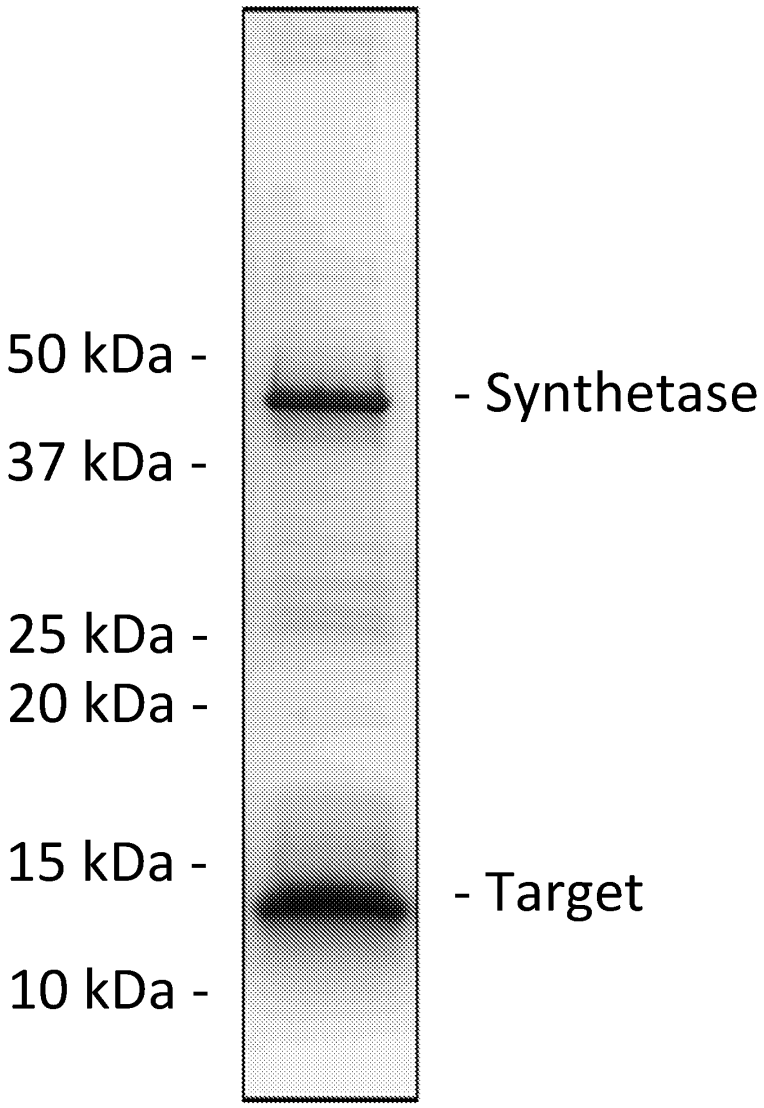



*FIG. 1*

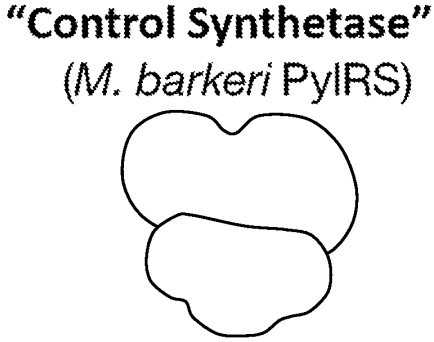



FIG. 2

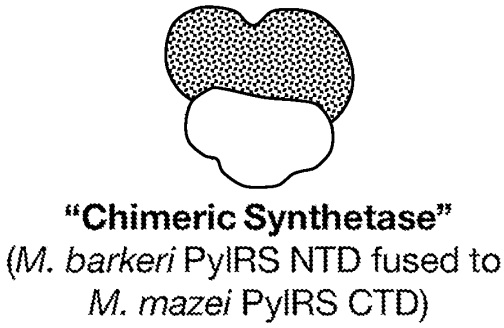



FIG. 3

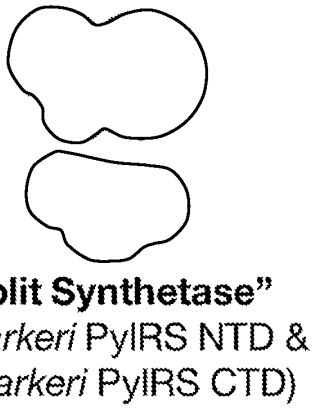



FIG. 4

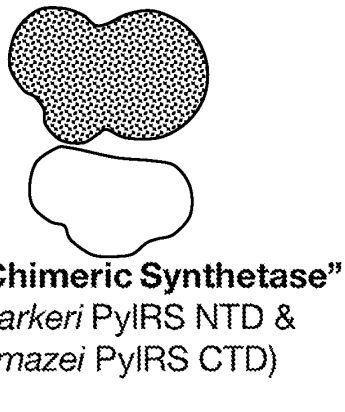



FIG. 5

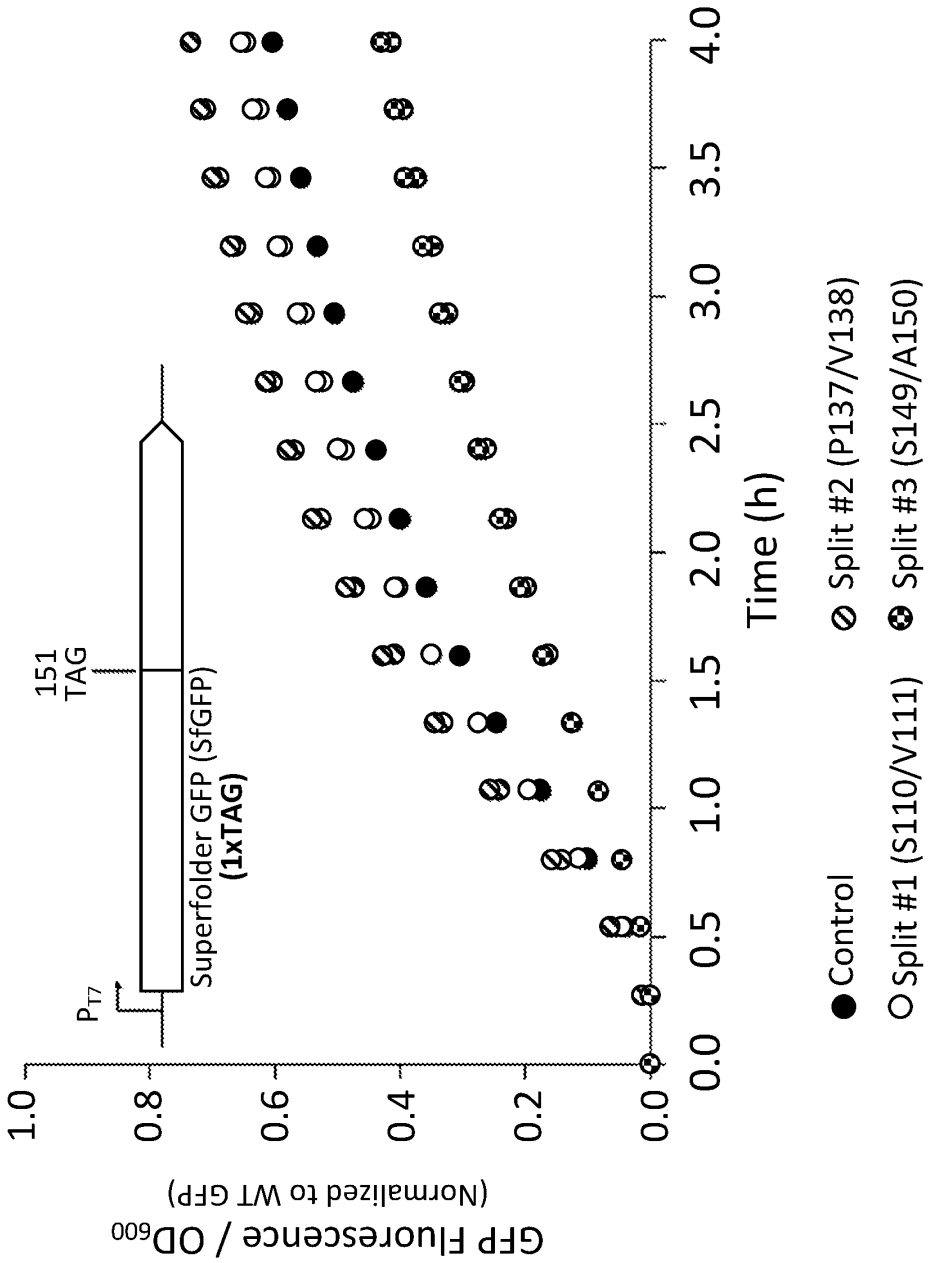

GFP Fluorescence / OD600
(Normalized to WT GFP)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Time (h)
PT7
Superfolder GFP (SfGFP)
(1xTAG)
151
TAG
Control
Split #1 (S110/V111)
Split #2 (P137/V138)
Split #3 (S149/A150)

*FIG. 6*

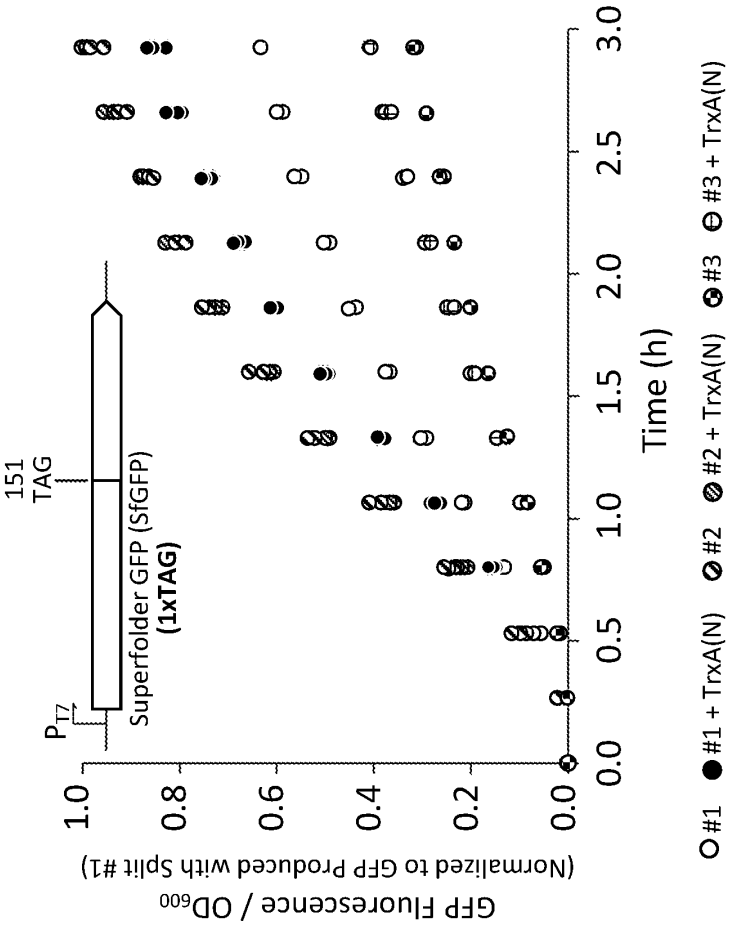

GFP Fluorescence / OD600
(Normalized to GFP Produced with Split #1)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Time (h)
PTZ
Superfolder GFP (SfGFP)
(1xTAG)
151
TAG
#1
#1 + TrxA(N)
#2
#2 + TrxA(N)
#3
#3 + TrxA(N)

*FIG. 8*

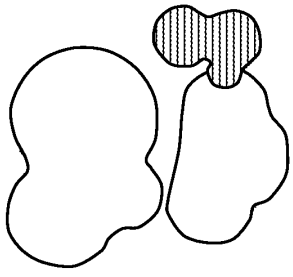

"Split Synthetase
with Fusion Partners"

*FIG. 7*

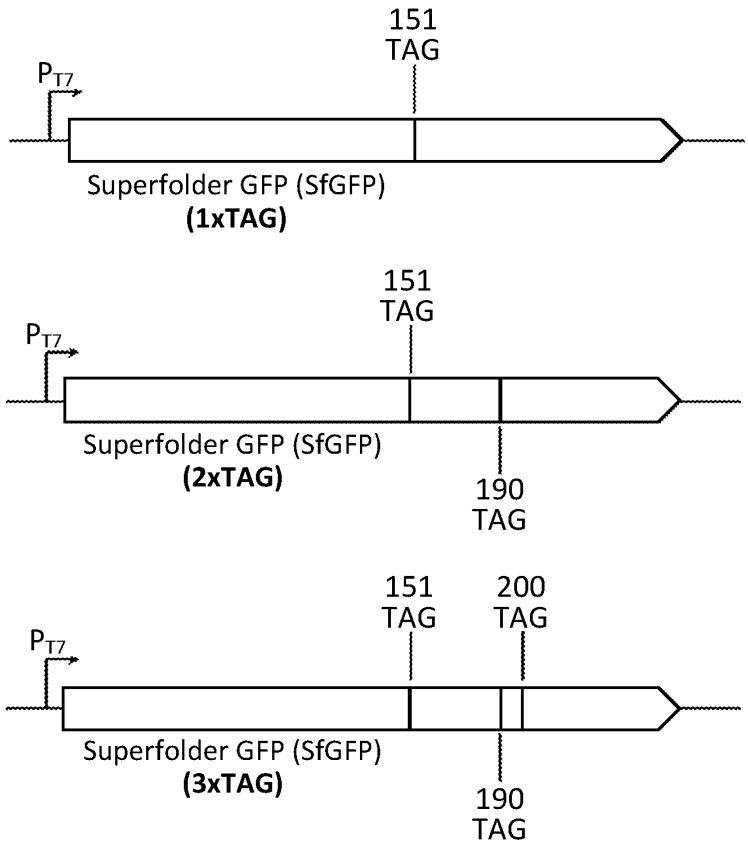

151
TAG
$P_{T7}$
Superfolder GFP (SfGFP)
(1xTAG)
151
TAG
$P_{T7}$
Superfolder GFP (SfGFP)
(2xTAG)
190
TAG
151
TAG
200
TAG
$P_{T7}$
Superfolder GFP (SfGFP)
(3xTAG)
190
TAG

*FIG. 9*

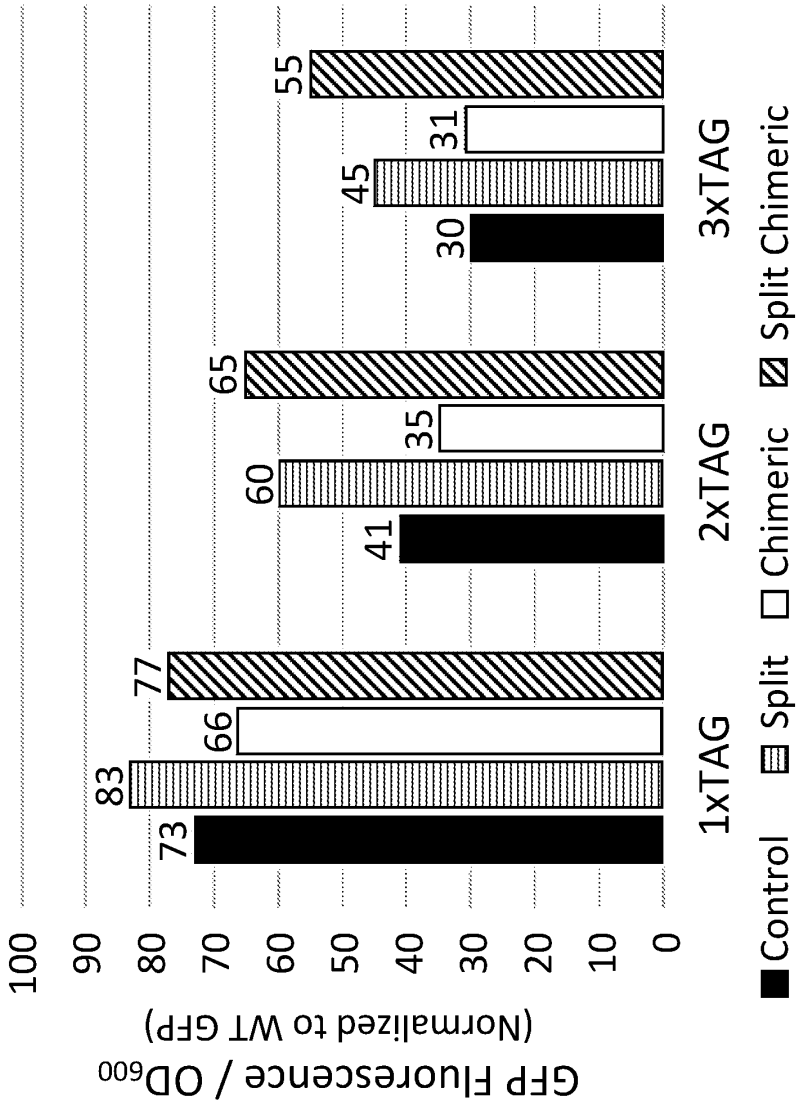

GFP Fluorescence
("High" Expression of Synthetase)
GFP Fluorescence / OD600
(Normalized to WT GFP)
100
90
80
70
60
50
40
30
20
10
0
73
83
66
77
41
60
35
65
30
45
31
55
1xTAG
2xTAG
3xTAG
Control
Split
Chimeric
Split Chimeric

*FIG. 10*

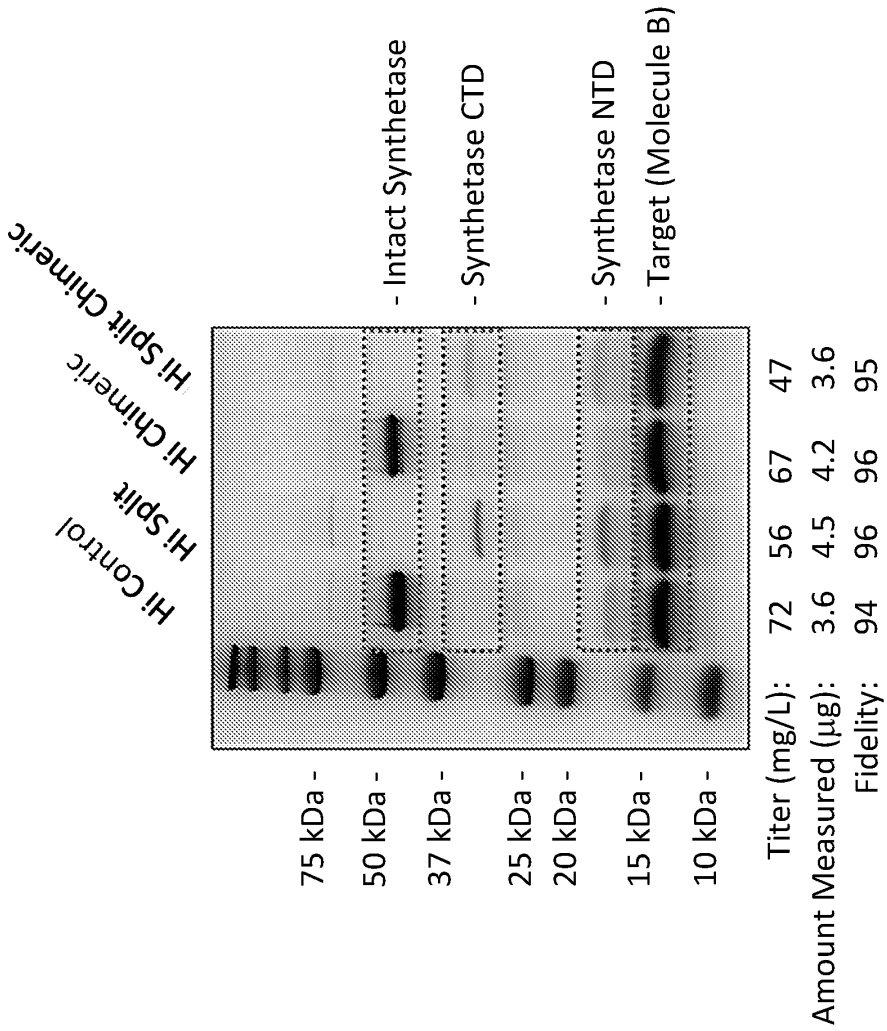

Hi Control
Hi Split
Hi Chimeric
Hi Split Chimeric
- Intact Synthetase
- Synthetase CTD
- Synthetase NTD
- Target (Molecule B)
75 kDa -
50 kDa -
37 kDa -
25 kDa -
20 kDa -
15 kDa -
10 kDa -
Titer (mg/L): 72 56 67 47
Amount Measured (µg): 3.6 4.5 4.2 3.6
Fidelity: 94 96 96 95

*FIG. 11*

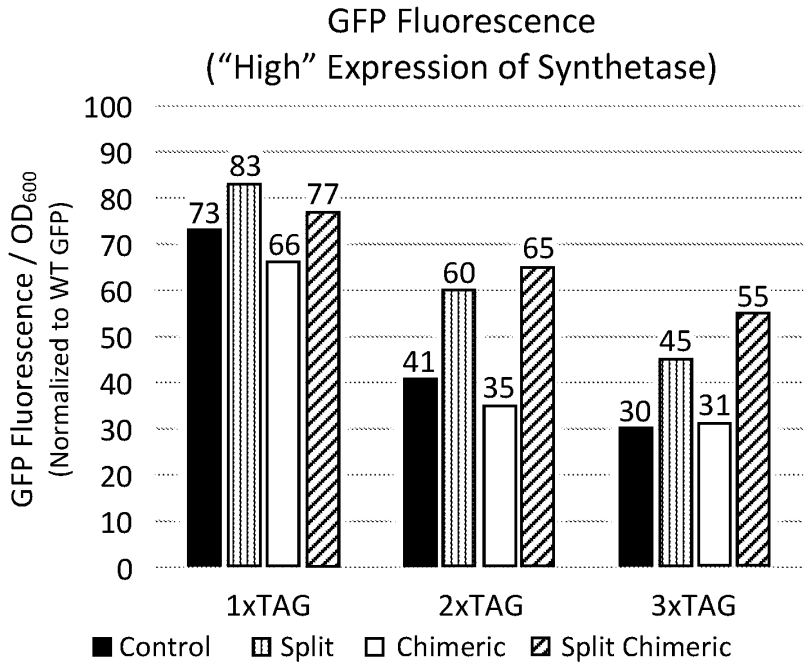

GFP Fluorescence
("High" Expression of Synthetase)
GFP Fluorescence / OD600
(Normalized to WT GFP)
100
90
80
70
60
50
40
30
20
10
0
73
83
66
77
41
60
35
65
30
45
31
55
1xTAG
2xTAG
3xTAG
Control
Split
Chimeric
Split Chimeric

*FIG. 12*

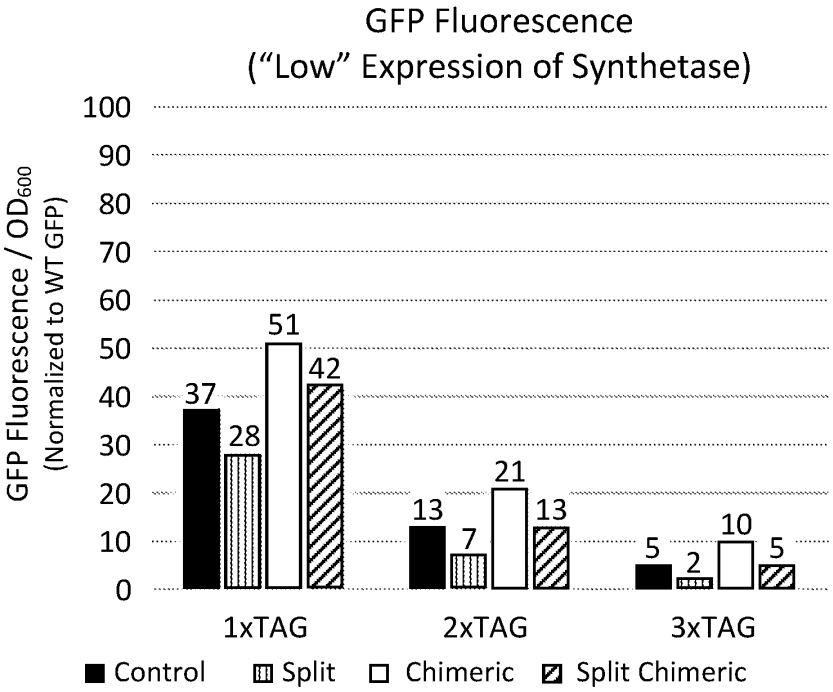

GFP Fluorescence
("Low" Expression of Synthetase)
GFP Fluorescence / OD600
(Normalized to WT GFP)
100
90
80
70
60
50
40
30
20
10
0
37
28
51
42
13
7
21
13
5
2
10
5
1xTAG
2xTAG
3xTAG
Control
Split
Chimeric
Split Chimeric

*FIG. 13*

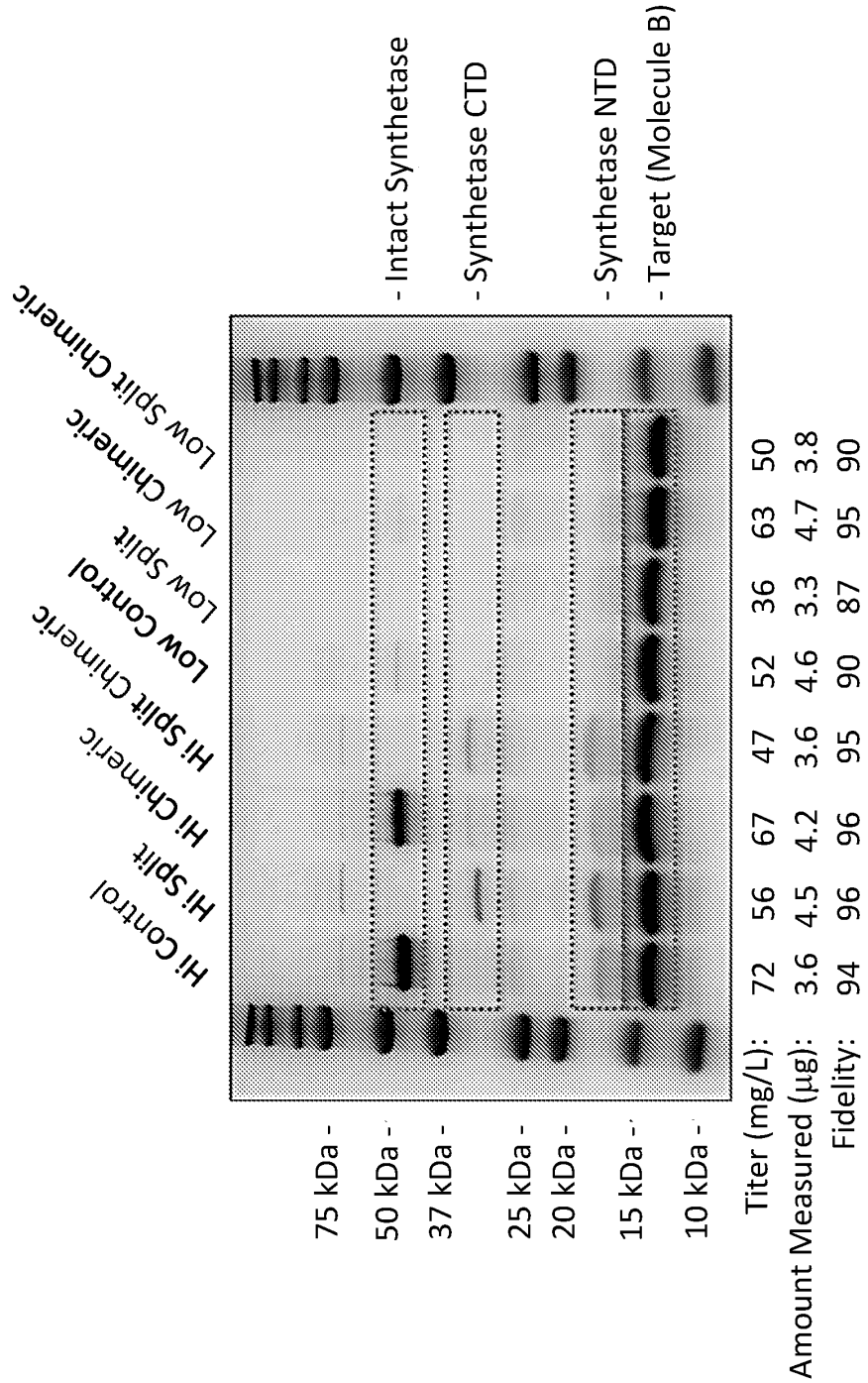

75 kDa -
50 kDa -
37 kDa -
25 kDa -
20 kDa -
15 kDa -
10 kDa -
- Intact Synthetase
- Synthetase CTD
- Synthetase NTD
- Target (Molecule B)
Hi Control
Hi Split
Hi Chimeric
Hi Split Chimeric
Low Control
Low Split
Low Chimeric
Low Split Chimeric
Titer (mg/L): 72 56 67 47 52 36 63 50
Amount Measured (µg): 3.6 4.5 4.2 3.6 4.6 3.3 4.7 3.8
Fidelity: 94 96 96 95 90 87 95 90

*FIG. 14*

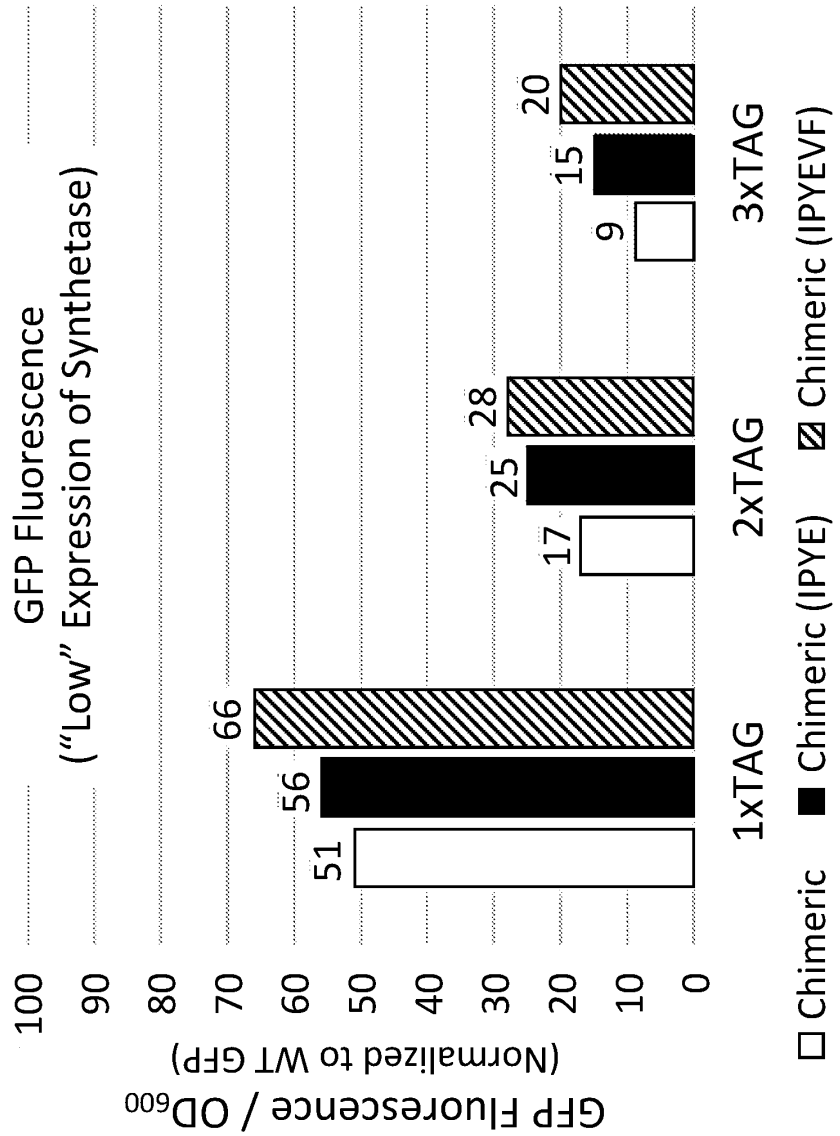

GFP Fluorescence
("Low" Expression of Synthetase)
GFP Fluorescence / OD600
(Normalized to WT GFP)
100
90
80
70
60
50
40
30
20
10
0
66
56
51
28
25
17
20
15
9
1xTAG
2xTAG
3xTAG
Chimeric
Chimeric (IPYE)
Chimeric (IPYEVF)

*FIG. 15*

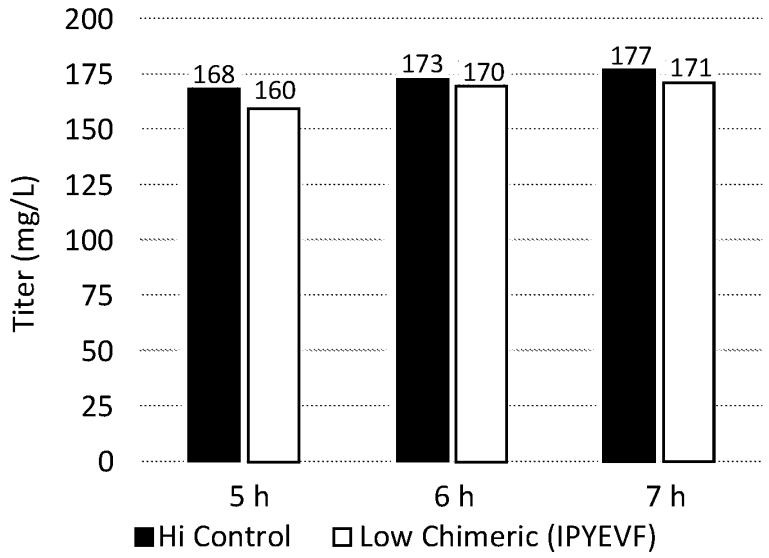

200
175
150
125
100
75
50
25
0
Titer (mg/L)
168
160
173
170
177
171
5 h
6 h
7 h
Hi Control
Low Chimeric (IPYEVF)

*FIG. 16*

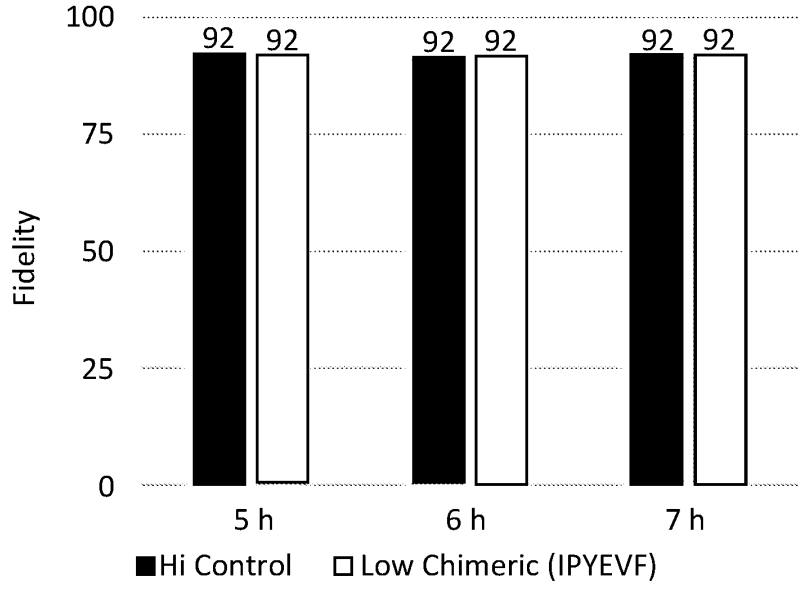

100
75
50
25
0
Fidelity
92
92
92
92
92
92
5 h
6 h
7 h
Hi Control
Low Chimeric (IPYEVF)

*FIG. 17*

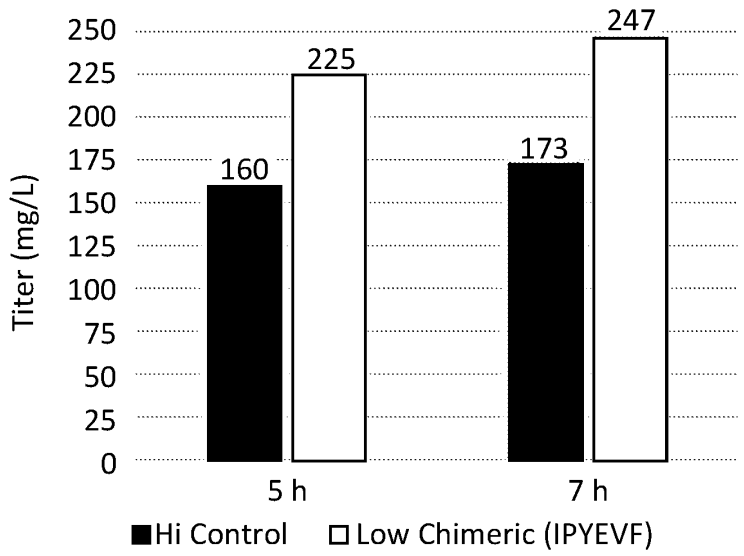

250
225
200
175
150
125
100
75
50
25
0
Titer (mg/L)
160
225
173
247
5 h
7 h
Hi Control
Low Chimeric (IPYEVF)

*FIG. 18*

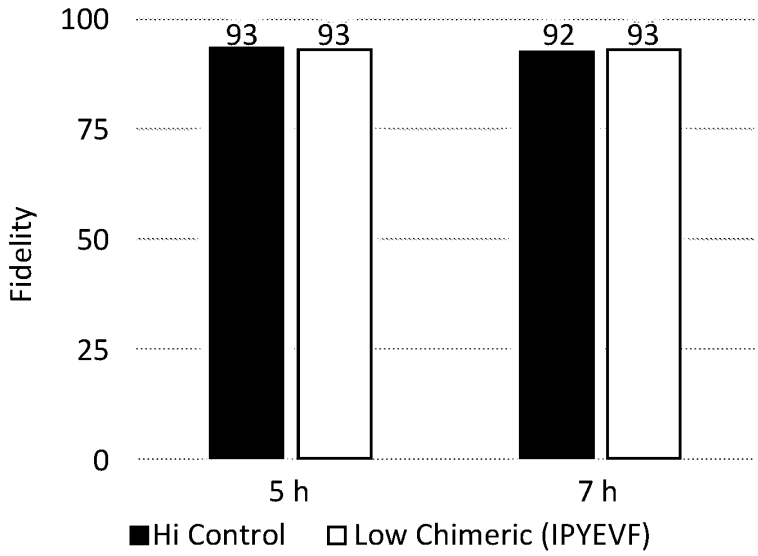

100
75
50
25
0
Fidelity
93
93
92
93
5 h
7 h
Hi Control
Low Chimeric (IPYEVF)

*FIG. 19*

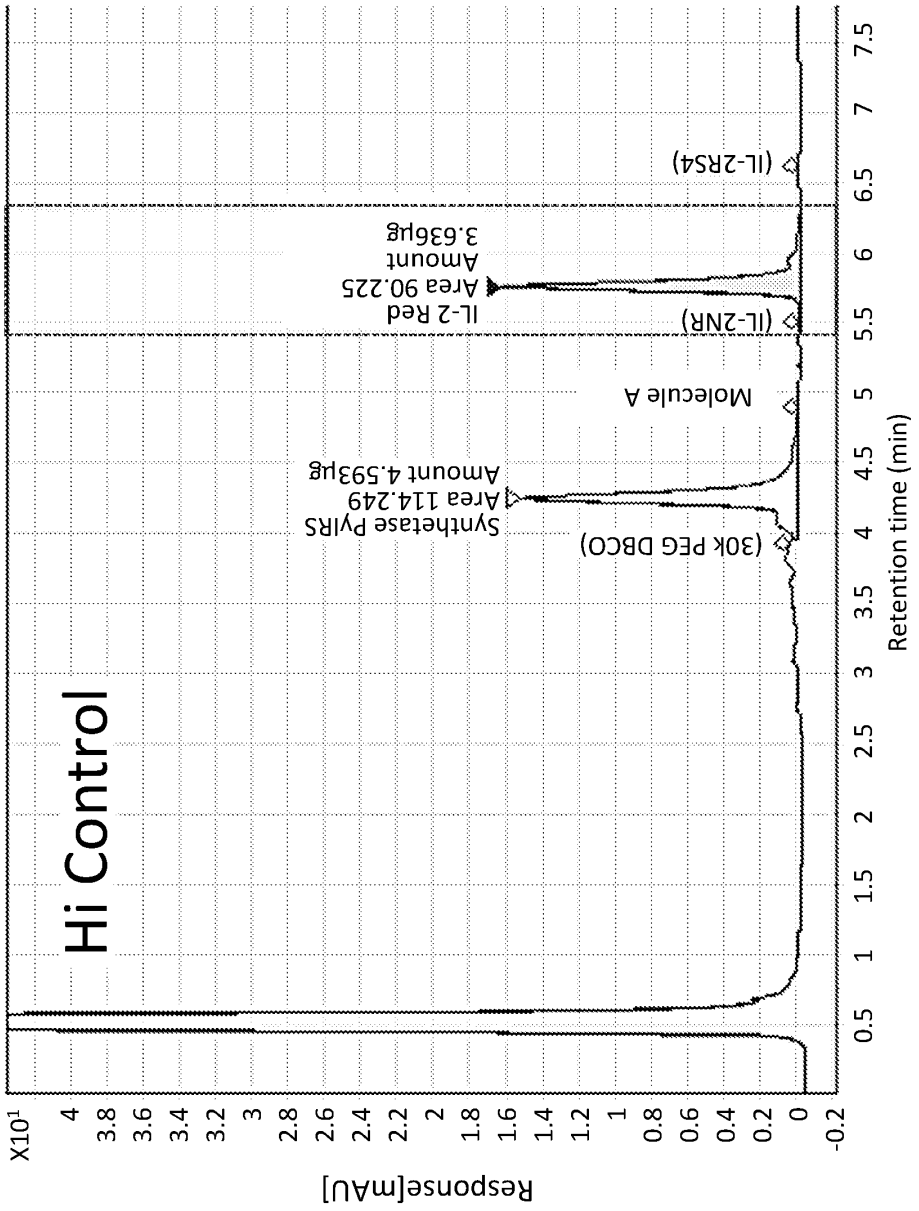

Hi Control
X10¹
Response[mAU]
Retention time (min)
(30k PEG DBCO)
Synthetase PylRS
Area 114.249
Amount 4.593µg
Molecule A
(IL-2NR)
IL-2 Red
Area 90.225
Amount
3.636µg
(IL-2RS4)

*FIG. 20*

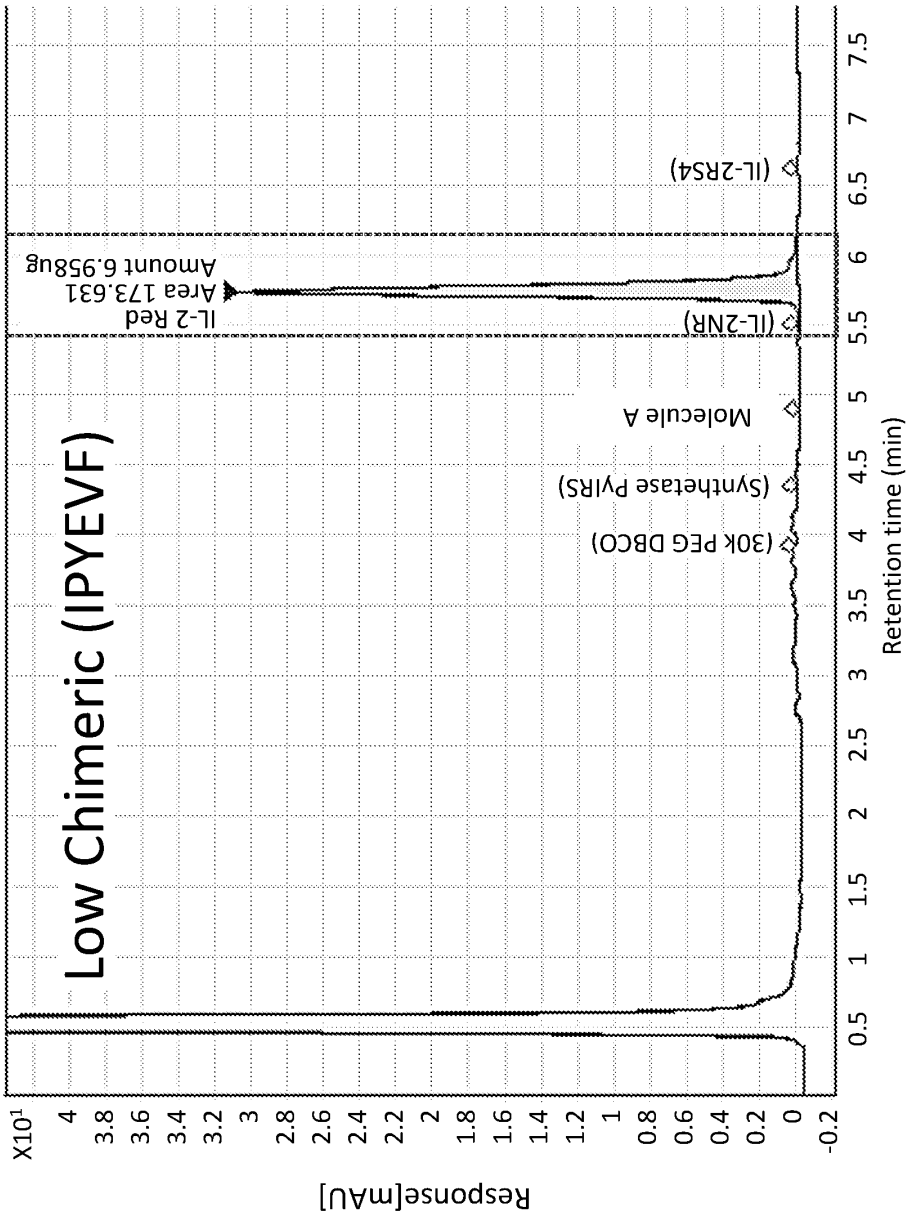

Low Chimeric (IPYEVF)
IL-2 Red
Area 173.631
Amount 6.958ug
(IL-2NR)
Molecule A
(Synthetase PylRS)
(30k PEG DBCO)
(IL-2RS4)
Response[mAU]
X10¹
Retention time (min)

FIG. 21

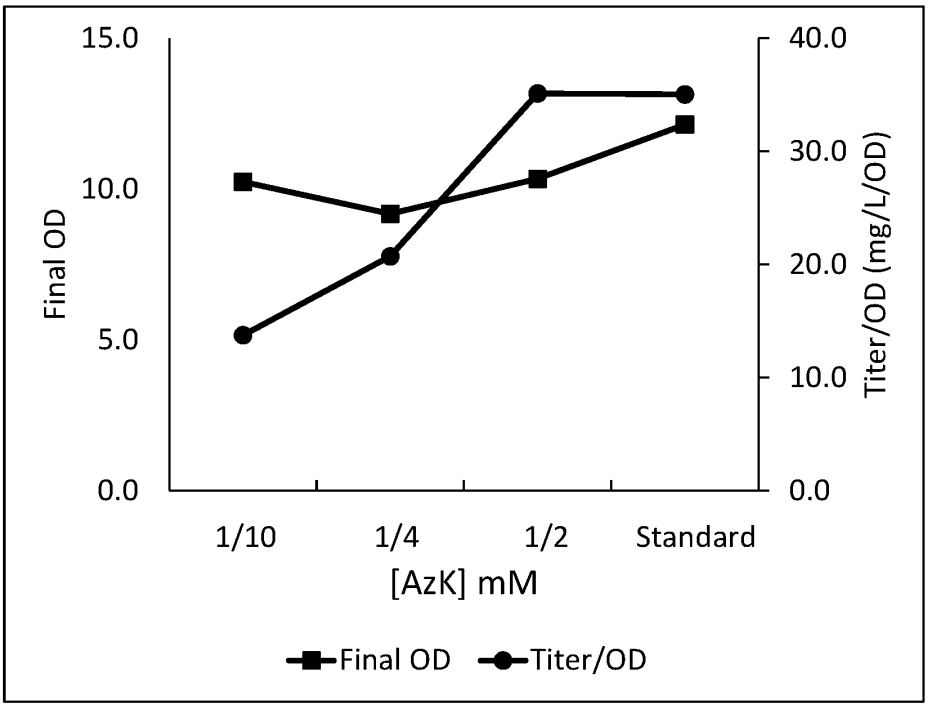

15.0
10.0
5.0
0.0
Final OD
40.0
30.0
20.0
10.0
0.0
Titer/OD (mg/L/OD)
1/10
1/4
1/2
Standard
[AzK] mM
Final OD
Titer/OD

FIG. 22

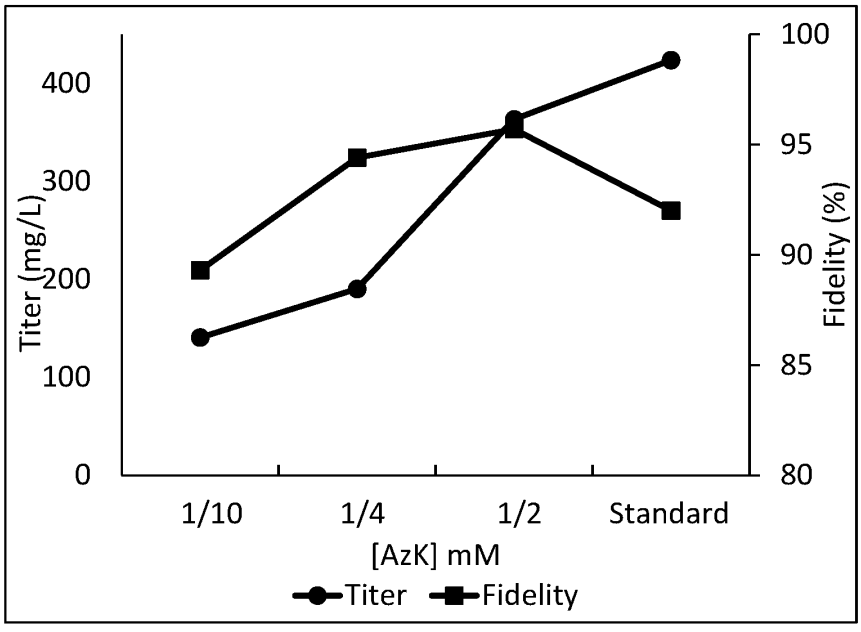

400
300
200
100
0
Titer (mg/L)
100
95
90
85
80
Fidelity (%)
1/10
1/4
1/2
Standard
[AzK] mM
Titer
Fidelity

FIG. 23

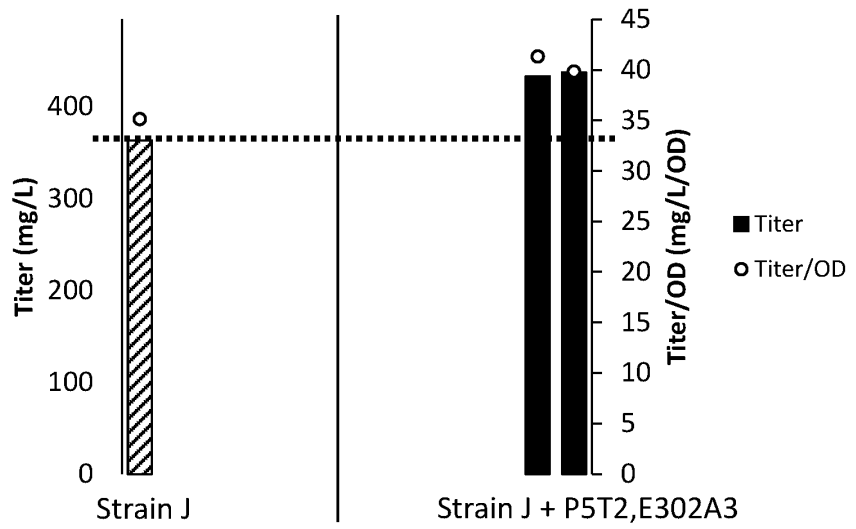

Titer (mg/L)
400
300
200
100
0
45
40
35
30
25
20
15
10
5
0
Titer/OD (mg/L/OD)
Titer
Titer/OD
Strain J
Strain J + P5T2,E302A3

FIG. 24

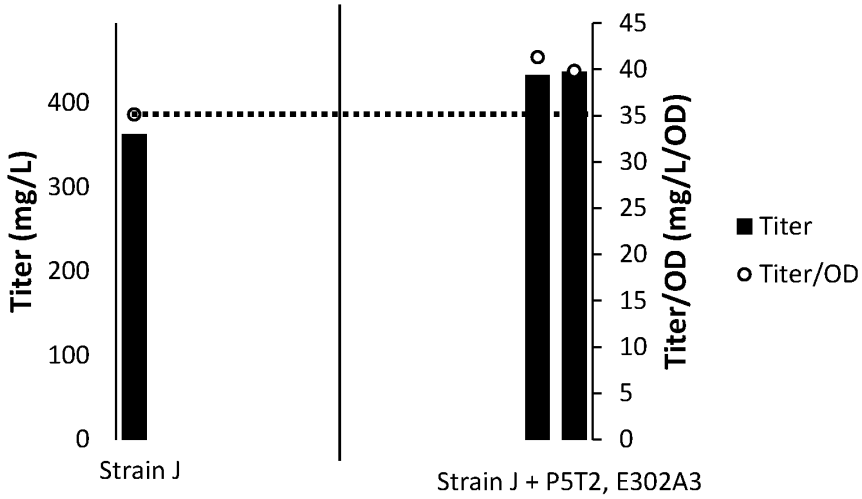

Titer (mg/L)
400
300
200
100
0
45
40
35
30
25
20
15
10
5
0
Titer/OD (mg/L/OD)
Titer
Titer/OD
Strain J
Strain J + P5T2, E302A3

FIG. 25

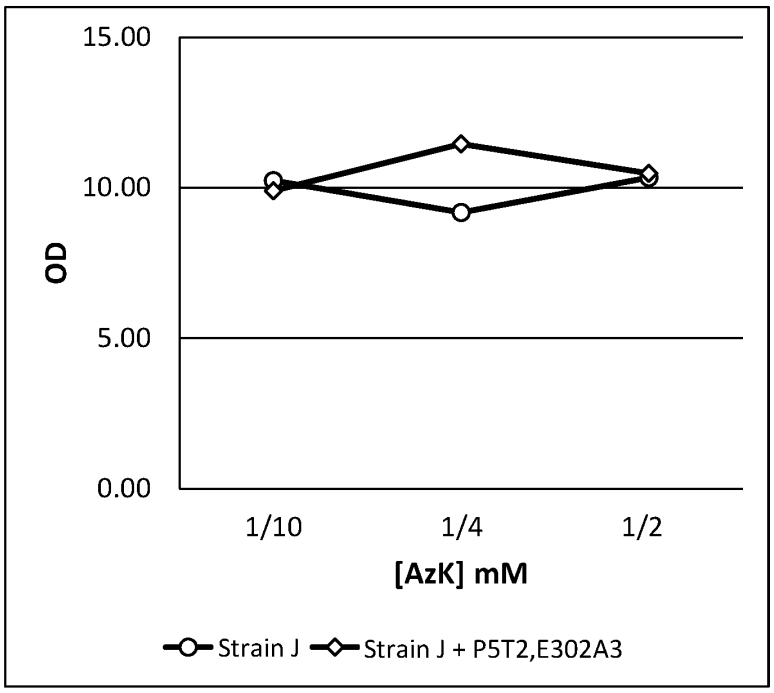

15.00
10.00
5.00
0.00
OD
1/10
1/4
1/2
[AzK] mM
Strain J
Strain J + P5T2,E302A3

FIG. 26

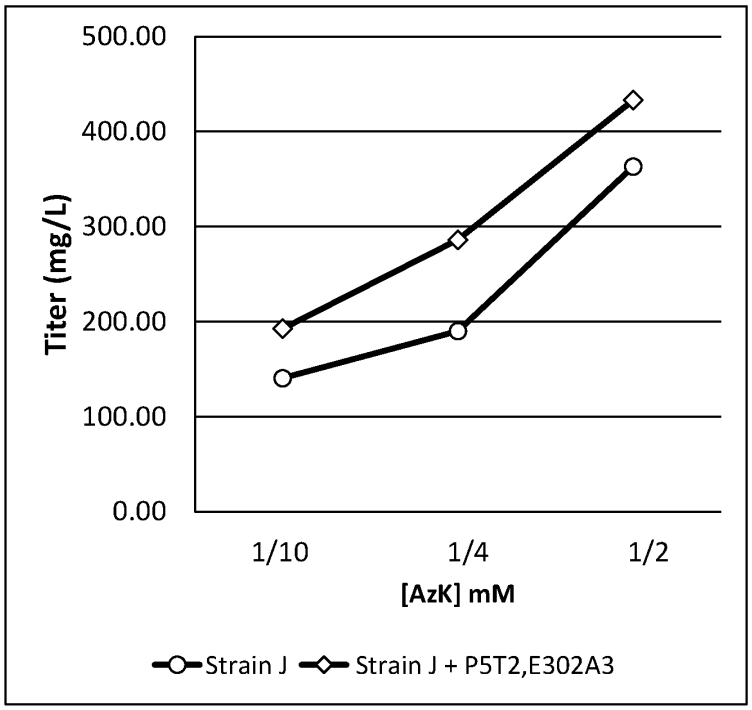

500.00
400.00
300.00
200.00
100.00
0.00
Titer (mg/L)
1/10
1/4
1/2
[AzK] mM
Strain J
Strain J + P5T2,E302A3

FIG. 27

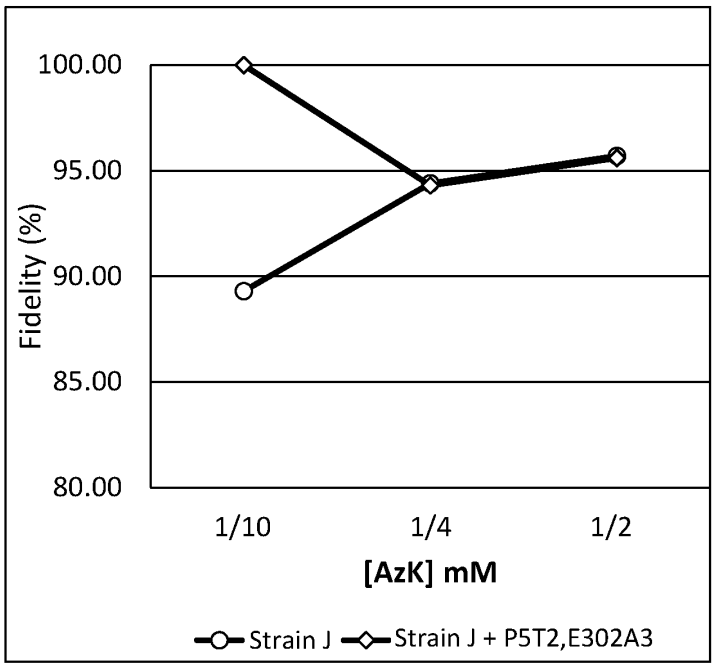

100.00
95.00
90.00
85.00
80.00
Fidelity (%)
1/10
1/4
1/2
[AzK] mM
Strain J
Strain J + P5T2,E302A3

*FIG. 28*

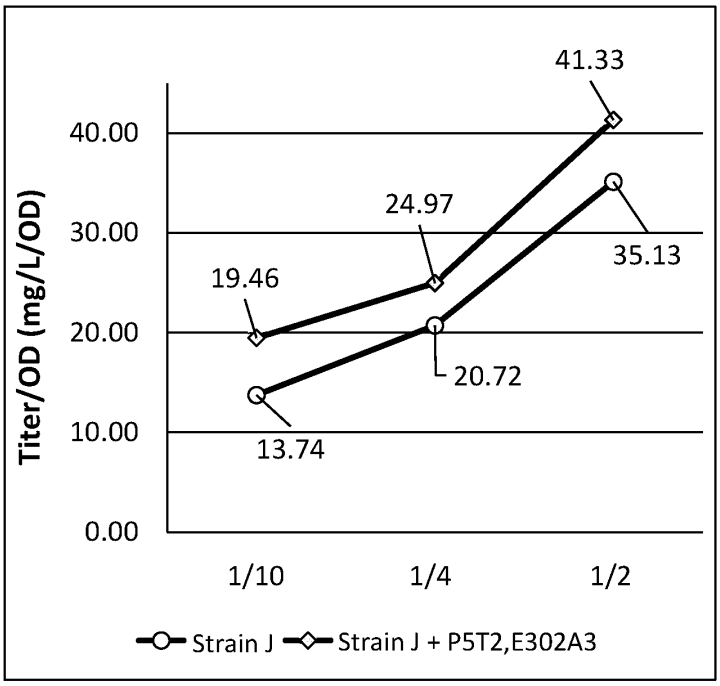

41.33
40.00
24.97
30.00
35.13
19.46
20.00
20.72
10.00
13.74
0.00
Titer/OD (mg/L/OD)
1/10
1/4
1/2
Strain J
Strain J + P5T2,E302A3

*FIG. 29*

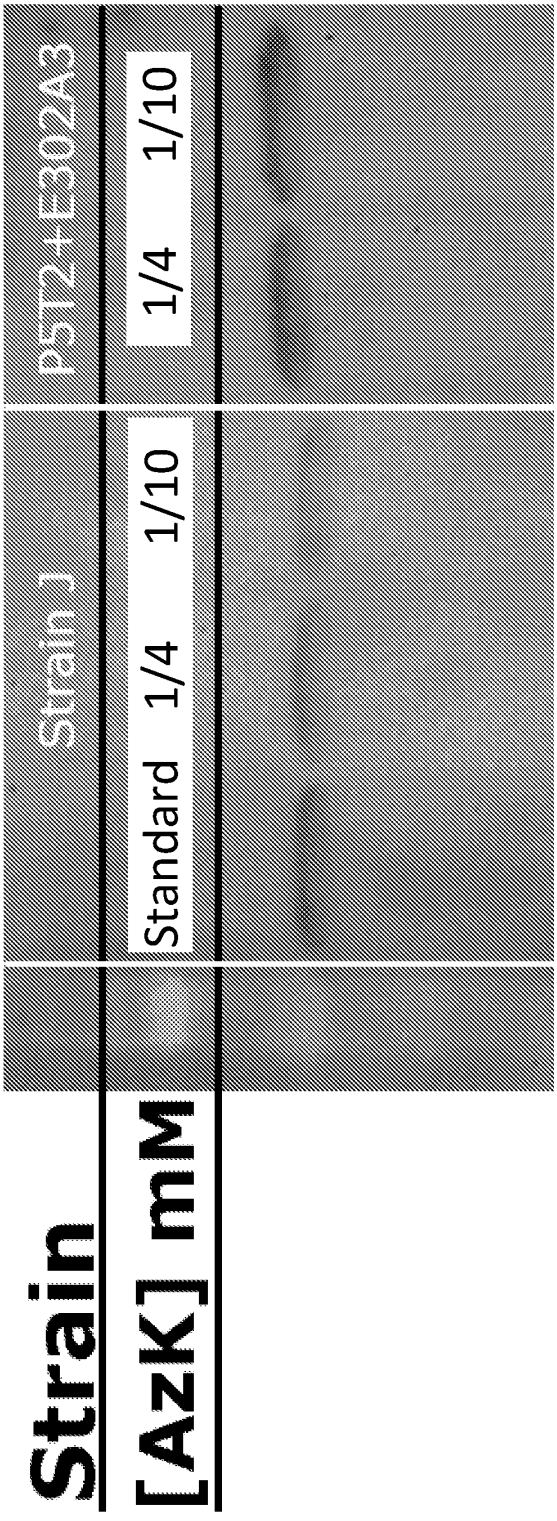



*FIG. 30*

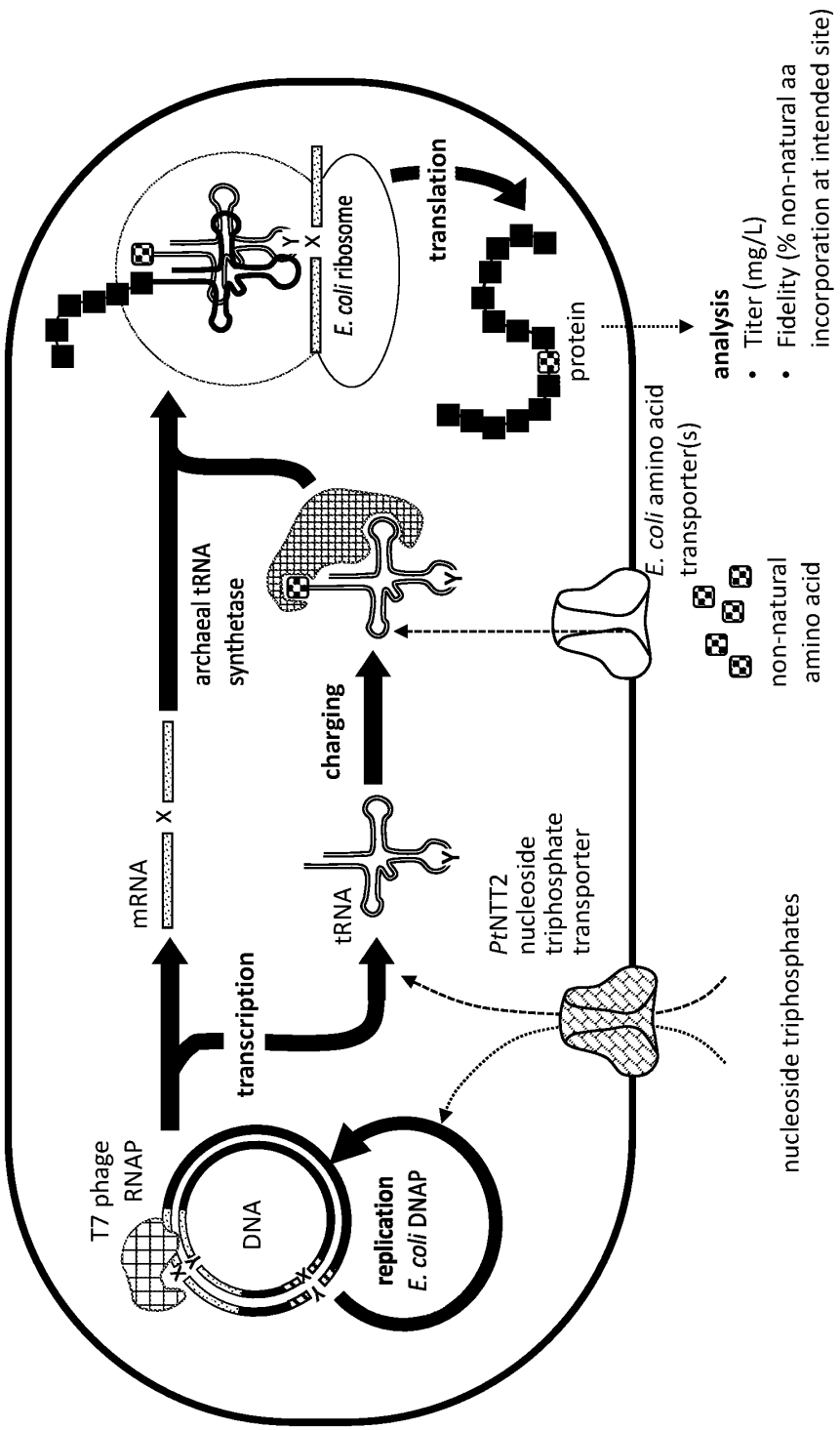

T7 phage RNAP
DNA
replication
E. coli DNAP
transcription
mRNA
X
tRNA
charging
archaeal tRNA synthetase
E. coli ribosome
translation
protein
PtNTT2 nucleoside triphosphate transporter
E. coli amino acid transporter(s)
non-natural amino acid
nucleoside triphosphates
analysis
• Titer (mg/L)
• Fidelity (% non-natural aa incorporation at intended site)

FIG. 31

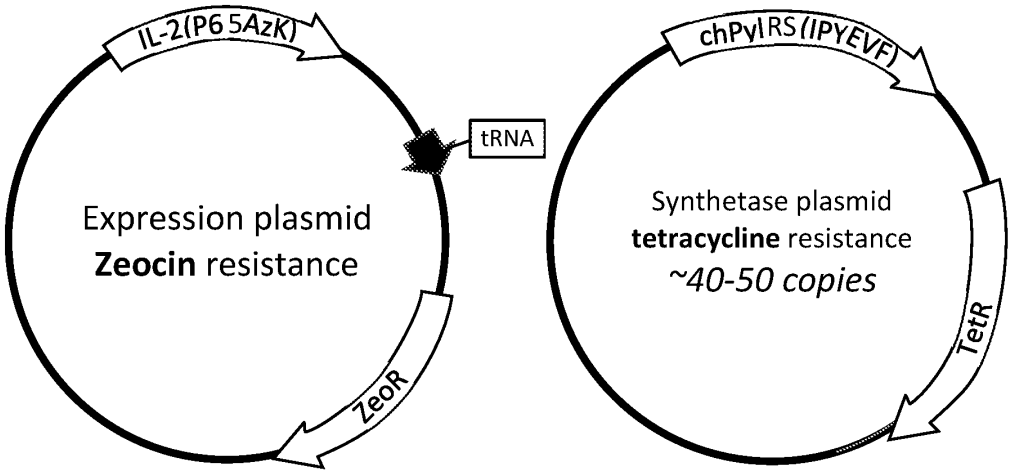

IL-2(P65AzK)
tRNA
Expression plasmid
**Zeocin** resistance
ZeoR
chPylRS(IPYEVF)
Synthetase plasmid
**tetracycline** resistance
*~40-50 copies*
TetR

*FIG. 32*

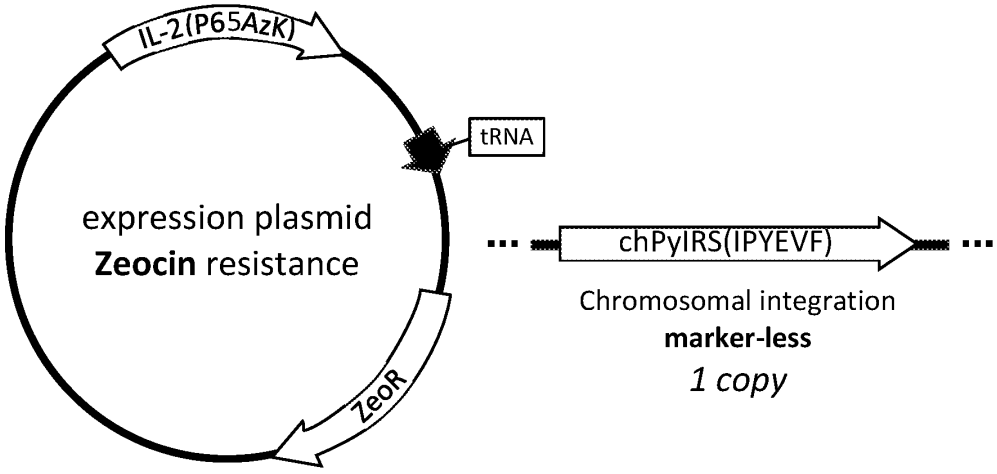

IL-2(P65AzK)
tRNA
expression plasmid
**Zeocin** resistance
ZeoR
chPylRS(IPYEVF)
Chromosomal integration
**marker-less**
*1 copy*

*FIG. 33*

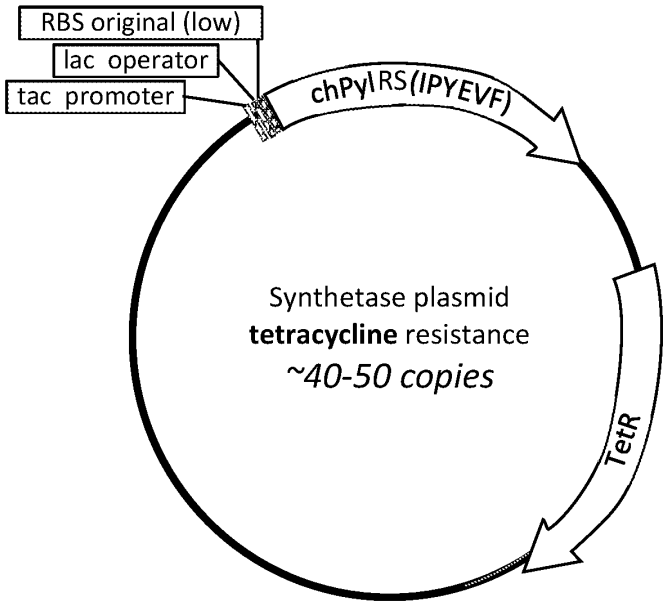
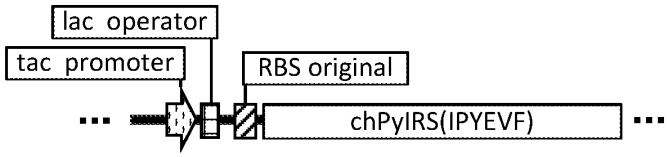
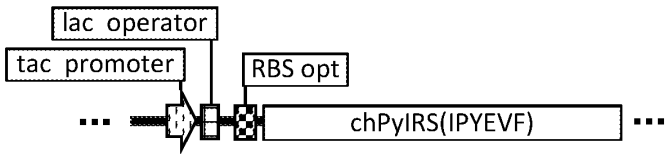



FIG. 34




Chromosomal integration
**marker-less**
*1 copy*

FIG. 35




Chromosomal integration
**marker-less**
*1 copy*

FIG. 36

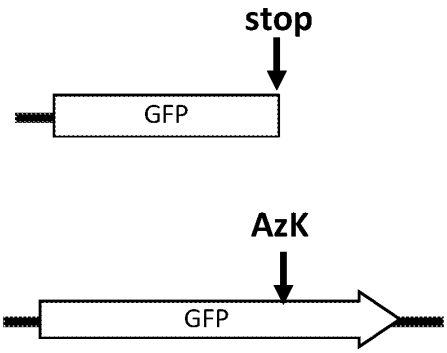

stop
GFP
AzK
GFP

*FIG. 37*

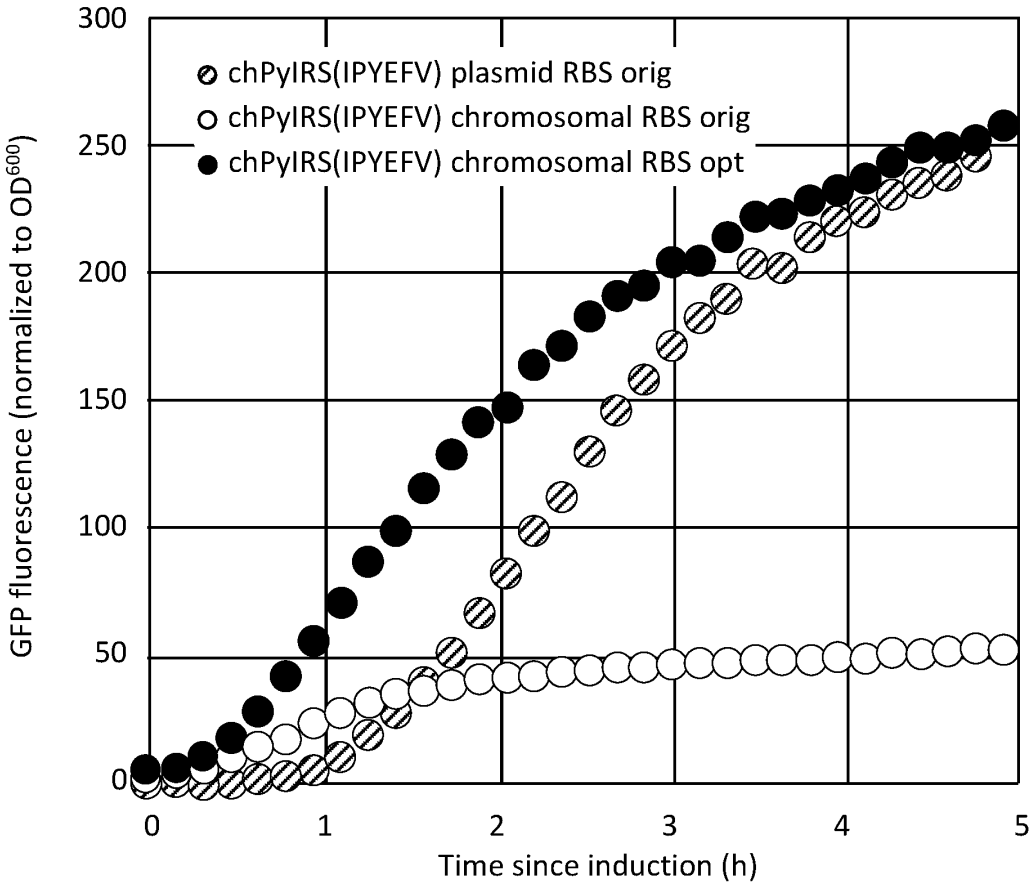

chPyIRS(IPYEFV) plasmid RBS orig
chPyIRS(IPYEFV) chromosomal RBS orig
chPyIRS(IPYEFV) chromosomal RBS opt
GFP fluorescence (normalized to OD600)
Time since induction (h)
300
250
200
150
100
50
0
0
1
2
3
4
5

*FIG. 38*

Chromosomal integration
**marker-less**
*1 copy*
X
~100-fold promoter strength
(LaclQ1)

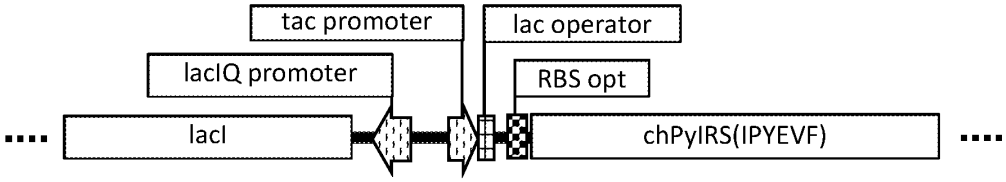



*FIG. 39*

Chromosomal integration
**marker-less**
*1 copy*
X
~10-fold promoter strength
(LaclQ1)

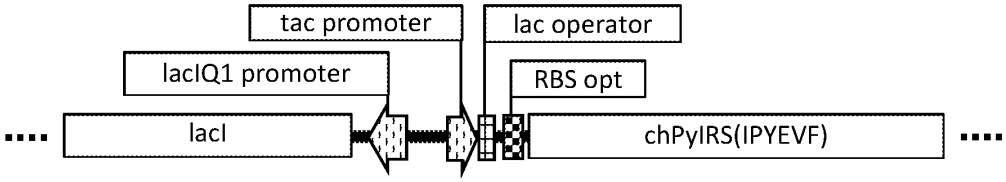



*FIG. 40*

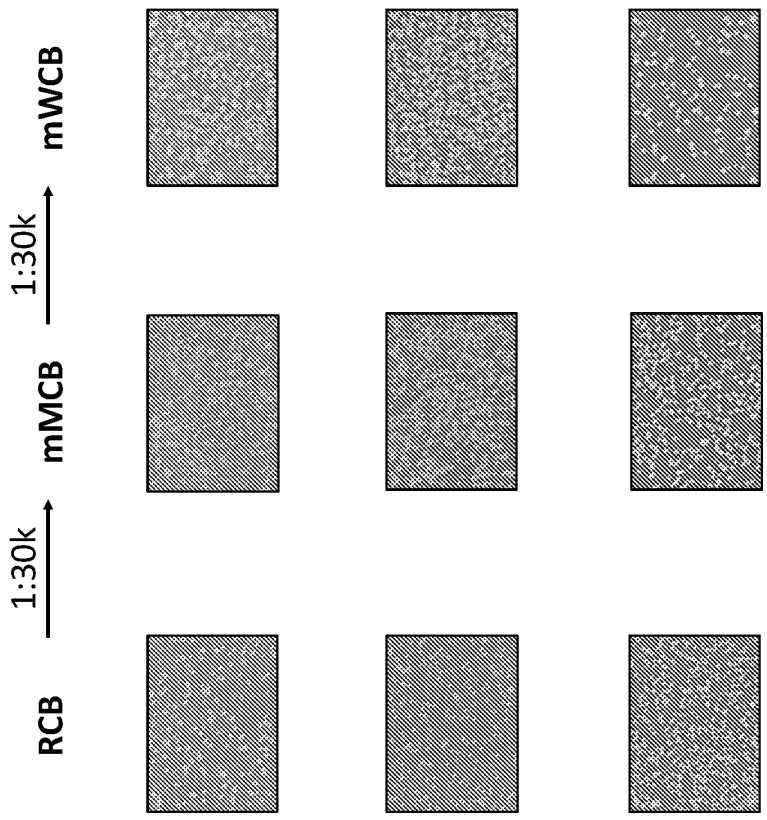

RCB
1:30k
mMCB
1:30k
mWCB
Strain A
Plasmid barkeri synthetase
Strain E
Integrated chimeric synthetase w/ PlacIQ
Strain F
Integrated chimeric synthetase w/ PlacIQ1

FIG. 41

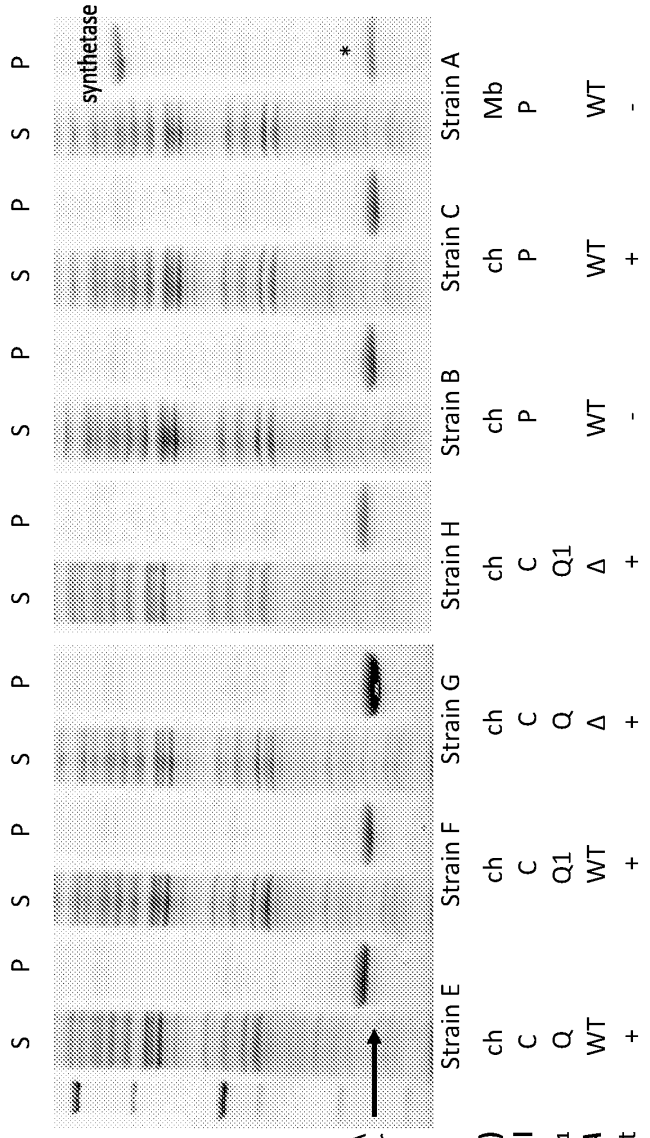

Soluble / Pellet (insoluble)
S P S P S P S P S P S P
synthetase
*
Molecule A precursor
Strain
MbPylRS / chPylRS(IPYE)
Plasmid / Chromosomal
lacI promoter PlacIQ / PlacIQ1
recA
Met39 RBS opt
Strain E
ch
C
Q
WT
+
Strain F
ch
C
Q1
WT
+
Strain G
ch
C
Q
Δ
+
Strain H
ch
C
Q1
Δ
+
Strain B
ch
P
WT
-
Strain C
ch
P
WT
+
Strain A
Mb
P
WT
-

*FIG. 42*

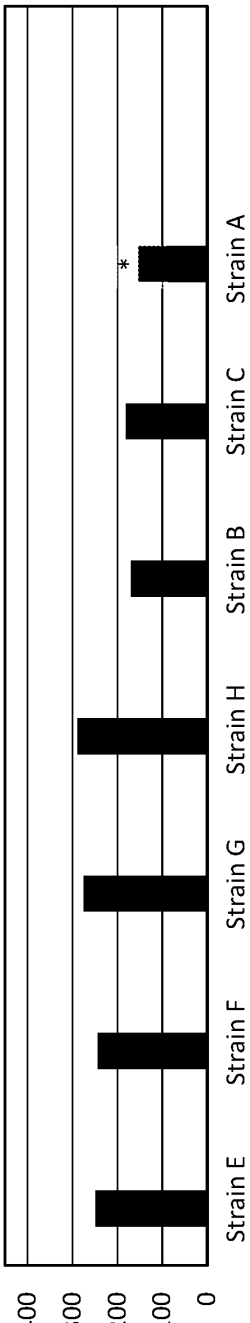



*FIG. 43A*

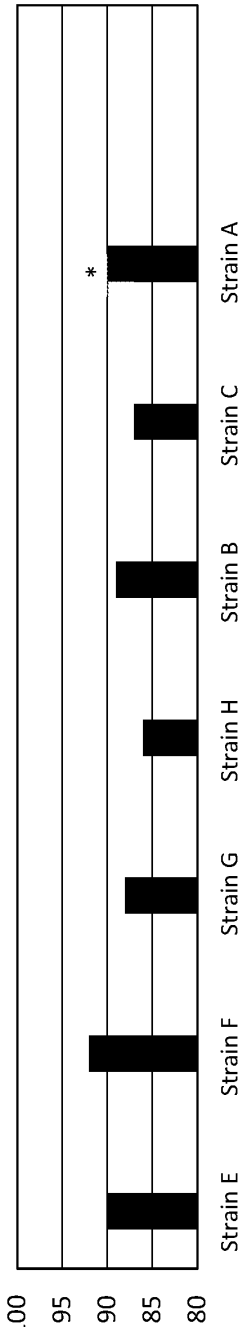



*FIG. 43B*

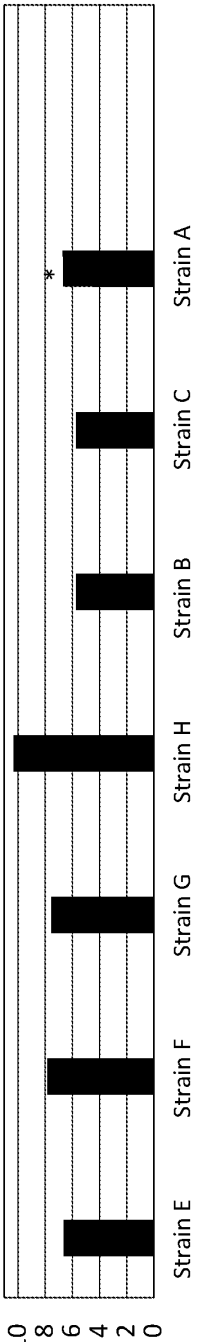



*FIG. 43C*

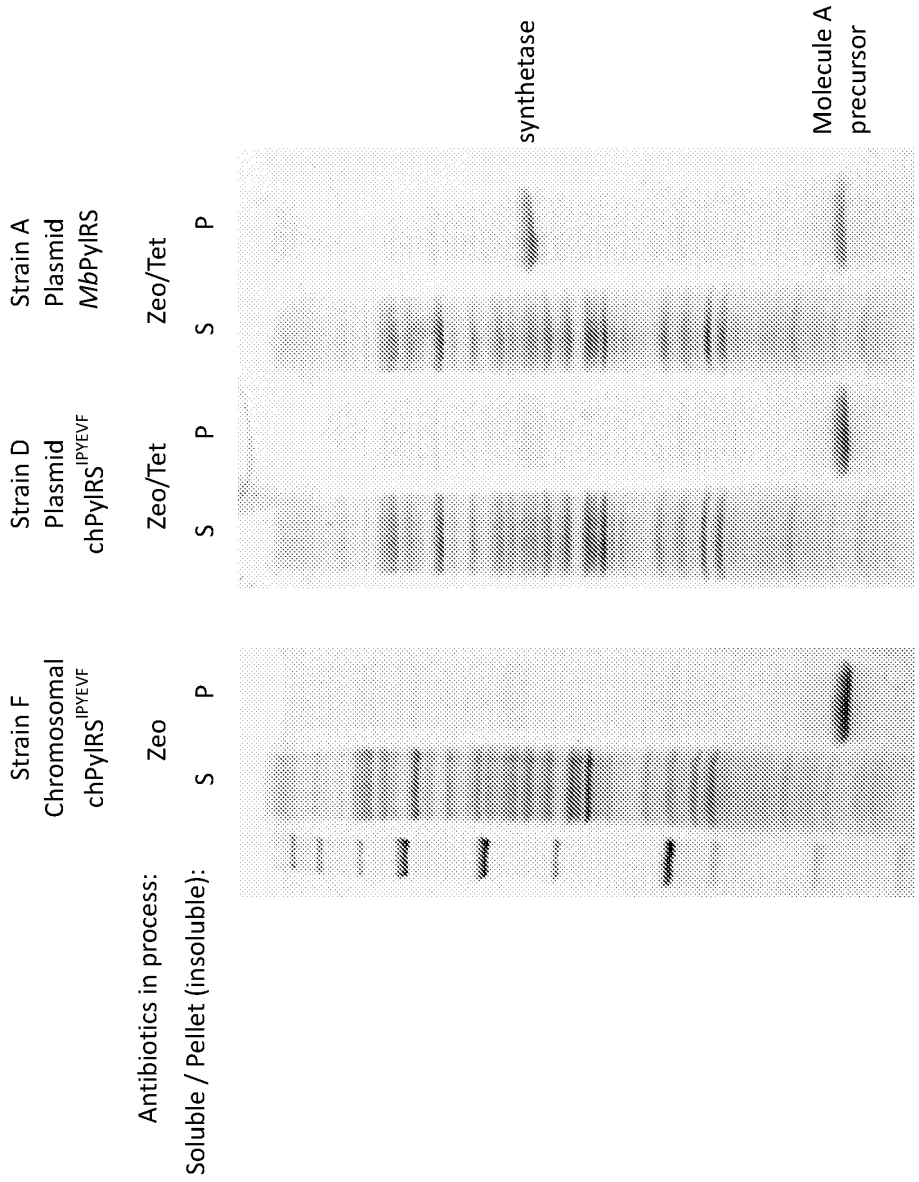

Strain F
Chromosomal
chPylRS^IPYEVF
Strain D
Plasmid
chPylRS^IPYEVF
Strain A
Plasmid
MbPylRS
Antibiotics in process:
Zeo
Zeo/Tet
Zeo/Tet
Soluble / Pellet (insoluble):
S
P
S
P
S
P
synthetase
Molecule A
precursor

FIG. 44

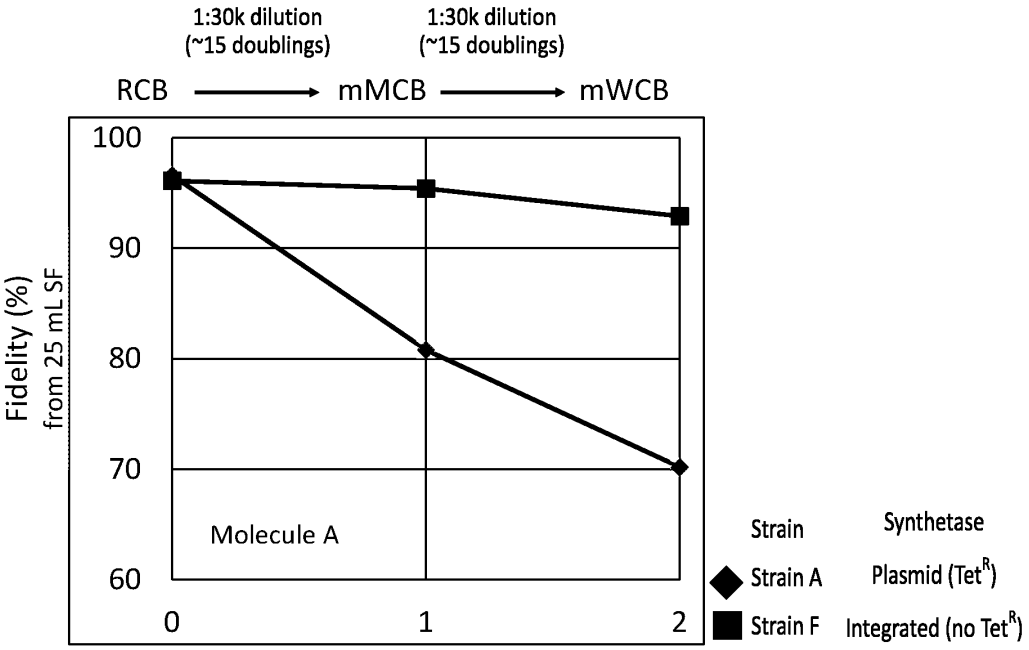

1:30k dilution
(~15 doublings)
1:30k dilution
(~15 doublings)
RCB
mMCB
mWCB
100
90
80
70
60
Fidelity (%)
from 25 mL SF
Molecule A
0
1
2
Strain
Synthetase
Strain A
Plasmid (Tet^R)
Strain F
Integrated (no Tet^R)

*FIG. 45*

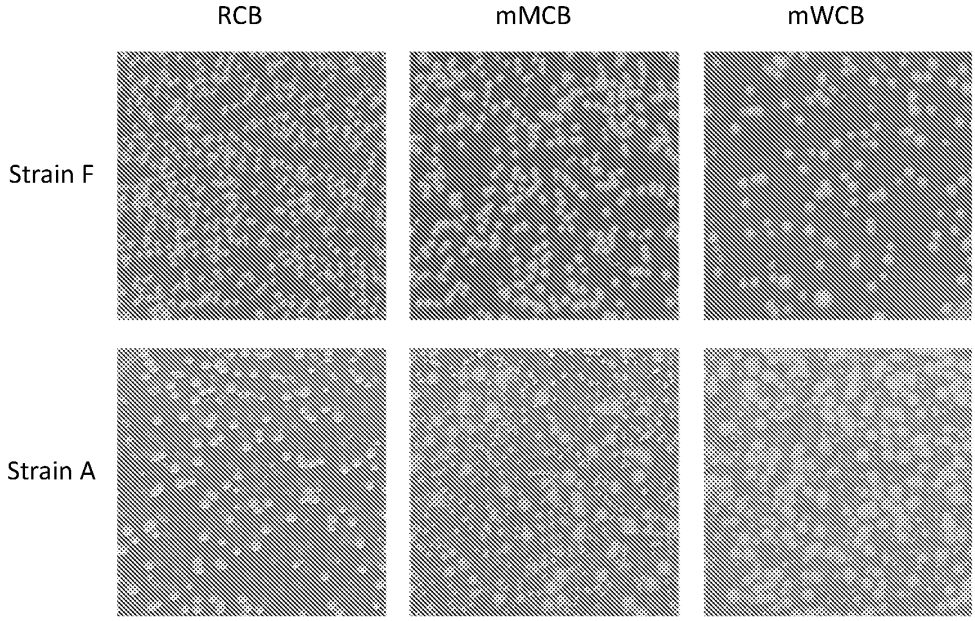

RCB
mMCB
mWCB
Strain F
Strain A

*FIG. 46*

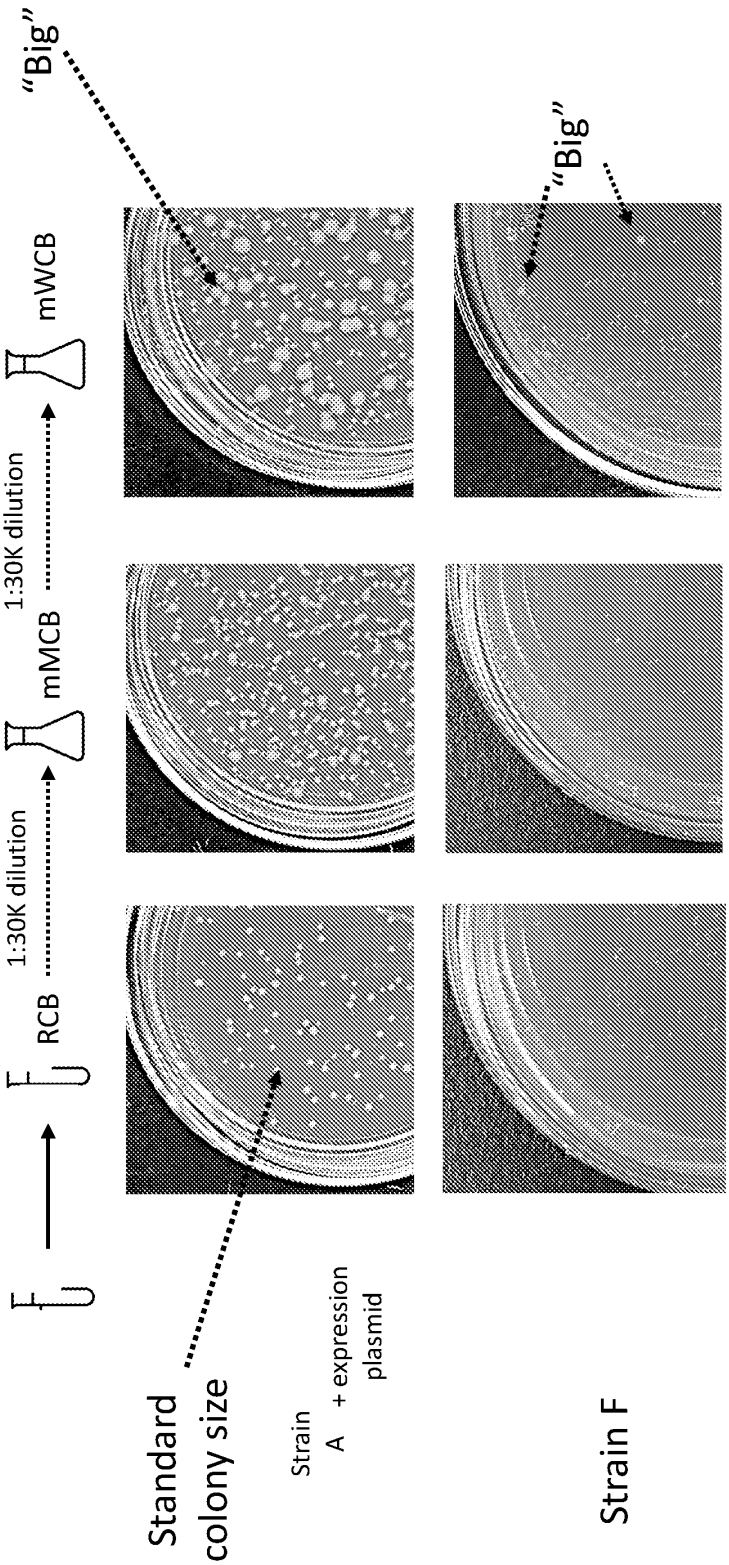

"Big"
"Big"
mWCB
1:30K dilution
mMCB
1:30K dilution
RCB
Standard colony size
Strain A + expression plasmid
Strain F

FIG. 47

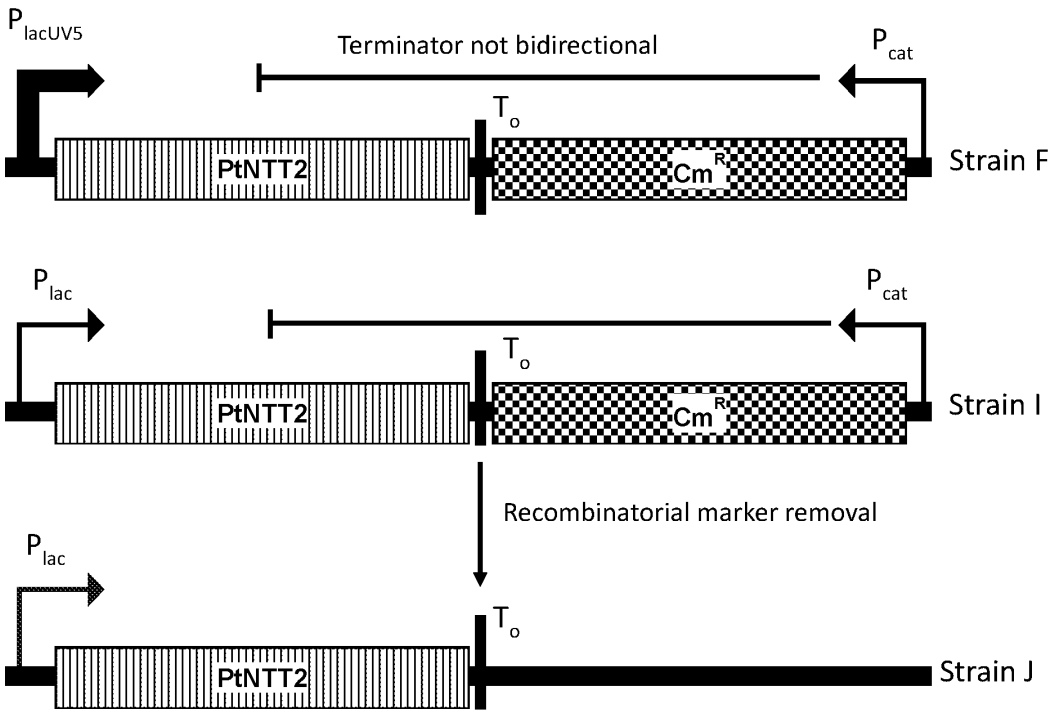

P lacUV5
Terminator not bidirectional
P cat
T o
PtNTT2
Cm R
Strain F
P lac
P cat
T o
PtNTT2
Cm R
Strain I
Recombinatorial marker removal
P lac
T o
PtNTT2
Strain J

FIG. 48

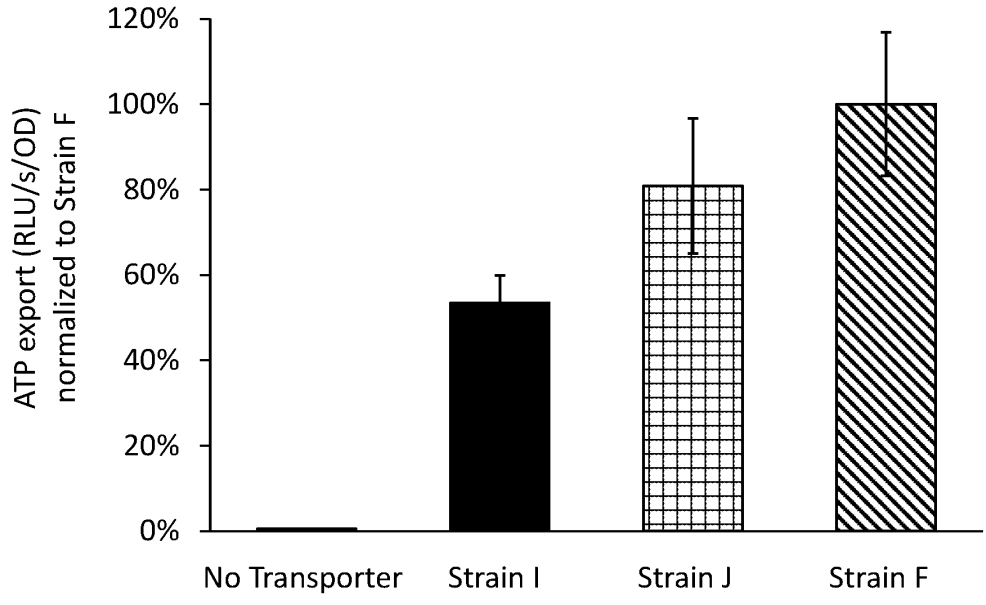

ATP export (RLU/s/OD) normalized to Strain F
120%
100%
80%
60%
40%
20%
0%
No Transporter
Strain I
Strain J
Strain F

FIG. 49

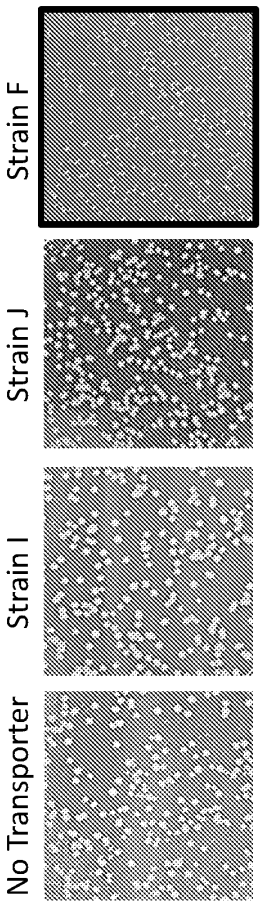

No Transporter
Strain I
Strain J
Strain F
Plating of indicated strain
Molecule B fidelity (%) of RCB produced with indicated strain in 25 mL SF expression w/ dNaMTP
n/a
84
93
93

*FIG. 50*

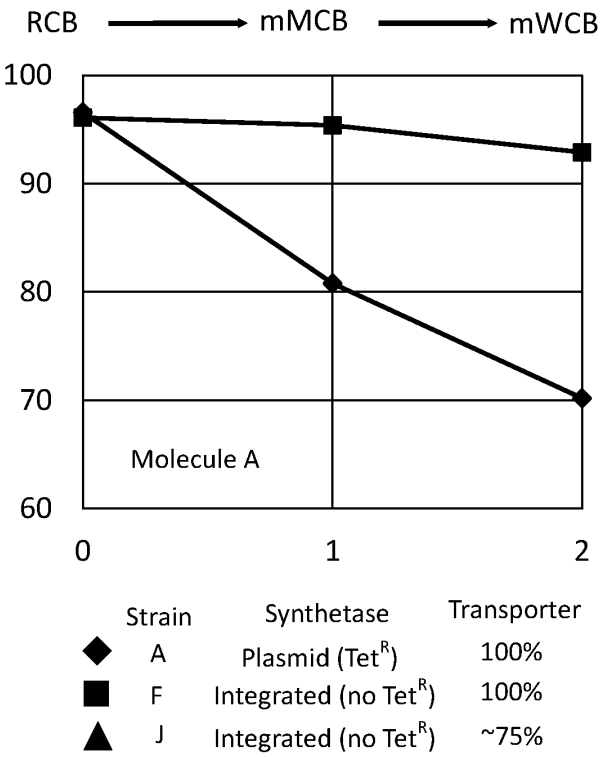



*FIG. 51A*

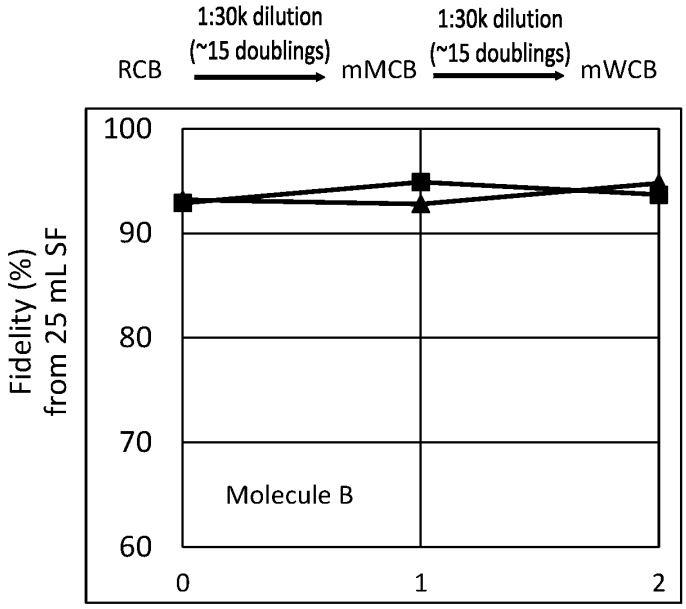



*FIG. 51B*

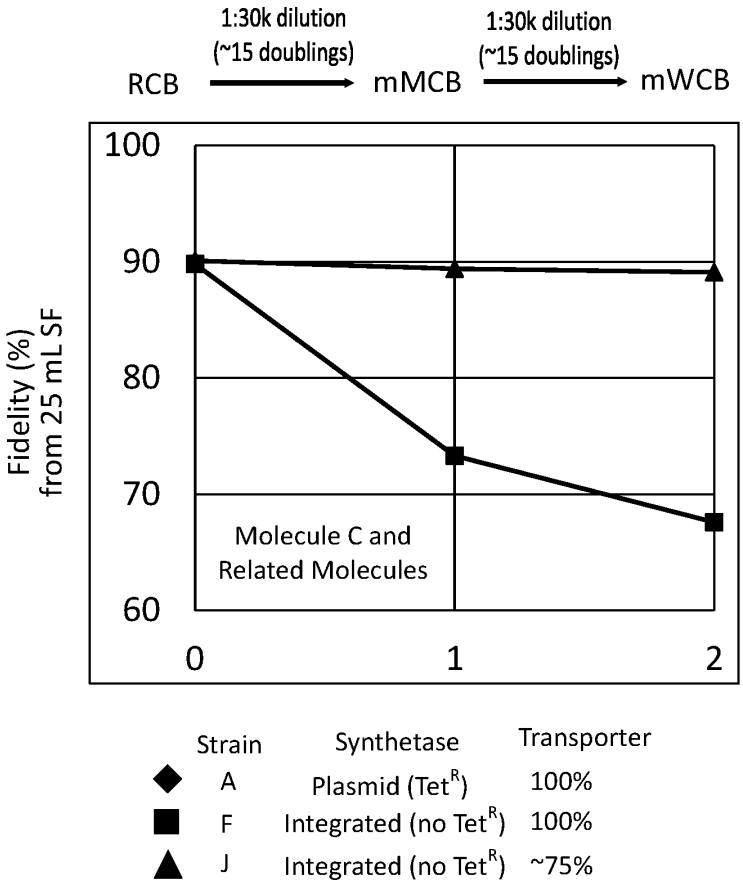

1:30k dilution
(~15 doublings)
1:30k dilution
(~15 doublings)
RCB
mMCB
mWCB
100
90
80
70
60
Fidelity (%)
from 25 mL SF
Molecule C and
Related Molecules
0
1
2
Strain
Synthetase
Transporter
A
Plasmid (Tet^R)
100%
F
Integrated (no Tet^R)
100%
J
Integrated (no Tet^R)
~75%

*FIG. 51C*

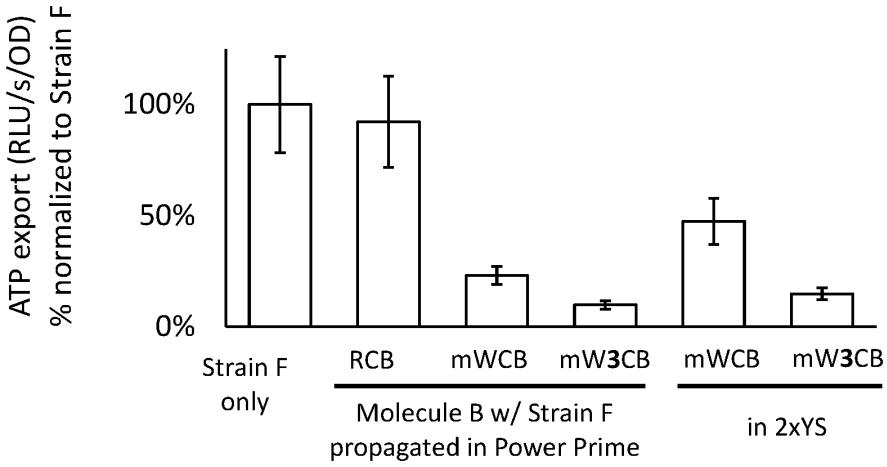



*FIG. 52A*

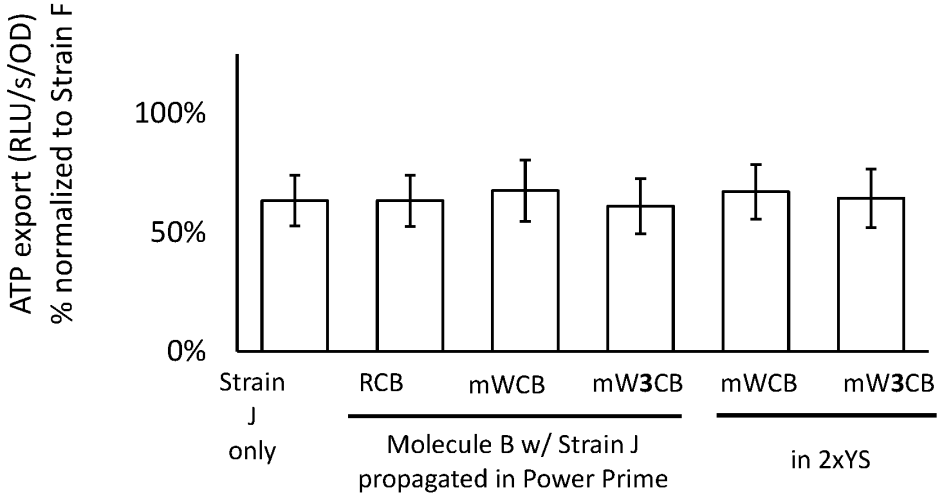



*FIG. 52B*

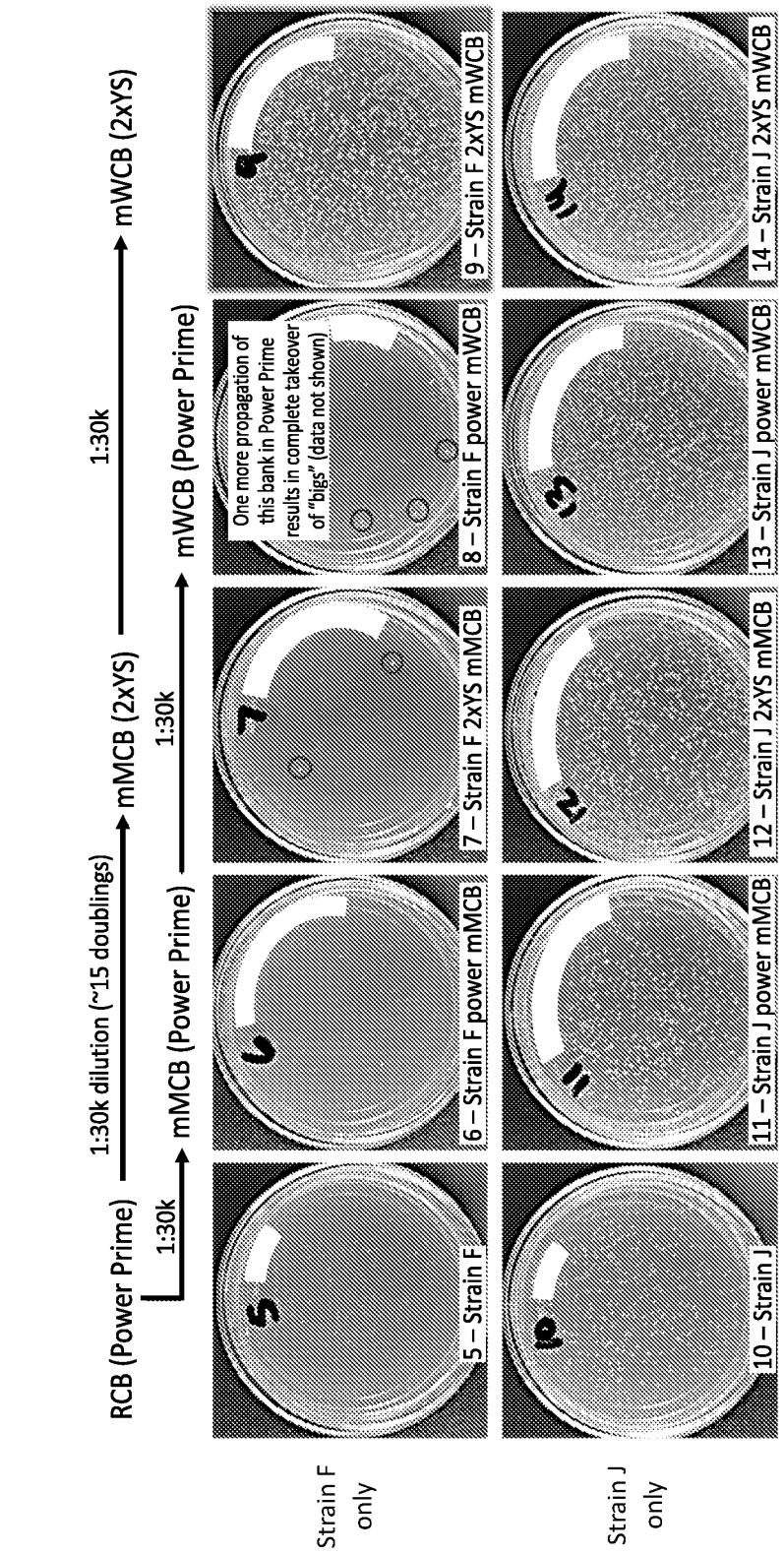

RCB (Power Prime)
1:30k
1:30k dilution (~15 doublings)
mMCB (Power Prime)
mMCB (2xYS)
1:30k
mWCB (Power Prime)
1:30k
mWCB (2xYS)
Strain F only
Strain J only
5 – Strain F
6 – Strain F power mMCB
7 – Strain F 2xYS mMCB
8 – Strain F power mWCB
9 – Strain F 2xYS mWCB
10 – Strain J
11 – Strain J power mMCB
12 – Strain J 2xYS mMCB
13 – Strain J power mWCB
14 – Strain J 2xYS mWCB
One more propagation of this bank in Power Prime results in complete takeover of "bigs" (data not shown)

*FIG. 53*

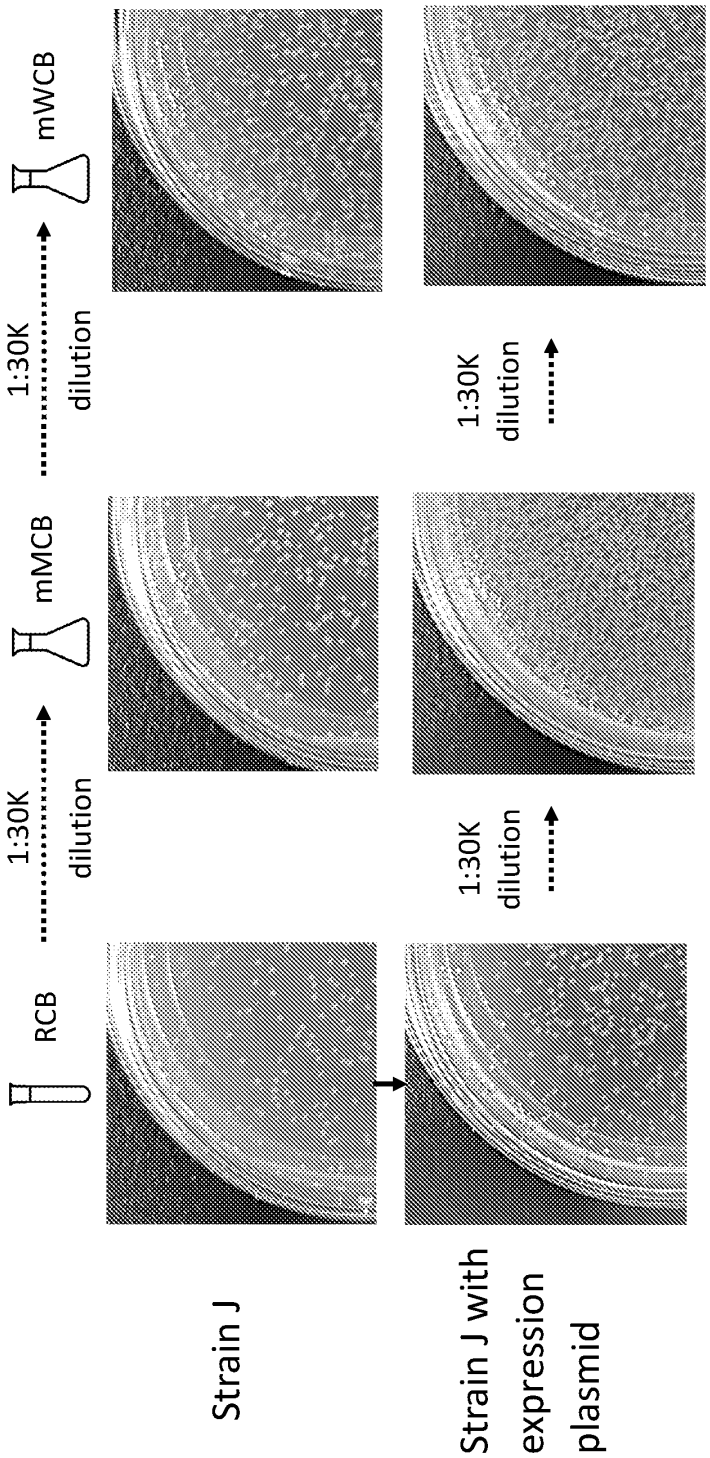

RCB
1:30K dilution
mMCB
1:30K dilution
mWCB
1:30K dilution
1:30K dilution
Strain J
Strain J with expression plasmid

*FIG. 54*

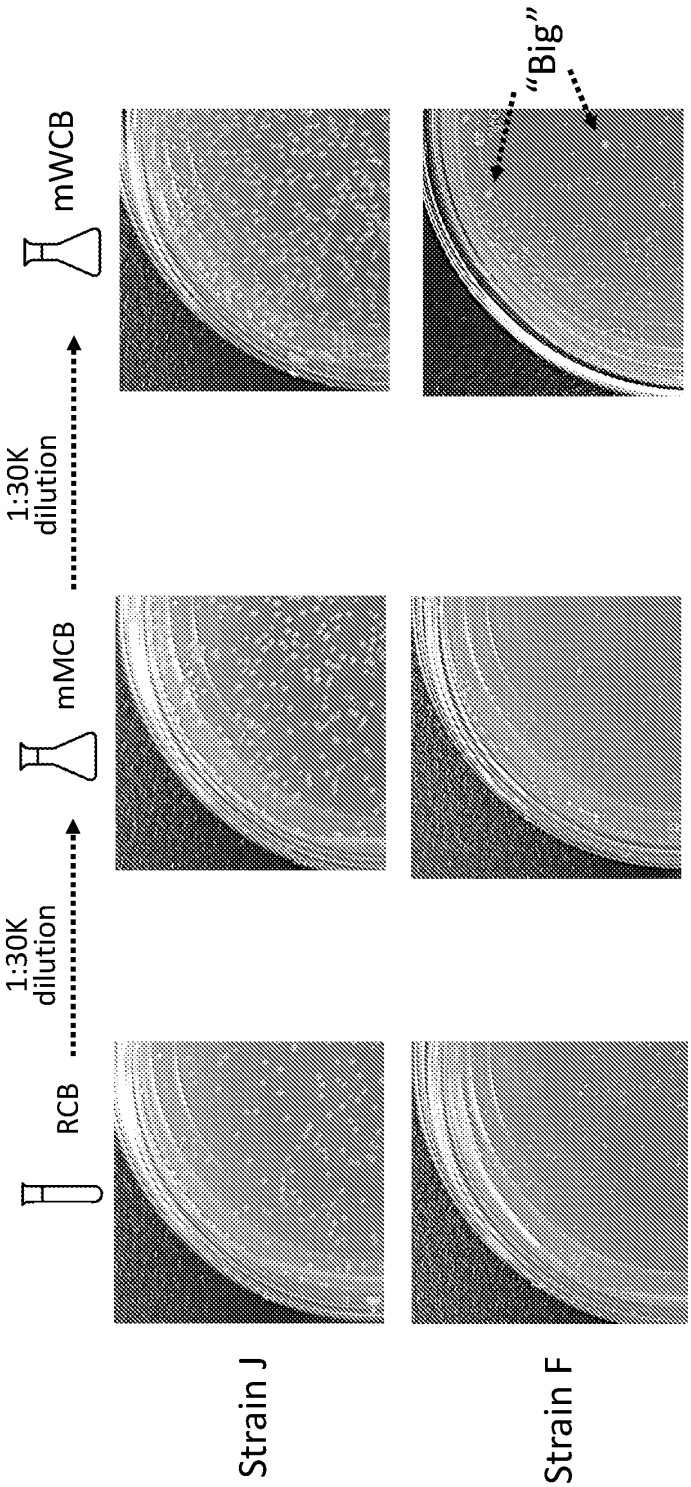

RCB
1:30K dilution
mMCB
1:30K dilution
mWCB
"Big"
Strain J
Strain F

*FIG. 55*

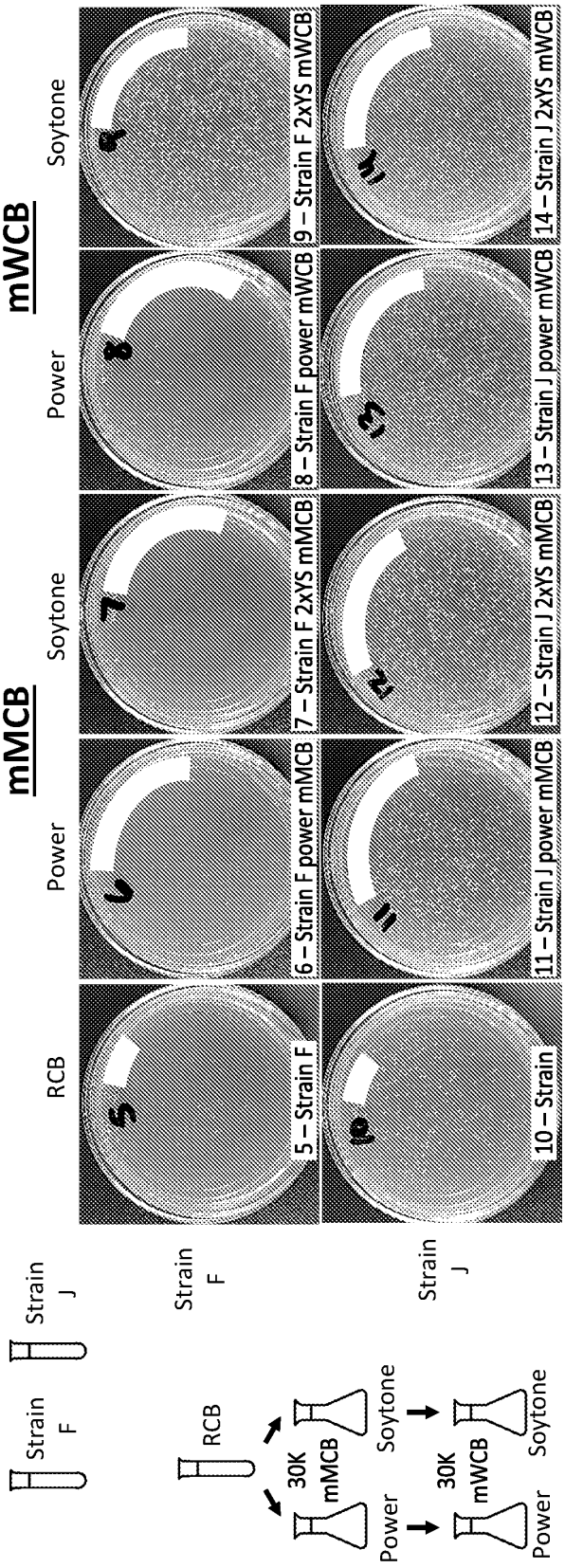

mMCB
mWCB
RCB
Power
Soytone
Power
Soytone
5 – Strain F
6 – Strain F power mMCB
7 – Strain F 2xYS mMCB
8 – Strain F power mWCB
9 – Strain F 2xYS mWCB
10 – Strain
11 – Strain J power mMCB
12 – Strain J 2xYS mMCB
13 – Strain J power mWCB
14 – Strain J 2xYS mWCB
Strain F
Strain J
Strain F
Strain J
RCB
30K mMCB
30K mWCB
Power
Soytone
Power
Soytone

*FIG. 56*

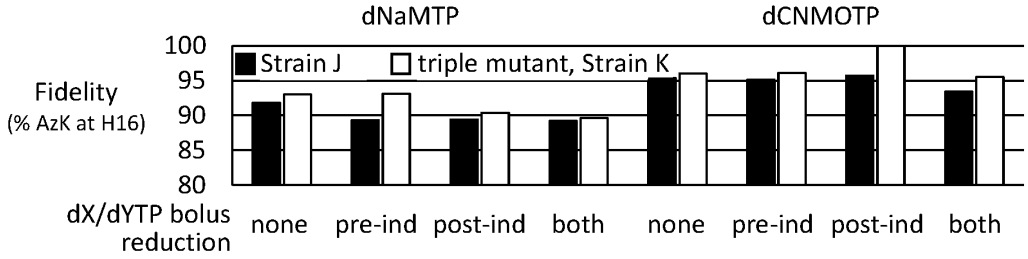



FIG. 57A

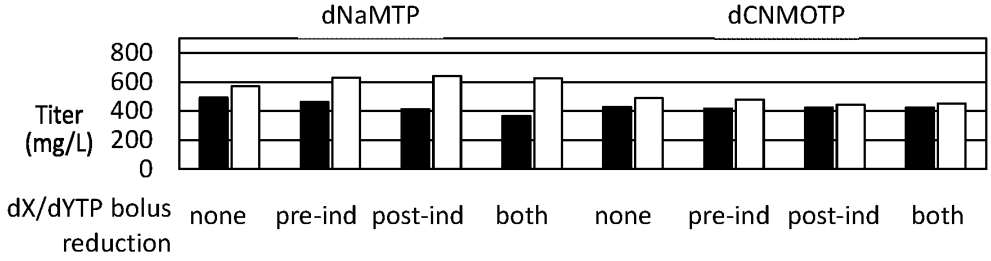



FIG. 57B

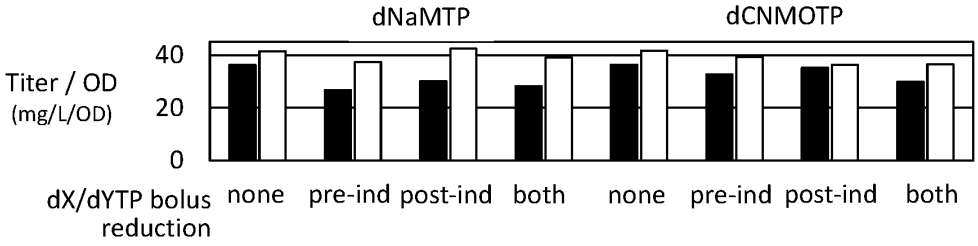



FIG. 57C

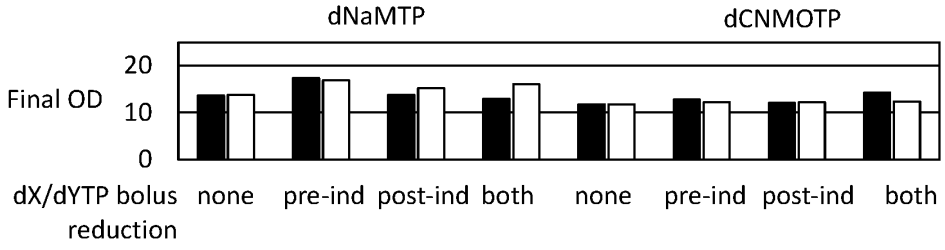



FIG. 57D

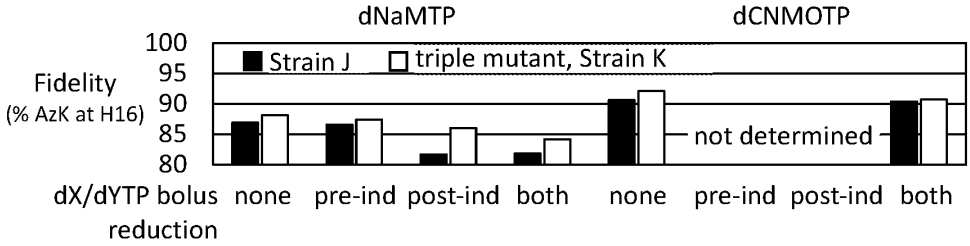



FIG. 58A

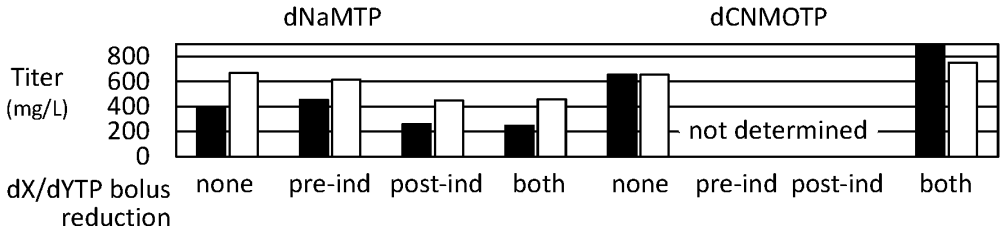



FIG. 58B

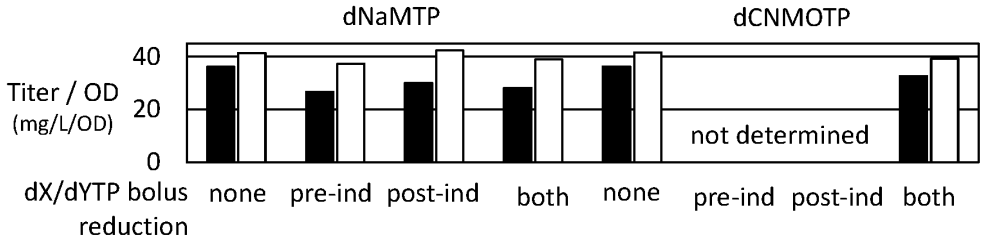



FIG. 58C

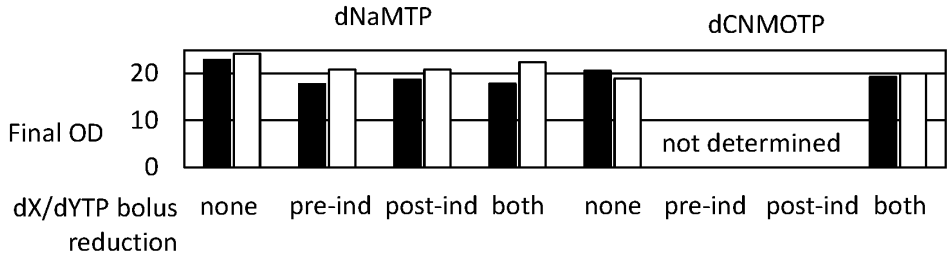
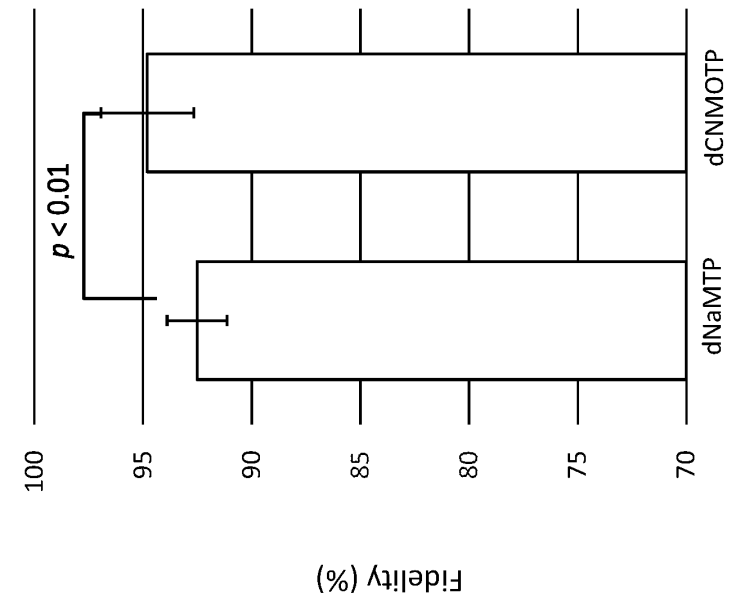
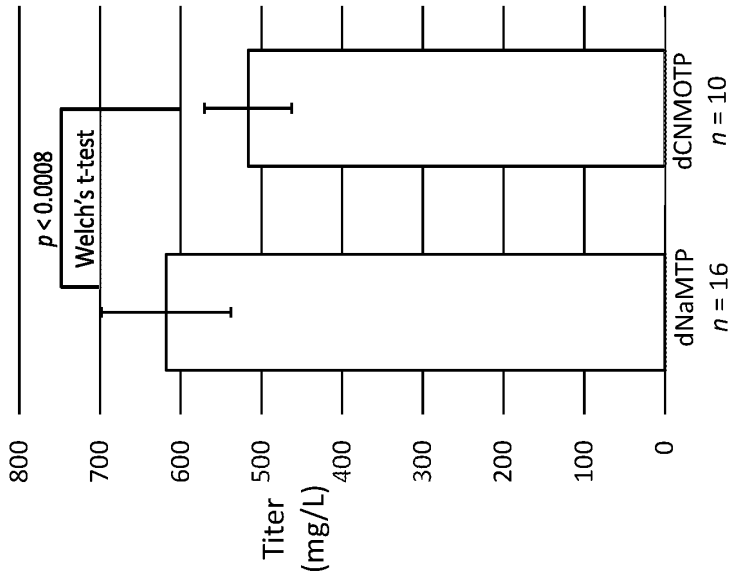



FIG. 58D


100
95
90
85
80
75
70
Fidelity (%)
p < 0.01
dNaMTP
dCNMOTP

F/G. 59B


800
700
600
500
400
300
200
100
0
Titer (mg/L)
p < 0.0008
Welch's t-test
dNaMTP
n = 16
dCNMOTP
n = 10

F/G. 59A

| Strain | Synthetase Promoter | [AzK] 1st bolus / [AzK] 2nd bolus (mM) | Induction OD | Final OD | OD Final / OD Induction | Titer (mg/L) | % Fidelity | Titer/OD |
|---|---|---|---|---|---|---|---|---|
| Strain J. Derivative | **TacO** | standard/standard (Platform Std) | 4.7 | 15.0 | 3.2 | 553 | 93.3 | 36.9 |
| | | standard/half (USP std) | 5.3 | 13.1 | 2.5 | 449 | 94.7 | 34.3 |
| | | half/half | 5.2 | 11.6 | 2.2 | 330 | 94.8 | 28.4 |
| | | half/quarter | 5.4 | 12.2 | 2.3 | 275 | 93.6 | 22.6 |
| Strain J. Derivative | **23119** (strongest known *E. coli* promoter) | standard/standard (Platform Std) | 5.4 | 14.9 | 2.8 | 526 | 93.5 | 35.2 |
| | | standard/half (USP std) | 5.5 | 12.3 | 2.7 | 394 | 93.7 | 32.0 |
| | | half/half | 4.6 | 12.6 | 2.3 | 358 | 93.1 | 28.3 |
| | | half/quarter | 4.9 | 10.3 | 2.1 | 242 | 93.4 | 23.4 |
| Strain J. Derivative | **0.26× T7** (~0.5× TacO) | standard/standard (Platform Std) | 4.8 | 13.0 | 2.7 | 459 | 92.9 | 35.4 |
| | | standard/half (USP std) | 4.8 | 13.1 | 2.7 | 396 | 92.4 | 30.1 |
| | | half/half | 4.9 | 11.8 | 2.4 | 297 | 91.2 | 25.2 |
| | | half/quarter | 4.7 | 11.6 | 2.5 | 239 | 90.7 | 20.7 |
| Strain J. Derivative | **3.65× T7** (~100× TacO) | standard/standard (Platform Std) | 5.0 | 12.8 | 2.5 | 387 | 91 | 30.2 |
| | | standard/half (USP std) | 4.9 | 10.5 | 2.3 | 269 | 91.5 | 25.6 |
| | | half/half | 4.6 | 9.7 | 2.0 | 233 | 89.2 | 24.0 |
| | | half/quarter | 4.5 | 10.8 | 2.4 | 166 | 90.1 | 15.3 |

*FIG. 60A*

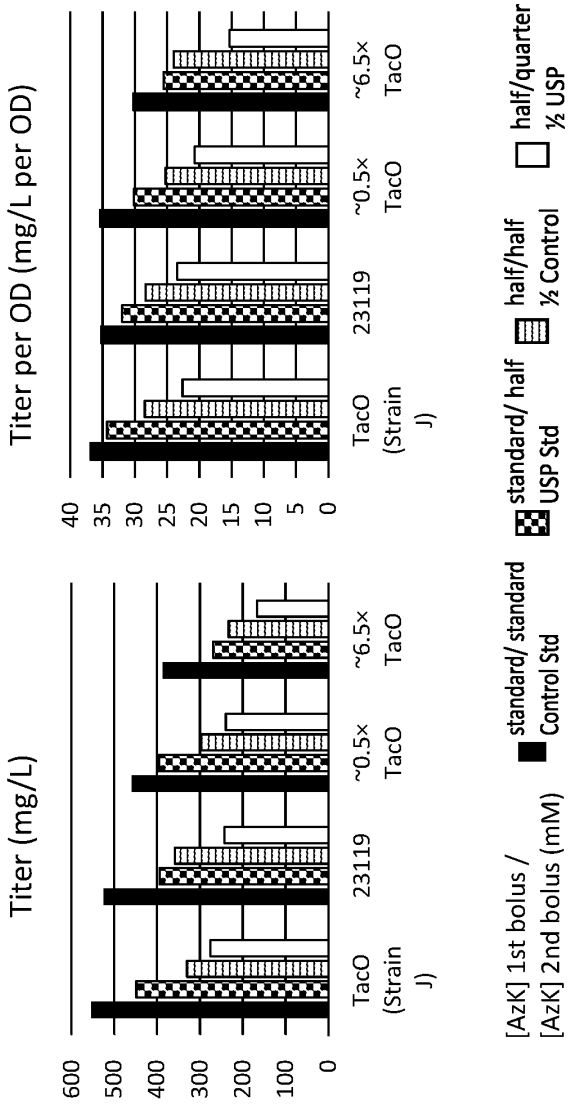



FIG. 60B




FIG. 60C

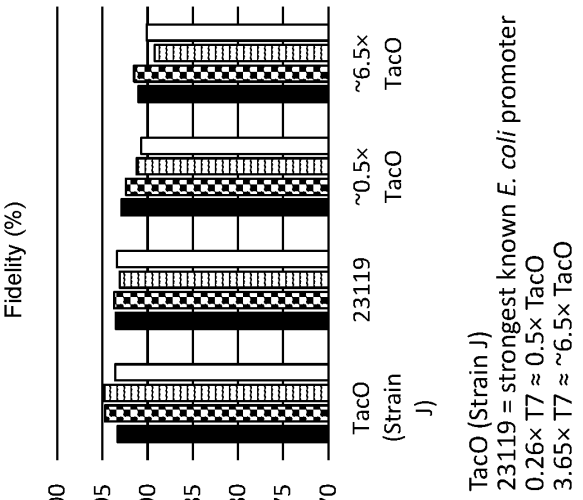



FIG. 60D