Orientation of Plasmid Origin of Replication (Ori) and its Impact on Inverted Terminal Repeat (ITR) Stability

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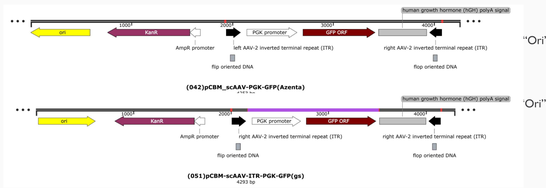
ABSTRACT

Adeno-associated virus 2 (AAV2). AAV2 ITRs are unique nucleotide sequences that support cross-packaging of cis-plasmids across all AAV natural or engineered serotypes. These GC-rich segments of DNA are ~145 nucleotides long, which consists of palindromic arms (A-A', B-B', and C-C') formed by first 125 nucleotides that fold back to form a T-shaped hairpin structure, which flanks the entire AAV genome and provides critical recognition sequences that are required for directing the AAV Rep and host cell proteins to replicate and encapsidate the viral DNA into pre-assembled AAV capsids. ITR modifications can affect gene transfer efficiency and transgene expression, and the B and C loops are found to be essential for vector genome replication. We sought to address the impact of the plasmid "Ori" orientation and its proximity to ITR on the stability of both single stranded (ss) and self complimentary (sc) AAV ITR plasmids. For this study, two scAAV plasmids, (042)pCBM_scAAV_PGK_GFP ("Ori" adjacent to ITR) and (051)pCBM_scAAV_ITR_PGK_GFP ("Ori" direction opposite the ITR, separated by the antibiotic resistance cassette), and two ssAAV plasmids (041)pCBM_AAV_CMV_GFP_IRES_FLuc ("Ori" adjacent to ITR) and (050)pCBM_AAV_ITR_CMV_GFP_IRES_FLuc ("Ori" direction opposite the ITR, separated by the antibiotic resistance cassette) were used (Fig.1a-1d). These plasmids were propagated in two *E.coli* strains (DH5**α** and Stbl3) under two different temperatures (30 & 37°C) and plasmid/ITR identity and integrity were confirmed by short read (250bp PE) Illumina MiSeq Next-Generation Sequencing. Read comparison to reference plasmid sequence map was done using an optimized bioinformatics pipeline.

RESULTS

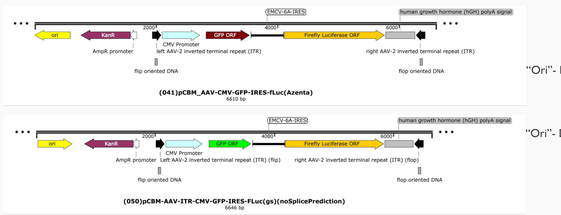
A. pCBM ITR self-complementary (sc) AAV plasmid (042 & 051)



"Ori"- Directed towards the proximal ITR

"Ori"- Directed opposite the proximal ITR

B. pCBM ITR single-stranded (ss) AAV plasmid (041 & 050)



"Ori"- Directed towards the proximal ITR

"Ori"- Directed opposite the proximal ITR

Figure 1. Schematic plasmid maps. The plasmids used in this study are shown above. A) scAAV or B) ssAAV maps showing the orientation of the Ori in relation to the ITRs

Clone	Relative Allele Frequency >/= 5%					
	Number of Variants	B loop deletion RAF %	C loop deletion RAF %	Complete ITR deletion RAF %	# SNP's	SNP RAF %
(042)pCBM_scAAV_ITR_PGK_GFP – Original	19	11.43	5.23	Nil	17	5.23 - 31.97
LB, 30°C, Stbl3 - colony 1	16	51.31	6.15	Nil	14	5.27-8.24
LB, 30°C, Stbl3 - colony 2	4	88.67	0	Nil	3	8.12-8.39
LB, 30°C, Stbl3 - colony 3	22	5.82	35.66	Nil	20	35.22- 48
LB, 37°C, Stbl3 - colony 1	6	7.02	0	Nil	5	5.04-5.15
LB, 37°C, Stbl3 - colony 2	0	0	0	Nil	0	0
LB, 37°C, Stbl3 - colony 3	17	6.25	0	Nil	16	5.12-9.06
TB, 30°C, Stbl3 - colony 1	22	10.3	16.34	Nil	20	12.42-65.2
TB, 30°C, Stbl3 - colony 2	25	59.21	5.34	Nil	23	5.34-56.77
TB, 30°C, Stbl3 - colony 3	34	11.69	6.46	Nil	32	5.02-18.82
TB, 37°C, Stbl3 - colony 1	15	8.21	0	Nil	14	5.04-8.21
TB, 37°C, Stbl3 - colony 2	16	6.89	0	Nil	15	5.26-7.87
TB, 37°C, Stbl3 - colony 3	21	0	11.57	Nil	20	10.16-79.85
LB, 37°C, DH5α - colony 1	8	51.69	0	Nil	7	6.58-85.68
LB, 37°C, DH5α - colony 2	8	51.48	0	Nil	7	6.61-86.09
LB, 37°C, DH5α - colony 3	21	16.12	5.78	Nil	19	5.35-9.14
TB, 37°C, DH5α - colony 1	23	18.6	5.03	Nil	21	5.03-10.41
TB, 37°C, DH5α - colony 2	4	14.2	26.75	Nil	2	5.14-6.28
TB, 37°C, DH5α - colony 3	15	20.41	15.84	Nil	13	5.03-39.81

Table 1. When the orientation of the "Ori" was adjacent to scAAV ITRs, significant deletions were detected in the proximal ITR. Both B loop, C loop and B-C loop deletions were observed in all clones except 1besides Single Nucleotide Polymorphisms (SNP's)in both ITR's.

Clone	Relative Allele Frequency >/= 5%					
	Number of Variants	B loop deletion RAF %	C loop deletion RAF %	Complete ITR deletion RAF %	# SNP's	SNP RAF %
LB, 37C, DH5α - colony 3a	22	14.6	7.7	Nil	20	7.16-19.11
LB, 37C, DH5α - colony 3b	4	0	90.03	Nil	3	6.26-6.34
LB, 37C, DH5α - colony 3c	21	13.3	5.39	Nil	19	5.09-13.3
LB, 37C, DH5α - colony 3d	21	13.2	8.42	Nil	19	7.55-21.64
LB, 37C, DH5α - colony 3e	18	41.66	0	Nil	17	7.87-9.64
LB, 37C, Stbl3 - colony 2a	4	0	93.55	Nil	3	5.37-5.44
LB, 37C, Stbl3 – colony 2b	4	0	92.96	Nil	3	5.88-5.98
LB, 37C, Stbl3 - colony 2c	7	0	91.48	Nil	6	5.37-89.85
LB, 37C, Stbl3 - colony 2d	7	89.45	0	Nil	6	6.92-89.92
LB, 37C, Stbl3 - colony 2e	2	6.33	0	Nil	1	5.32

Table 2. Repropagation of clones selected with lower mutations (corresponding highlights from Table1.) did not maintain ITR integrity under same conditions.

Clone	Relative Allele Frequency >5%					
	Number of Variants	B loop deletion RAF %	C loop deletion RAF %	Complete ITR deletion RAF %	# SNP's	SNP RAF %
(041)pCBM_AAV_ITR_CMV_GFP_IRES_fluc – Original	1	8.25	0	Nil	0	0
LB, 37°C, DH5α - colony 1	25	50.53	11.42	Nil	23	7.49 - 40.79
LB, 37°C, DH5α - colony 2	25	49.1	12.34	Nil	23	6.66 -38.28
LB, 37°C, DH5α - colony 3	26	43.83	14.4	5.6	24	8.28 -17.45
TB, 37°C, DH5α - colony 1	25	34	16.89	Nil	23	10.47-18.02
TB, 37°C, DH5α - colony 2	26	31.04	19.48	5.02	24	10.27-19.91
TB, 37°C, DH5α - colony 3	26	41.17	14.35	5.02	24	8.05 - 21.93
LB, 37°C, Stbl3- colony 1	26	13.73	7.19	5.09	24	6.31 -20.13
LB, 37°C, Stbl3- colony 2	25	17.11	7.74	Nil	24	6.73 -18.57
LB, 37°C, Stbl3- colony 3	25	13.45	11.19	Nil	23	5.47 -16.41
TB, 37°C, Stbl3- colony 1	25	39.48	6.19	Nil	23	5.93 -14.26
TB, 37°C, Stbl3- colony 2	25	25.97	13.69	Nil	23	8.96 14.04
TB, 37°C, Stbl3- colony 3	25	21.52	6.93	Nil	23	6.21 -16.72

Table 3. For ssAAV plasmids, both B loop, C loop and B-C loop deletions were observed in all clones. 1 of 6 clones in Stbl3 and 3 of 6 clones in DH5**α** had complete proximal ITR deletions in addition to SNP's identified in both ITR's.

Clone	Relative Allele Frequency >/= 5%		
	Number of Variants	# SNP's	SNP RAF %
(051)pCBM_scAAV_ITR_PGK_GFP - Original	0	0	0
Stbl3_med/large_37°C – colony 1	0	0	0
Stbl3_med/large_37°C – colony 2	0	0	0
Stbl3_med/large_37°C – colony 3	0	0	0
Stbl3_med/large_37°C – colony 4	0	0	0
Stbl3_med/large_37°C – colony 5	0	0	0
Stbl3_med/large_30°C – colony 1	0	0	0
Stbl3_med/large_30°C – colony 2	1	1	0
Stbl3_med/large_30°C – colony 3	0	0	0
Stbl3_med/large_30°C – colony 4	0	0	0
Stbl3_med/large_30°C – colony 5	1	1	5.5
Stbl3_small_37°C – colony 1	0	0	0
Stbl3_small_37°C – colony 2	0	0	0
Stbl3_small_37°C – colony 3	0	0	0
Stbl3_small_37°C – colony 4	1	1	5
Stbl3_small_37°C – colony 5	0	0	5.1
Stbl3_small_30°C – colony 1	0	0	5.0-6.0
Stbl3_small_30°C – colony 2	0	0	0
Stbl3_small_30°C – colony 3	6	6	0
Stbl3_small_30°C – colony 4	0	0	0
Stbl3_small_30°C – colony 5	0	0	0
DH5α_med/large_37°C – colony 1	0	0	0
DH5α_med/large_37°C – colony 2	18	18	5.3-7.3
DH5α_med/large_37°C – colony 3	13	13	5.1-8.1
DH5α_med/large_37°C – colony 4	3	3	5.7-6.1
DH5α_med/large_37°C – colony 5	10	10	5.0-6.0
DH5α_med/large_30°C – colony 1	2	2	5.0-5.4
DH5α_med/large_30°C – colony 2	0	0	0
DH5α_med/large_30°C – colony 3	4	4	5.1-5.3
DH5α_med/large_30°C – colony 4	16	16	5.1-7.9
DH5α_med/large_30°C – colony 5	0	0	0
DH5α_small_37°C – colony 1	16	16	5.2-6.5
DH5α_small_37°C – colony 2	4	4	5.0-5.4
DH5α_small_37°C – colony 3	1	1	6
DH5α_small_37°C – colony 4	0	0	0
DH5α_small_37°C – colony 5	6	6	5.2-5.4
DH5α_small_30°C – colony 1	0	0	0
DH5α_small_30°C – colony 2	1	1	5
DH5α_small_30°C – colony 3	17	17	5.6-8.7
DH5α_small_30°C – colony 4	12	12	5.0-6.6
DH5α_small_30°C – colony 5	0	0	0

Clone	Relative Allele Frequency >/= 5%		
	Number of Variants	# SNP's	SNP RAF %
(050)pCBM-AAV-ITR-CMV-GFP-IRES-Fluc - Original	0	0	0
Stbl3_med/large_37°C – colony 1	0	0	6.2
Stbl3_med/large_37°C – colony 2	1	1	5.2-6.1
Stbl3_med/large_37°C – colony 3	4	4	5.1-6.3
Stbl3_med/large_37°C – colony 4	3	3	5.2-7.4
Stbl3_med/large_37°C – colony 5	7	7	0
Stbl3_med/large_30°C – colony 1	0	0	0
Stbl3_med/large_30°C – colony 2	0	0	0
Stbl3_med/large_30°C – colony 3	0	0	0
Stbl3_med/large_30°C – colony 4	0	0	0
Stbl3_med/large_30°C – colony 5	0	0	0
Stbl3_small_37°C – colony 1	0	0	0
Stbl3_small_37°C – colony 2	0	0	0
Stbl3_small_37°C – colony 3	0	0	0
Stbl3_small_37°C – colony 4	2	2	5.1-5.7
Stbl3_small_37°C – colony 5	0	0	0
Stbl3_small_30°C – colony 1	0	0	0
Stbl3_small_30°C – colony 2	1	1	5.3
Stbl3_small_30°C – colony 3	1	1	5.6
Stbl3_small_30°C – colony 4	3	3	5.1-5.7
Stbl3_small_30°C – colony 5	0	0	0
DH5α_med/large_37°C – colony 1	26	26	5.0-9.2
DH5α_med/large_37°C – colony 2	12	12	5.4-6.5
DH5α_med/large_37°C – colony 3	23	23	5.2-8.6
DH5α_med/large_37°C – colony 4	31	31	5.0-8.1
DH5α_med/large_37°C – colony 5	34	34	5.4-10.3
DH5α_med/large_30°C – colony 1	20	20	5.1-9.9
DH5α_med/large_30°C – colony 2	12	12	5.1-7.1
DH5α_med/large_30°C – colony 3	1	1	5.5
DH5α_med/large_30°C – colony 4	3	3	5.1
DH5α_med/large_30°C – colony 5	3	3	5.4-7.0
DH5α_small_37°C – colony 1	29	29	5.0-7.6
DH5α_small_37°C – colony 2	31	31	5.0-8.2
DH5α_small_37°C – colony 3	30	30	5.1-8.5
DH5α_small_37°C – colony 4	11	11	5.0-6.2
DH5α_small_37°C – colony 5	21	21	5.1-9.0
DH5α_small_30°C – colony 1	0	0	0
DH5α_small_30°C – colony 2	6	6	5.1-7.6
Stbl3_med/large_37°C – colony 1	7	7	5.0-6.4
Stbl3_med/large_37°C – colony 2	32	32	5.1-8.4
Stbl3_med/large_37°C – colony 3	2	2	5.3-5.5

Table 4. We examined the correlation, if any, the ITR mutation frequency based on colony size. For both the scAAV (Left) and ssAAV (right) plasmids, no B loop, C loop, B-C loop or complete ITR deletions were observed in any of the clones under the conditions tested.

Two different colony size

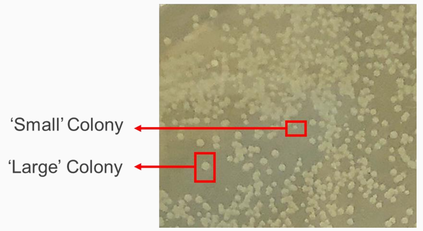


Figure 2. Colony Morphology of Bacteria. Representative example of colony size differences taken as representative examples to compare ITR stability across conditions.

CONCLUSION

- For ITR plasmids with the "Ori" orientation directed opposite to the proximal ITR and separated by the antibiotic resistance cassette yielded vastly superior ITR integrity in terms of retention of ITR structural elements (no loop deletions) and significantly low SNP allele frequency in the ITR region.irrespective of the two plasmid types studied.
- The orientation of the "Ori" did not have any impact on distal ITR integrity as there were no deletions observed in this ITR regardless of the Ori orientation, plasmid type, *E.coli* strain, or growth conditions.
- Ori orientation had equal effects on both sc and ssAAV ITRs.