- Sequencing of plasmid DNA performed by GATC Biotech
- E.coli T7 cells transformed with His-tag containing cas1 and cas2 plasmids, induced with IPTG, centrifuged and sonicated. Nickel HisTrap column used for cas1 and cas2 protein purification with step increase of imidazole concentration, dialysis.
- Cation exchange chromatography to purify Cas1 wild-type and H208A (AKTA)
- Anion exchange chromatography to purify Cas2 (AKTA), Novagen thrombin cleavage kit to remove His-tag from Cas2

Results

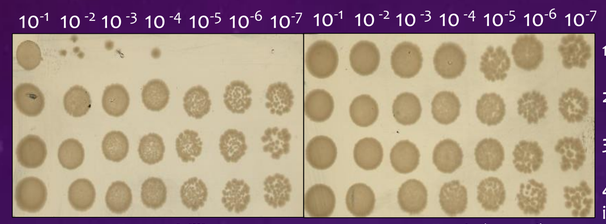


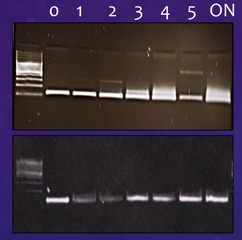
Figure 1. **In vivo experimental system set-up to show plasmid kinetics after 5 hours of CRISPR/Cas induction**

Induction with IPTG and arabinose -> CRISPR/Cas system activated

g8 (wild-type protospacer) recognition and interference	g8_C1T (escape mutation) not recognised

Plasmid is

destroyed, ampicillin resistance is lost, colonies die on ampicillin	intact, ampicillin resistance persists, colonies survive on ampicillin



PCR and gel electrophoresis analysis of **CRISPR cassette expansion** after induction of CRISPR/Cas system to detect **spacer acquisition** of mutated protospacer (top) and wild-type protospacer (bottom)
Figure 2. Induced KD263 with g8_C1T plasmid (top) CRISPR cassette expansion increases as the band of higher MW intensifies in time-dependent manner after the induction (every hour for 5 hours and overnight). Primed spacer acquisition of mutated protospacer takes place.
KD263 with g8 plasmid (bottom) CRISPR cassette has not shown expansion.

Transposon library candidates screening
Figure 3. After creation of KD263+g8 Tn5 library, it was tested by inducing it with 1mM IPTG and arabinose for 5 hours in liquid media prior to plating.
Induced colonies (bottom) grown on LB media with ampicillin, along with the non-induced library colonies (top).
600 potential candidates that survive on ampicillin (due to their g8 plasmid being intact) were selected and then tested separately for resistance to kanamycin and ampicillin (without and with inducers present). All of them survived.

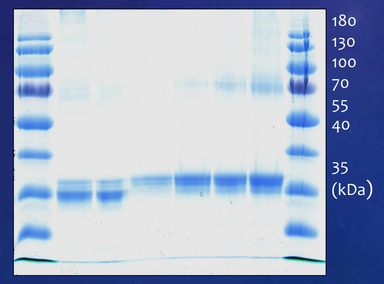


Figure 4. 12% (acrylamide) SDS-PAGE showing **purified mutated and wild-type cas1** present in different amounts shown.

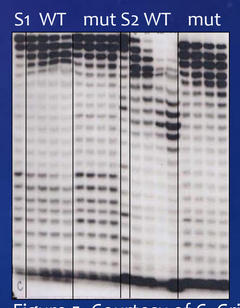
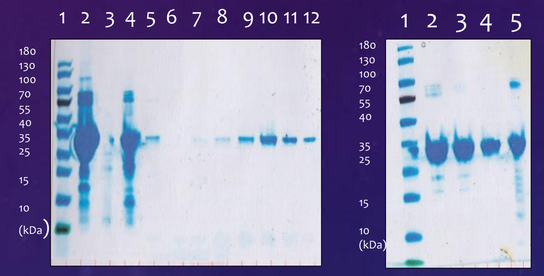


Figure 5. Courtesy of C. Griffiths.
Activity assay of cas1 using 5'radiolabelled ssDNA. In theory cas1 H208A must have a loss in nuclease activity due to a mutation (CAT changes to GCT, its presence confirmed by plasmid encoding Cas1 H208A sequencing) at its active site, but this autoradiograph shows that mutant cleaves S1 and S2 strands of the short 33bp DNA substrate.

Nickel chromatography results of cas1 purification and co-elution of cas1 (His-tag) and cas2 (no His-tag)



Figures 6. SDS-PAGE scan: 1 – MW ladder, 2 – Cas1 (Ni column only), 3 – Cas2 with His-tag cleaved, 4 – sample load Cas1 and Cas2, 5 – unbound fraction, 6-12 – step gradient of imidazole concentration increasing from 0mM to 200mM.
Figure 7. Shows SDS-PAGE gel: 1 – MW ladder and Cas1 and Cas2 concentrated from eluates at 2 – 100mM, 3 – 150mM, 4 – 200mM imidazole, 5 – eluates above pooled.

Conclusions and further investigation

- Shown that induced KD263 cells lose their g8-containing plasmid, that encodes ampicillin resistance, via activated CRISPR interference that recognises g8 protospacer and as a result becoming sensitive to ampicillin. Non-induced KD263 cells shown dormant CRISPR/Cas system that is characteristic of E.coli in laboratory conditions.
- Demonstrated that CRIPSR cassette expansion happens when a protospacer with an escape point mutation (g8_C1T) is recognised and adapted by an induced CRIPSR/Cas system, via primed acquisition in time-dependent manner post-induction, indicating roles of Cas3 and Cascade proteins in this process.
- Successfully purified Cas1 protein and assessed its activity of wild-type and H208A mutants *in vitro*. Concluded that anion exchange chromatography is not suitable for Cas2 purification as it was eluting in all fractions collected during gradient elution, therefore there is a need to consider a new purification method.
- Created two separate transposon 5 insertion libraries of KD263 on its own (~100,000 colonies) and KD263 transformed with g8-carrying plasmid (~65,000 colonies) of sufficient coverage (to achieve high enough number of independent transposition events).
- These libraries can be used to discover new genes and their products that play role at different stages of CRISPR adaptive immunity of type I-E in E.coli. Utilising colony PCR is considered at this stage to select for the candidates that have CRISPR/Cas loci size unchanged compared to the wild-type, therefore must have a transposon insertion somewhere else in the genome, that can be of interest.
- Gel bands that are observed in SDS-PAGE close to purified cas1 wild-type and cas1 H208A can be analysed by mass spectrometry to determine whether they might be different post-translational modifications of cas1.