Fig. 1

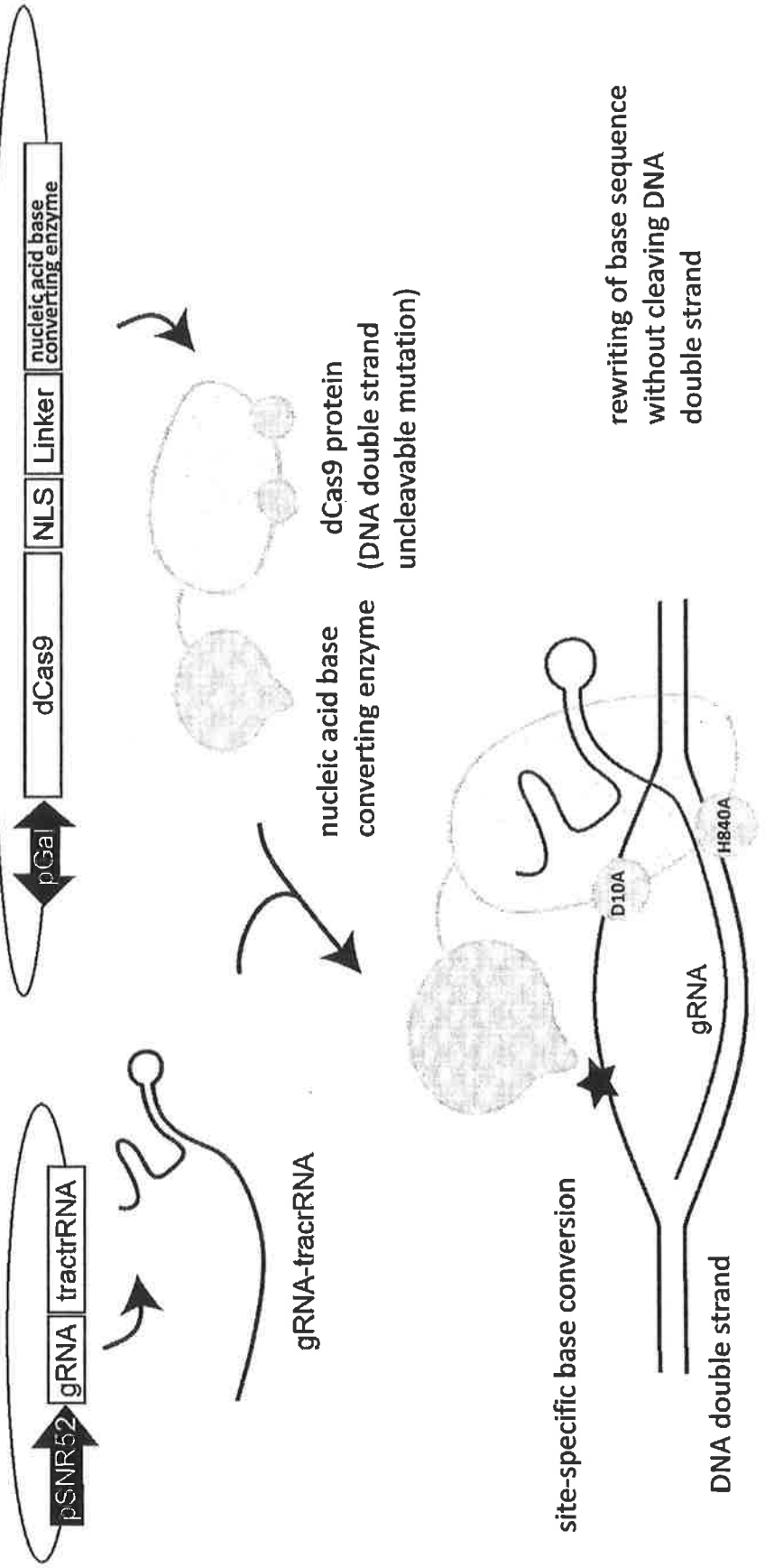

pSNR52
gRNA
tractrRNA
gRNA-tracrRNA
pGal
dCas9
NLS
Linker
nucleic acid base converting enzyme
nucleic acid base converting enzyme
dCas9 protein (DNA double strand uncleavable mutation)
rewriting of base sequence without cleaving DNA double strand
site-specific base conversion
D10A
H840A
gRNA
DNA double strand
target sequence (12～20 b)

Fig. 2

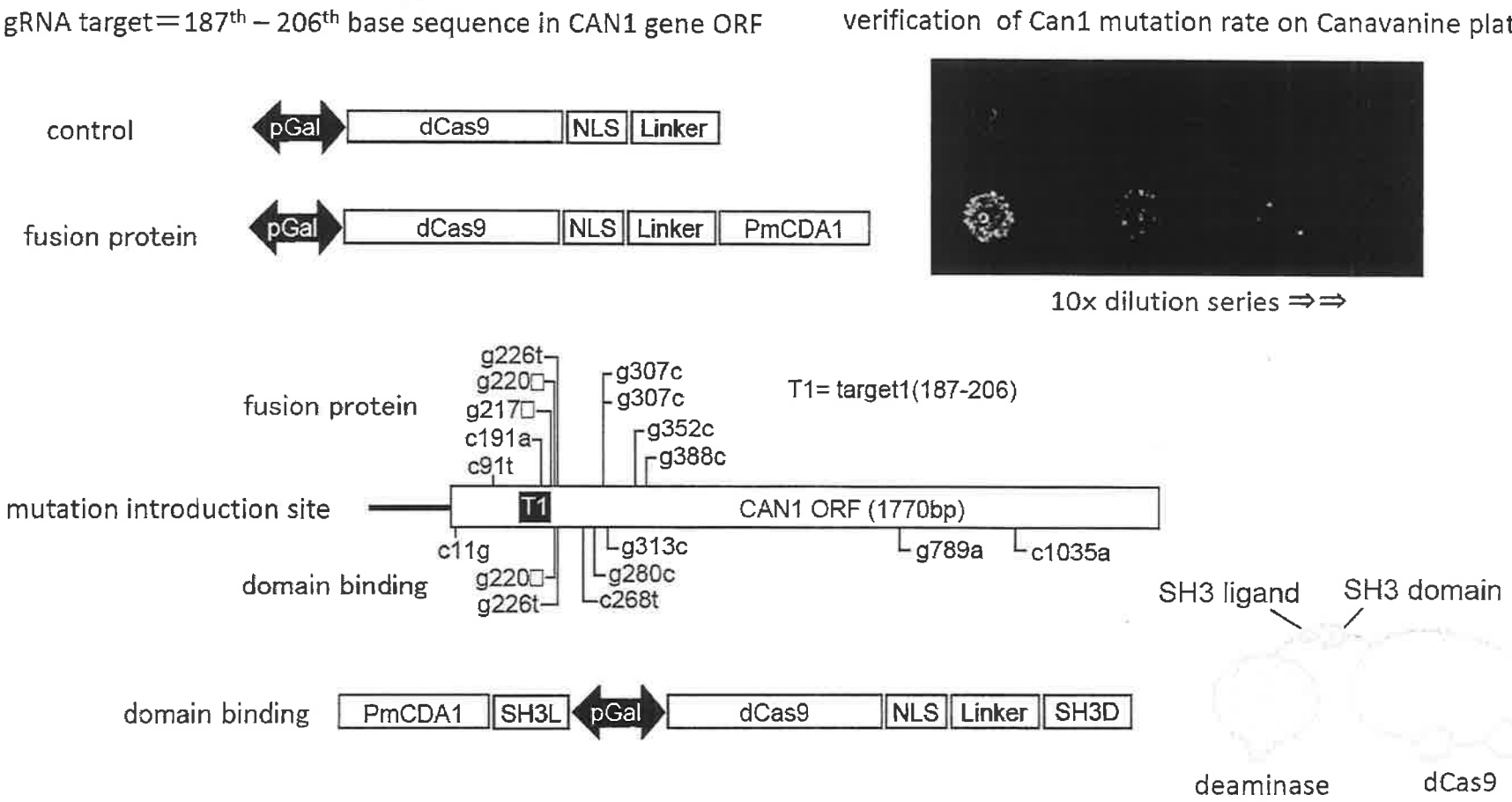

gRNA target＝187th – 206th base sequence in CAN1 gene ORF
verification of Can1 mutation rate on Canavanine plate
control
pGal
dCas9
NLS
Linker
fusion protein
pGal
dCas9
NLS
Linker
PmCDA1
10x dilution series ⇒⇒
fusion protein
g226t
g220□
g217□
c191a
c91t
g307c
g307c
g352c
g388c
T1= target1(187-206)
mutation introduction site
T1
CAN1 ORF (1770bp)
c11g
g220□
g226t
g313c
g280c
c268t
g789a
c1035a
domain binding
SH3 ligand
SH3 domain
domain binding
PmCDA1
SH3L
pGal
dCas9
NLS
Linker
SH3D
deaminase
dCas9

Fig. 3

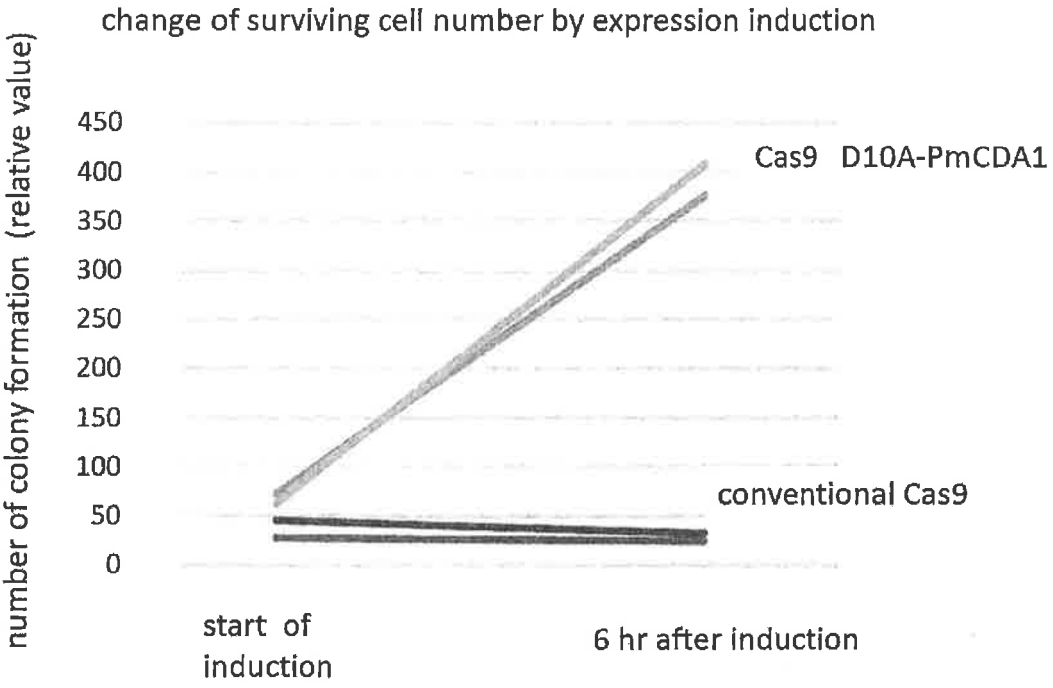

change of surviving cell number by expression induction
number of colony formation (relative value)
450
400
350
300
250
200
150
100
50
0
Cas9 D10A-PmCDA1
conventional Cas9
start of induction
6 hr after induction

Fig. 4

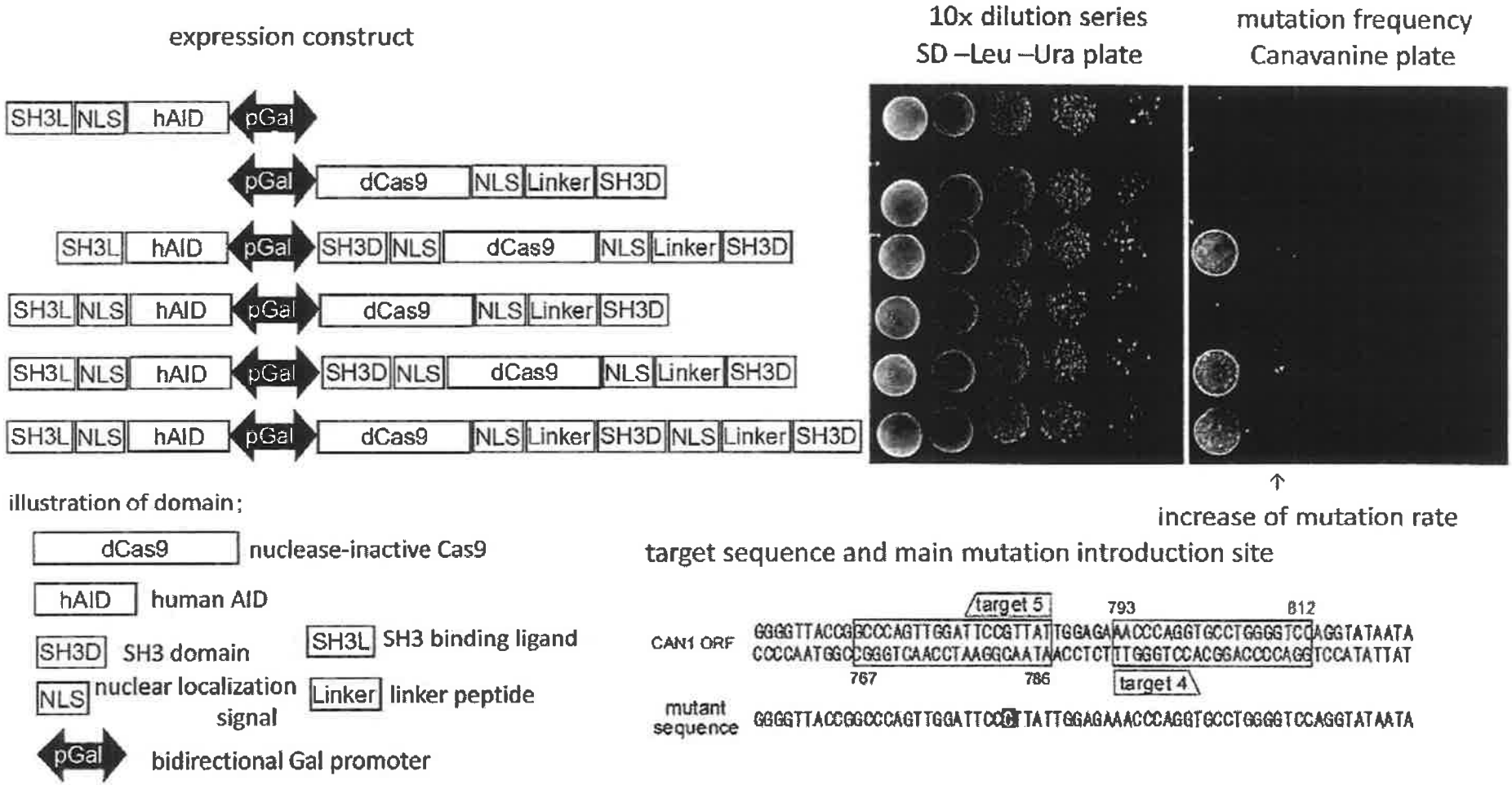

expression construct
10x dilution series
SD –Leu –Ura plate
mutation frequency
Canavanine plate
SH3L NLS hAID pGal
pGal dCas9 NLS Linker SH3D
SH3L hAID pGal SH3D NLS dCas9 NLS Linker SH3D
SH3L NLS hAID pGal dCas9 NLS Linker SH3D
SH3L NLS hAID pGal SH3D NLS dCas9 NLS Linker SH3D
SH3L NLS hAID pGal dCas9 NLS Linker SH3D NLS Linker SH3D
↑
increase of mutation rate
illustration of domain;
dCas9 nuclease-inactive Cas9
hAID human AID
SH3D SH3 domain
SH3L SH3 binding ligand
NLS nuclear localization signal
Linker linker peptide
pGal bidirectional Gal promoter
target sequence and main mutation introduction site
target 5
target 4
793
812
767
786
CAN1 ORF
GGGGTTACCGGCCCAGTTGGATTCCGTTATTGGAGAAACCCAGGTGCCTGGGGTCCAGGTATAATA
CCCCAATGGCCGGGTCAACCTAAGGCAATAACCTCTTTGGGTCCACGGACCCCAGGTCCATATTAT
mutant sequence
GGGGTTACCGGCCCAGTTGGATTCCGTTATTGGAGAAACCCAGGTGCCTGGGGTCCAGGTATAATA

Fig. 5

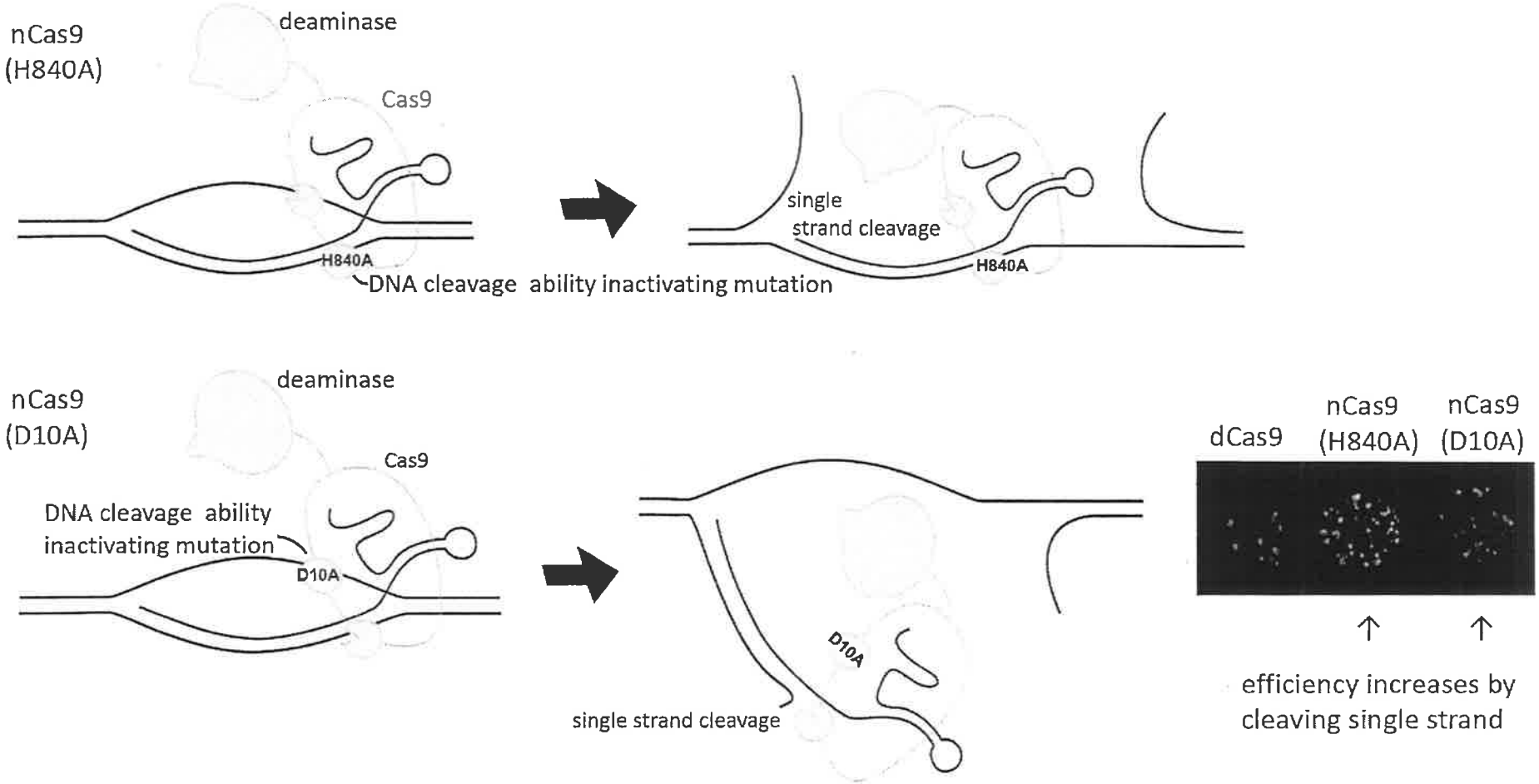
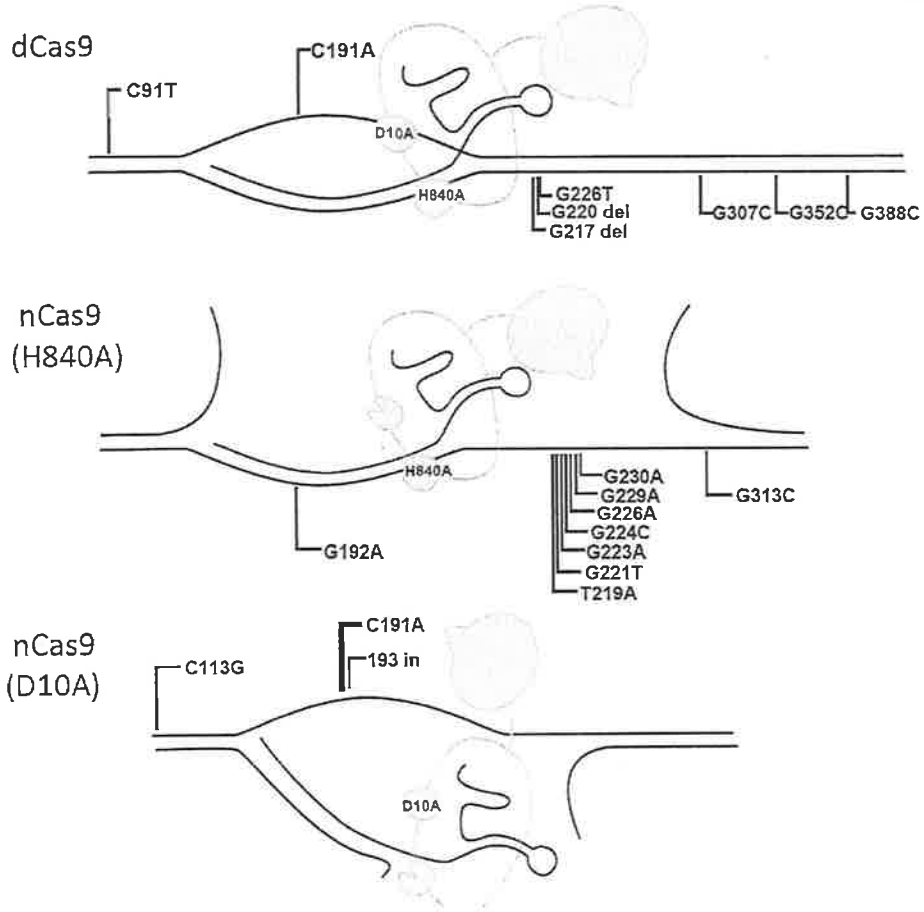

nCas9
(H840A)
deaminase
Cas9
H840A
DNA cleavage ability inactivating mutation
single
strand cleavage
H840A
nCas9
(D10A)
deaminase
Cas9
DNA cleavage ability
inactivating mutation
D10A
single strand cleavage
D10A
dCas9
nCas9
(H840A)
nCas9
(D10A)
efficiency increases by
cleaving single strand

Fig. 6

Change of mutation introduction pattern



target sequence

187 206

AAATGGCGAGGATACGTTCTCTATGGAGGATGGCATAGGT

TTTACCGCTCCTATGCAAGAGATACCTCCTACCGTATCCA

target 1

1024-T1					
1118-9	C191A				
1118-10	G226T				
1118-11	C091T				
1130-9	G226T				
1130-10	G307C				
1130-11	G352C				
1130-13	G217del				
1130-14	G307C				
1130-15	G388C				
1130-16	G220del				

1043-T1					
0110-1	G223C	G313C			
0110-2	G223A	G226A			
0110-3	T219A	G221T	G224C		
0110-4	C191T	G223A	G226AA	G229A	G230A
0111-1	G313C				
0111-2	C191T	C011G			
0111-5	G215C	G223C	G229T	G247A	
0111-6	G257C				

1042-T1		
0110-6	C191A	
0110-7	in 194	
0110-8	C113G	C191A
0110-10	C191A	
0110-11	C191A	
0110-12	C191A	
0110-13	In 193	
0111-7	del 188-209	
0111-8	In 192	
0111-9	del 188-209	
0111-10	C191T	
0111-11	C191A	

Fig. 7

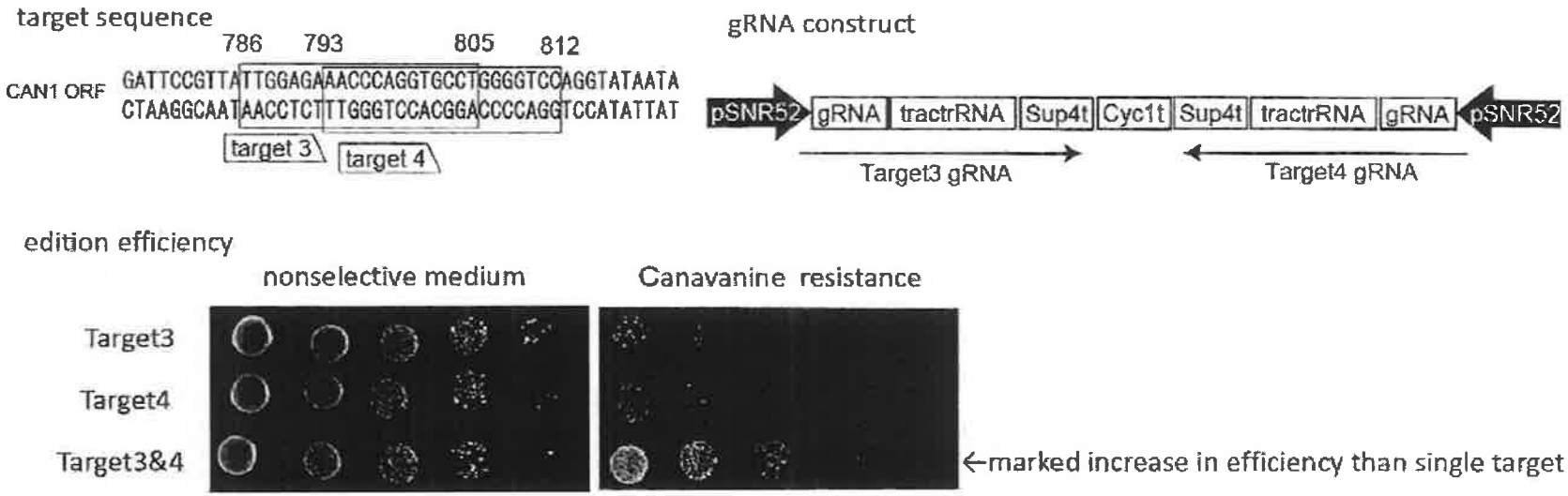
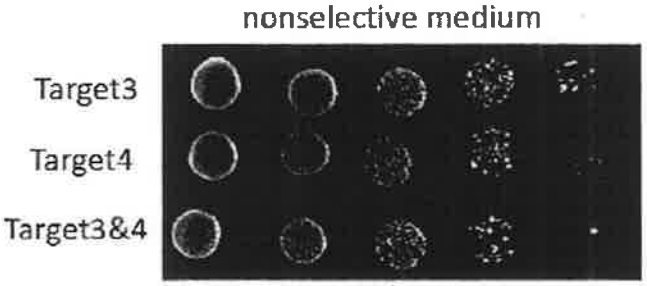

target sequence
786
793
805
812
CAN1 ORF
GATTCCGTTATTGGAGAAACCCAGGTGCCTGGGGTCCAGGTATAATA
CTAAGGCAATAACCTCTTTGGGTCCACGGACCCCAGGTCCATATTAT
target 3
target 4
gRNA construct
pSNR52
gRNA
tractrRNA
Sup4t
Cyc1t
Sup4t
tractrRNA
gRNA
pSNR52
Target3 gRNA
Target4 gRNA
edition efficiency
nonselective medium
Canavanine resistance
Target3
Target4
Target3&4
←marked increase in efficiency than single target

Fig. 8



Clone No.	Mutation	Amino acid	Canavanine resistance
0114-3	CC795TT	P266S	
0114-5	CC795AAT	Frame	CanR
0114-7	CC795TT	P266S	
0114-9	CC796TT	P266L	CanR
0114-11	CC795AT	P266S	
0114-12	CC795AAT	Frame	CanR
0115-12	CC796TT	P266L	CanR
0127-5	CC795AT	P266S	
0128-8	CC796TT	P266L	CanR
0128-14	CC795TT	P266S	

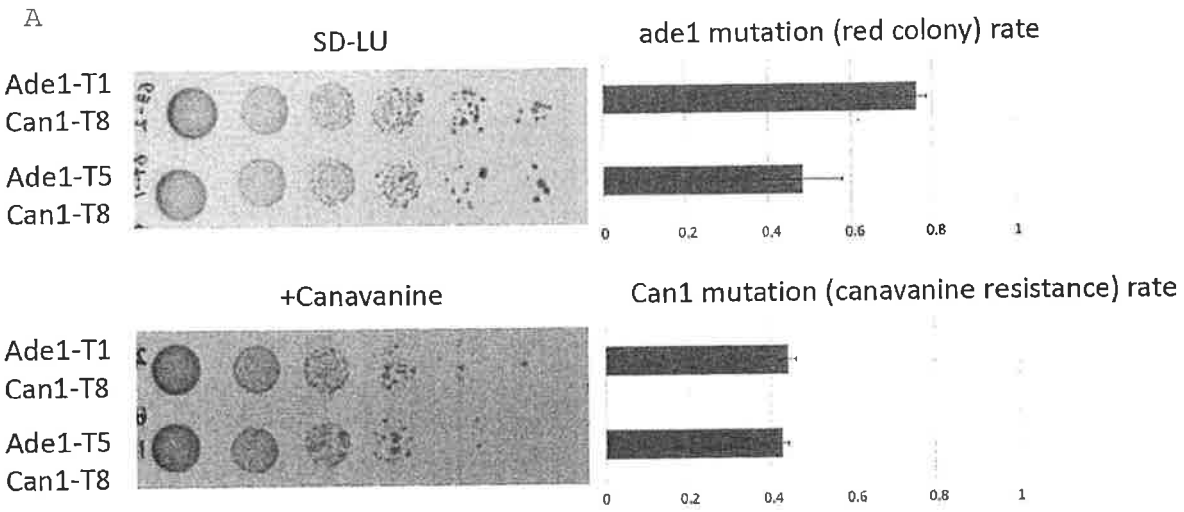
mutation introduction confirmed in all 10 strains randomly taken from nonselective medium

highly efficient gene manipulation is possible without using selection marker

Fig. 9

Ade1
Can1
3
767
TAACG ATA ATG TCA ATT ACG AAG ACT GAA CTG GAC GGT AT
GGG GTT ACC GGC CCA GTT GGA TTC CGT TAT TGG AGA AAC C
M S I T K T E L D G I
G V T G P V G F R Y W R N
Red/ White
CanR
R R TAACG ATA ATG TCA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CCT TAT TGG AGA AAC C
R R TAACG ATA ATG TCA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CCT TAT TGG AGA AAC C
R R TAACG ATA ATG TCA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CCT TAT TGG AGA AAC C
R S TAACG ATA ATG TCA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CAT TAT TGG AGA AAC C
W R TAACG ATA ATG TTA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GAA TTC CCT TAT TGG AGA AAC C
W S TAACG ATA ATG TTA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CCT TAT TGG AGA AAC C
W S TAACG ATA ATG TTA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CAT TAT TGG AGA AAC C
W R TAACG ATA ATG TTA ATT ACG AAG ACT GAA CTG GAC GGT AT GGG GTT ACC GGC CCA GTT GGA TTC CCT TAT TGG AGA AAC C

Fig. 10


A
SD-LU
ade1 mutation (red colony) rate
Ade1-T1
Can1-T8
Ade1-T5
Can1-T8
0
0.2
0.4
0.6
0.8
1
+Canavanine
Can1 mutation (canavanine resistance) rate
Ade1-T1
Can1-T8
Ade1-T5
Can1-T8
0
0.2
0.4
0.6
0.8
1

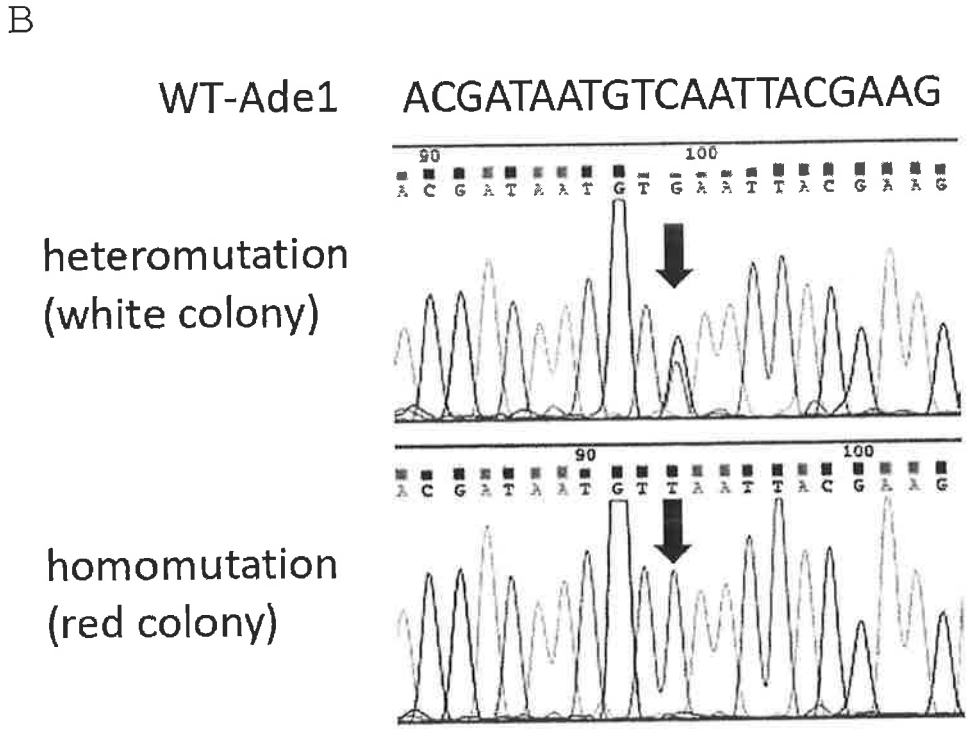
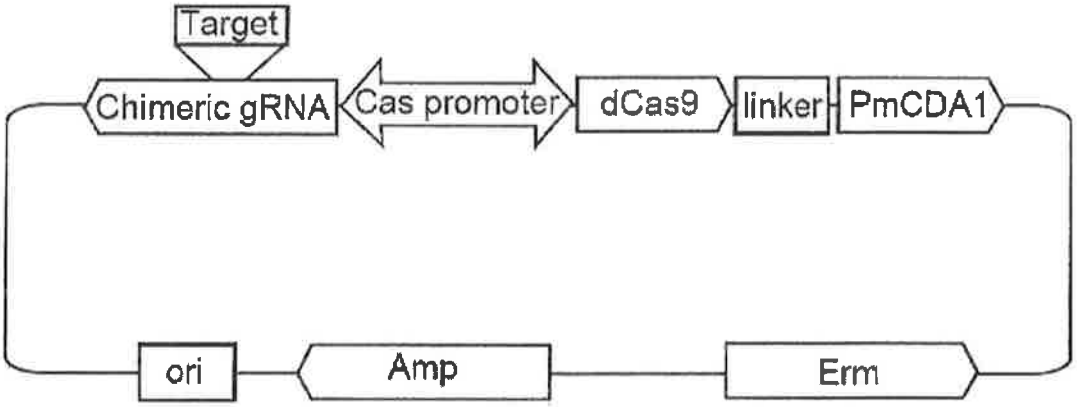

B
WT-Ade1
ACGATAATGTCAATTACGAAG
heteromutation
(white colony)
homomutation
(red colony)
90
100

Fig. 11

A



B

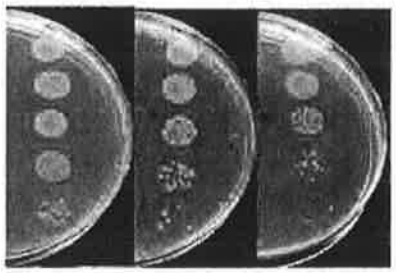
galK TCAATGGGCTAACTACGTTC-3′
1 TCAATGGGCTAACTACGTTC
2 TTAATGGGCTAACTACGTTC
3 TTAATGGGCTAACTACGTTC

C

rpoB GCTGTCTCAGTTTATGGACC-3′
[CGACAGAGTCAAATACCTGG-5′]

none[1 GCTGTCTCAGTTTATGGACC
2 GCTGTCTCAGTTTATGGACC
Rif25[1 GCTGTCTCAGTTTATGAACC
2 GCTGTCTCAGTTTATGAACC
Rif50[1 GCTGTCTCAGTTTATAAACC
2 GCTGTCTCAGTTTATGAACC
[CGACAGAGTCAAATACTTGG-5′]

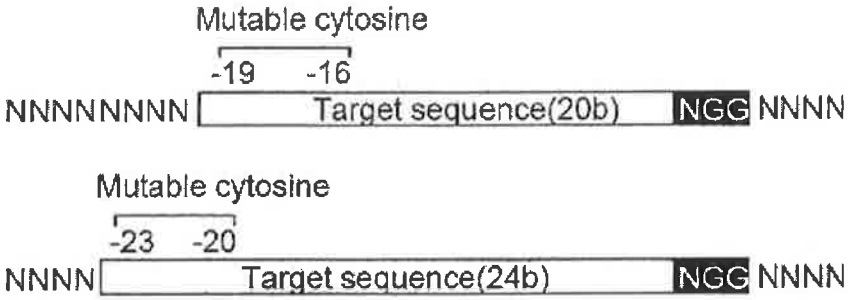
DH5α



none Rif 25 (ug/ml) Rif 50 (ug/ml)

Fig. 12

A


Mutable cytosine
-19
-16
NNNNNNNN
Target sequence(20b)
NGG
NNNN
Mutable cytosine
-23
-20
NNNN
Target sequence(24b)
NGG
NNNN

B

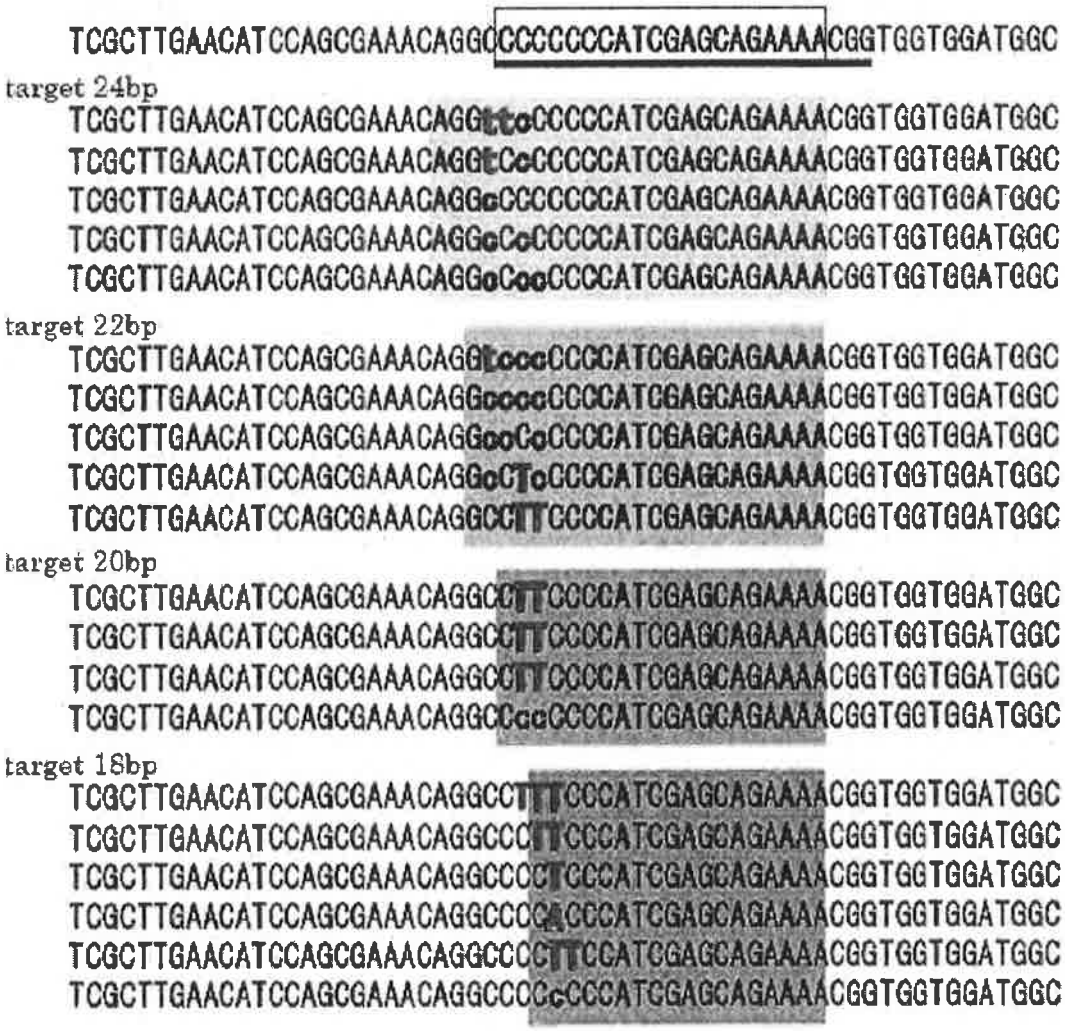

TCGCTTGAACATCCAGCGAAACAGGCCCCCCCCATCGAGCAGAAAACGGTGGTGGATGGC
target 24bp
target 22bp
target 20bp
target 18bp

Fig. 13

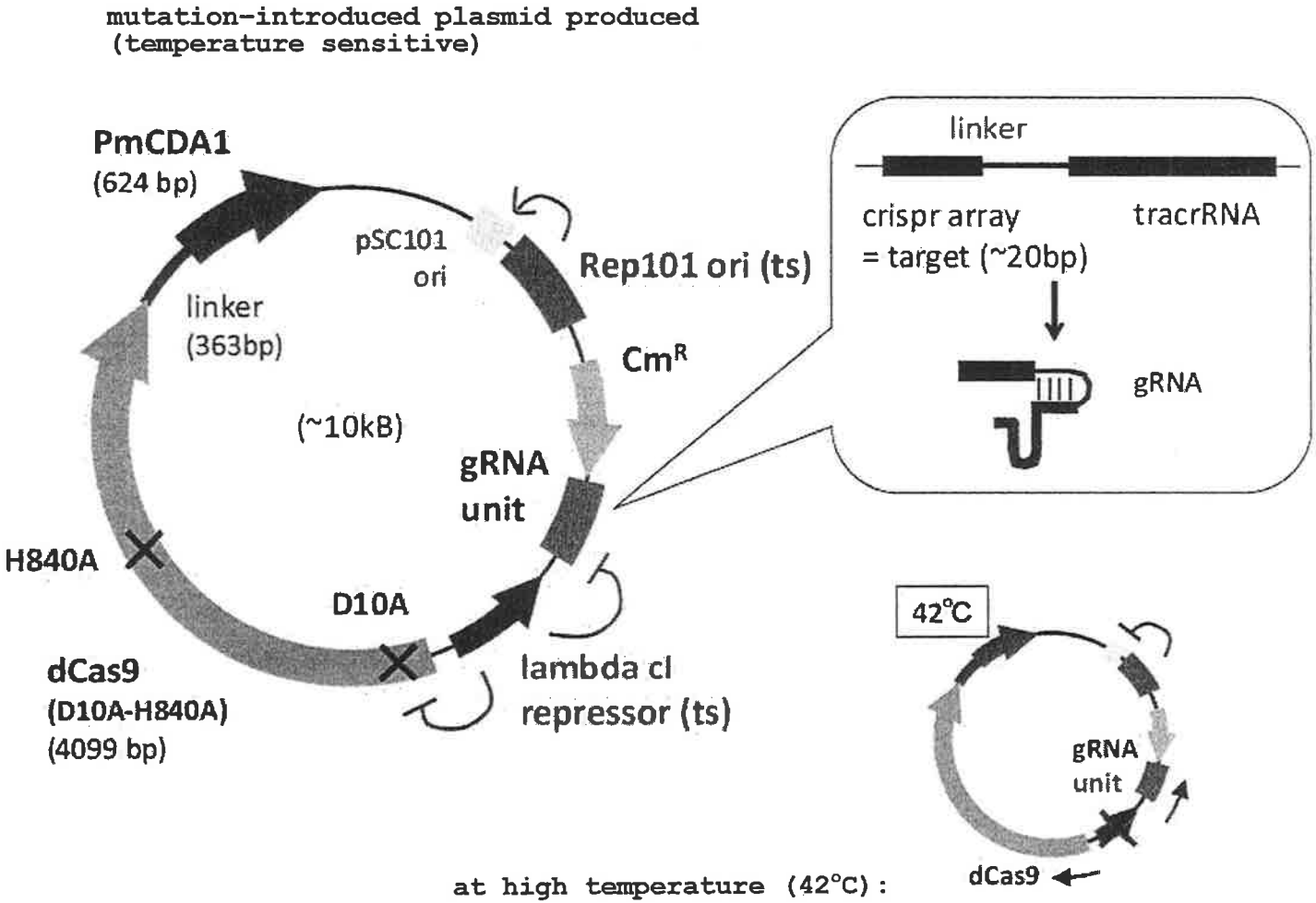

mutation-introduced plasmid produced
(temperature sensitive)
PmCDA1
(624 bp)
pSC101
ori
Rep101 ori (ts)
linker
(363bp)
Cm^R
(~10kB)
gRNA
unit
H840A
D10A
dCas9
(D10A-H840A)
(4099 bp)
lambda cI
repressor (ts)
linker
crispr array
= target (~20bp)
tracrRNA
gRNA
42°C
gRNA
unit
dCas9
at high temperature (42°C):
• dCas9-PmCDA1 expression • gRNA transcription are induced
• replication of plasmid ceases

Fig. 14

mutation introduction method (temperature sensitive plasmid)

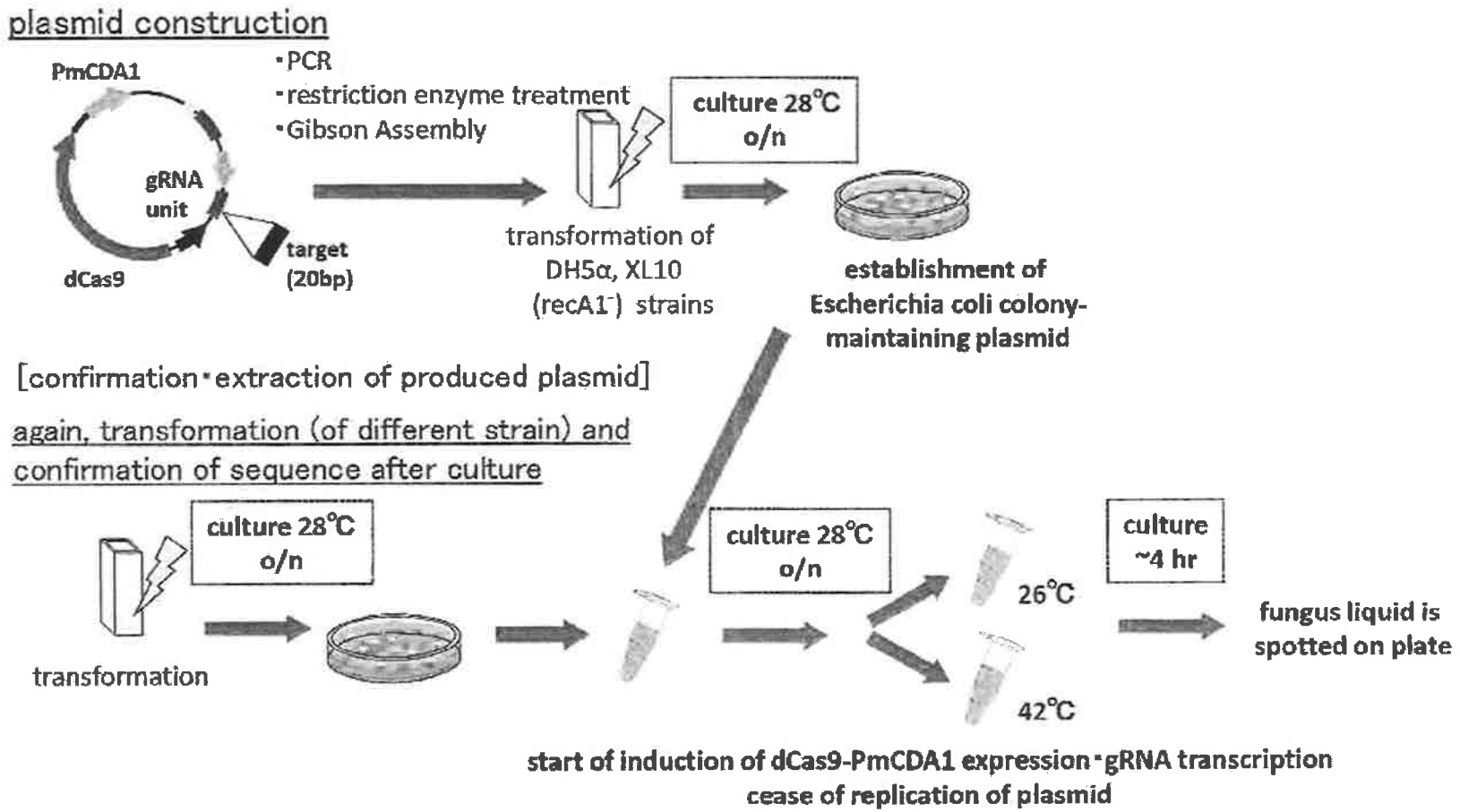
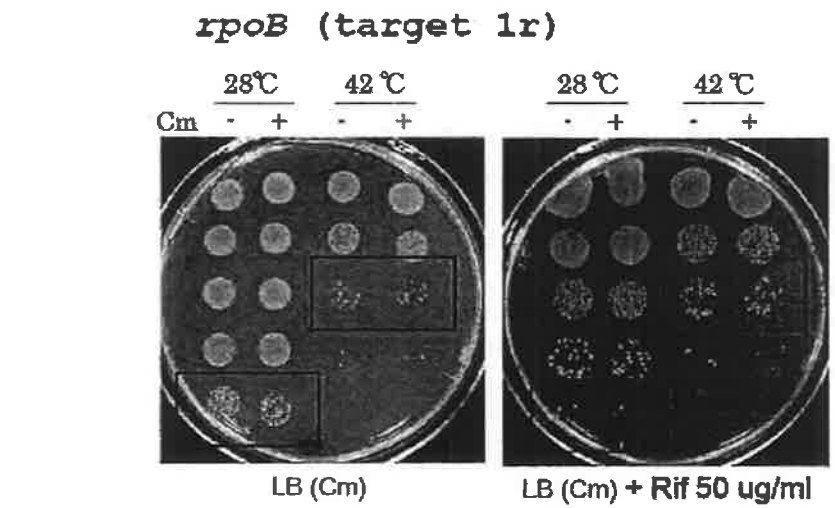
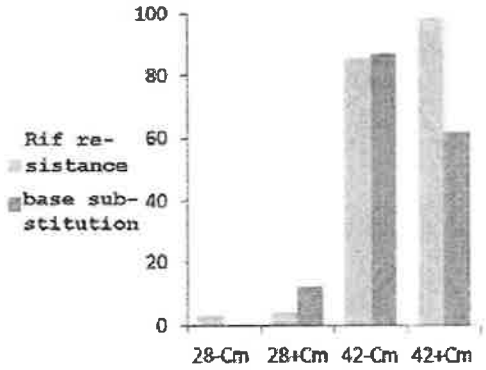


Fig. 15





rate (%) of Rifampicin resistance	4.4	3.0	86	>95
rete (%) of colony base substitution on LB plate	0	13	83	63

rpoB1r GCTGTCTCAGTTTATGGACC-3'

colony1 GCTGTCTCAGTTTATGAACC
colony2 GCTGTCTCAGTTTATGAACC (c→t)

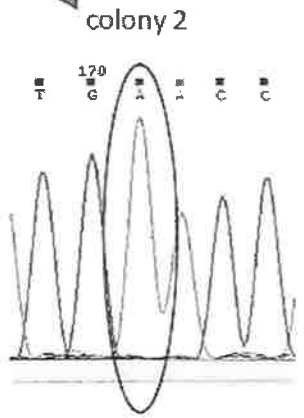
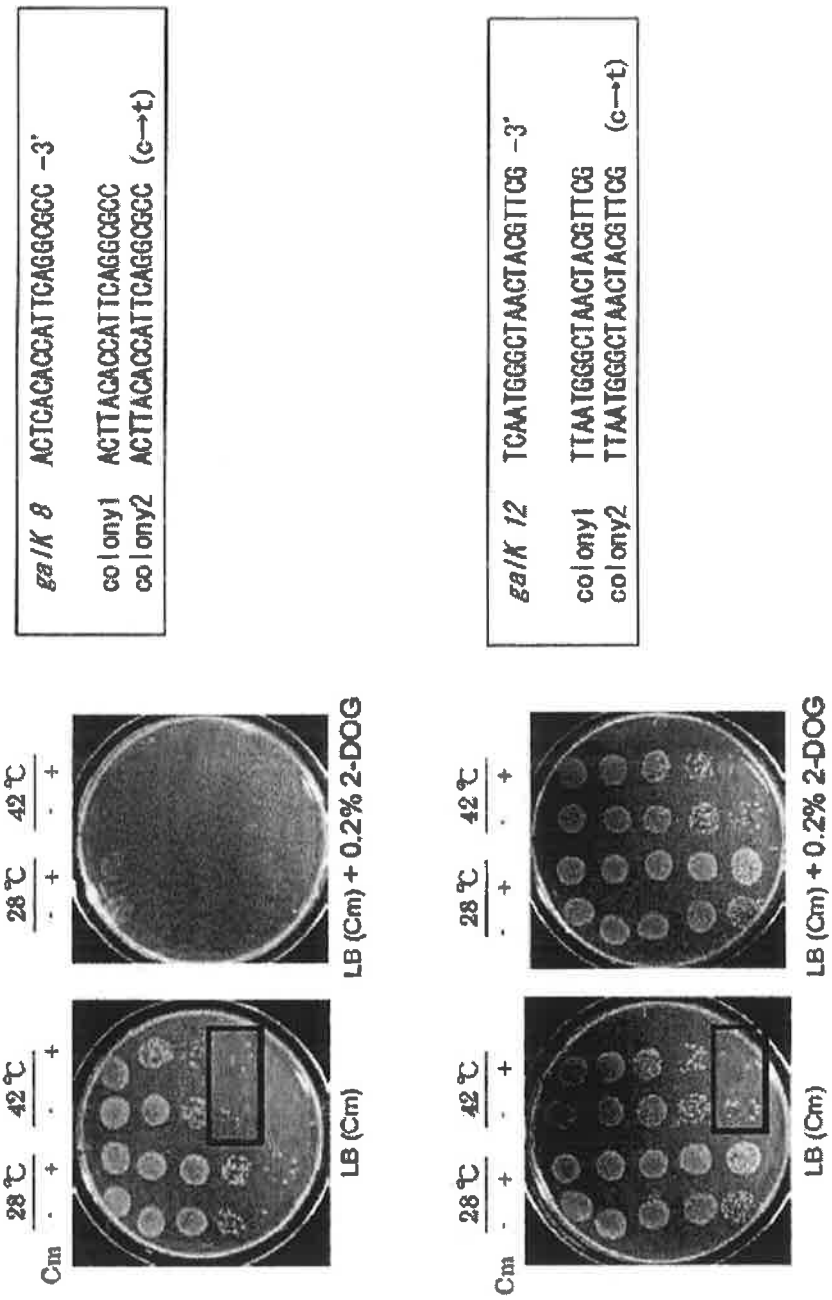


Fig. 16

galK (target 8, target 12)