Kinetic comparison of Cas9 homologs recognizing diverse PAM sequences

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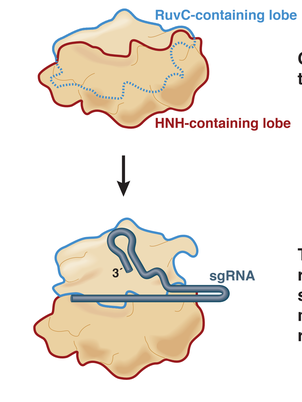
Introduction

Cas9 nuclease is the key effector of type II CRISPR adaptive immune systems found in bacteria. The nuclease can be programmed by a single guide RNA (sgRNA) to cleave DNA in a sequence-specific manner. This property has led to its widespread adoption as a genome editing tool in research laboratories and holds great promise for biotechnological and therapeutic applications. The general mechanistic features of catalysis by Cas9 homologs are comparable; however, a high degree of diversity exists among the protein sequences, which may result in subtle mechanistic differences. *S. aureus* (SauCas9) and especially *S. pyogenes* (SpyCas9) are among the best-characterized Cas9 proteins and share about 17% sequence identity. A notable feature of SpyCas9 is an extremely slow rate of reaction turnover, which is thought to limit the amount of substrate DNA cleavage. Using *in vitro* biochemistry and enzyme kinetics we directly compare SpyCas9 and SauCas9 activities. In contrast to SpyCas9, SauCas9 is a multiple-turnover enzyme, which to our knowledge is the first report of such activity in a Cas9 homolog. We also show that DNA cleaved with SauCas9 does not undergo any detectable single-stranded degradation after the initial double-stranded break observed previously with SpyCas9, thus providing new insights and considerations for future design of CRISPR/Cas9-based applications.

Overview of Cas9 catalysis



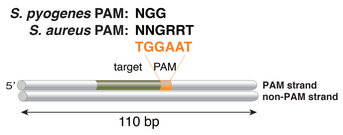
Cas9 consists of two major lobes and is in an apo state in the absence of RNA.

The fold of the single guide RNA (sgRNA) 3'-terminal ~80 ribonucleotides is recognized by Cas9. Upon binding the sgRNA, Cas9 undergoes a large conformational change, marked by rotation of the RuvC domain, forming a stable ribonucleoprotein complex (RNP).

The Cas9 RNP searches the DNA for a protospacer adjacent motif (PAM), which is NGG for SpyCas9 and NNGRRT for SauCas9. Locating the PAM poises the complex to form a hybrid duplex between the "reverse" strand of the DNA and the 5'-terminal ~20 ribonucleotides of the sgRNA. If the DNA is complementary, an R-loop is formed, and the DNA is cut by RuvC- and HNH-like domains. Upon DNA clevage, *S. pyogenes* Cas9 is known to have extremely slow product release and may exhibit additional DNase activity.

Comparison of *S. pyogenes* and *S. aureus* Cas9 homologs

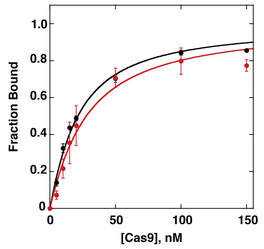
Image adapted from Nishimasu *et al.*, 2015.

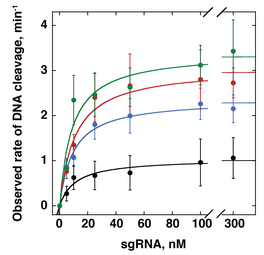


***S. pyogenes* and *S. aureus* recognize different protospacer adjacent motiffs (PAM) but TGGAAT satisfies the requirements for both proteins.**

Results

S. aureus and *S. pyogenes* Cas9 bind respective sgRNA with comparable affinities and form active RNPs





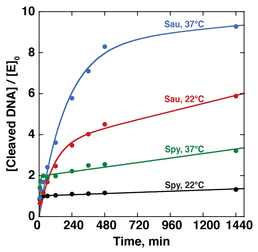
Unlabeled Cas9 was titrated in the presence of Cy5-labeled sgRNAs and fraction bound was calculated from changes in fluorescence anisotropy.

Cas9	K_D, nM
S. pyogenes	21 ± 1
S. aureus	30 ± 10

Observed rates of DNA cleavage in the presence of various concentrations of sgRNA

	SpyCas9		SauCas9	
	PAM strand	non-PAM strand	PAM strand	non-PAM strand
k_{max}, min^{-1}	2.3 ± 0.4	3.0 ± 0.6	1.0 ± 0.5	3.3 ± 0.5
$K_{1/2}$ (nM)	8 ± 1	9 ± 1	10 ± 1	8 ± 2

S. aureus Cas9 turns over faster than *S. pyogenes* Cas9

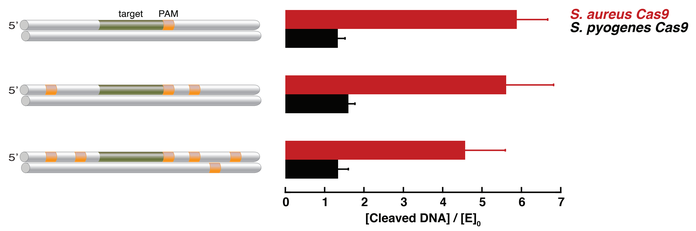


DNA cleavage at 22 °C or 37 °C was measured with SpyCas9 or SauCas9

	SpyCas9			SauCas9		
	Amp	k_{exp} (min^{-1})	k_{lin} (min^{-1})	Amp	k_{exp} (min^{-1})	k_{lin} (min^{-1})
22 °C	1.0 ± 0.1	ND	$2.4x10^{-4}$ ± $9x10^{-5}$	3.6 ± 0.3	0.010 ± 0.001	$1.6x10^{-3}$ ± $5x10^{-4}$
37 °C	2.0	ND	$9.2x10^{-4}$	8.4	0.005	$6.1x10^{-4}$

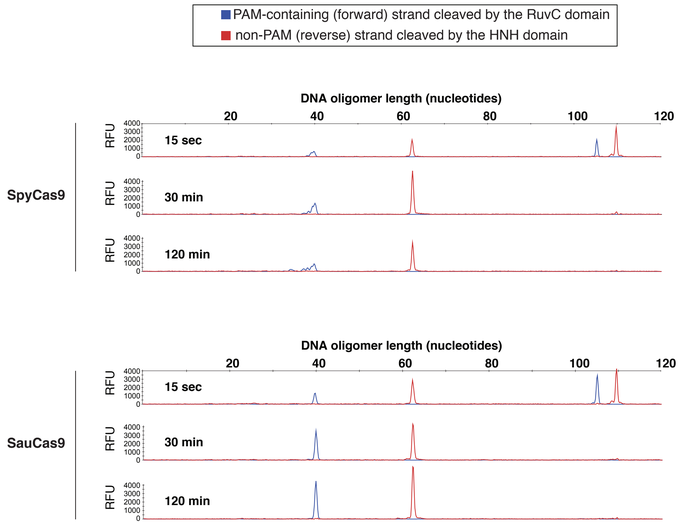
Decoy PAMs modestly inhibit the degree of DNA cleavage

DNA cleaved, *in vitro*, after 24 hrs., at 22 °C, normalized to the amount of Cas9 RNP in reaction



SauCas9 RuvC domain does not exhibit any detectable post-cleavage DNase activity observed in SpyCas9

Cas9 cleavage reactions separated via capillary electrophoresis



Summary

***S. aureus* and *S. pyogenes* Cas9 tighly bind their respective sgRNAs (K_D ~ 20 nM) and form active ribonucleoprotein complexes that cleave DNA at maximal rates (k_{max}) between 1 - 3 min^{-1}.**

***S. aureus* Cas9 turns over significantly faster than *S. pyogenes* Cas9. The effect was observed at 22°C and 37°C. Presence of "decoy" PAMs that are not adjacent to a target sequence modestly decreased the amount of DNA cleaved in 24 hours for *S. aureus* but not *S. pyogenes* Cas9.**

***S. pyogenes* Cas9 partially degrades the (forward) PAM-containing strand while *S. aureus* Cas9 does not have any detectable post-cleavage DNase activity.**

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