Highly Thermostable Kanamycin Resistance Marker Expands the Toolkit for Genetic Manipulation of *Caldicellulosiruptor bescii*

Gina L. Lipscomb[a], Jonathan M. Conway[b], Sara E. Blumer-Schuette[b]*, Robert M. Kelly[b] and Michael W. W. Adams[a]#

[a]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA
[b]Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695-7905

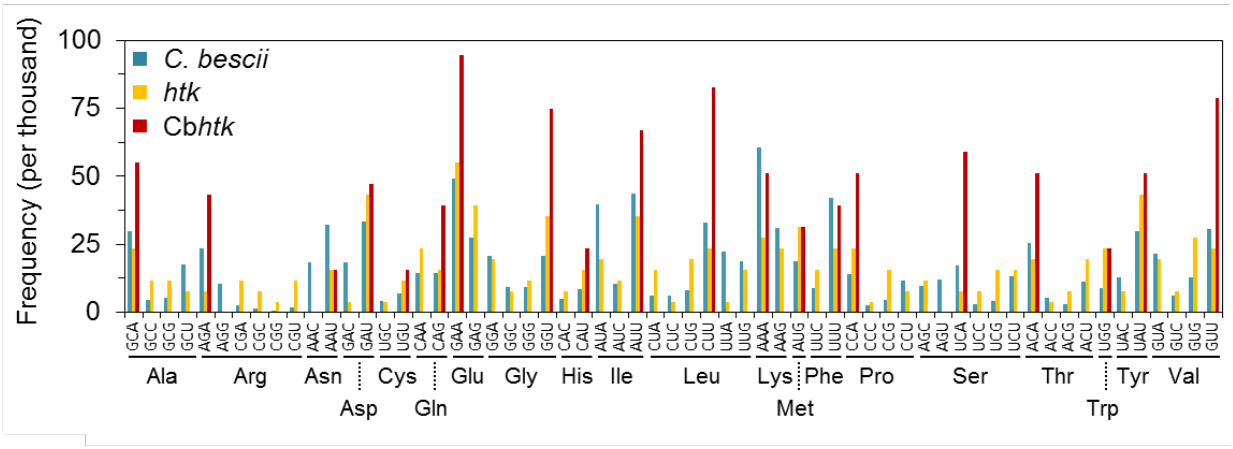
#Address correspondence to Michael W. W. Adams, adams@bmb.uga.edu.

*Current address: Department of Biological Sciences, Oakland University, Rochester, MI.

Supplemental Material:

Figure S1

A



B

```
ATGAAAGGTCCAATTATTATGACAAGAGAAGAAAGAATGAAAATTGTTCATGAAATTAAAGAAAGAATTCTTGATAAAT
ATGGTGATGATGTTAAAGCAATTGGTGTTTATGGTTCACTTGGTAGACAGACAGATGGTCCATATTCAGATATTGAAAT
GATGTGTGTTCTTTCAACAGAAGGTGTTGAATTTTCATATGAATGGACAACAGGTGAATGGAAAGCAGAAGTTAATTTT
TATTCAGAAGAAATTCTTCTTGATTATGCATCAAGAGTTGAACCAGATTGGCCACTTACACATGGTAGATTTTTTTCAA
TTCTTCCAATTTATGATCCAGGTGGTTATTTTGAAAAAGTTTATCAGACAGCAAAATCAGTTGAAGCACAGAAATTTCA
TGATGCAATTTGTGCACTTATTGTTGAAGAACTTTTTGAATATGCAGGTAAATGGAGAAATATTAGAGTTCAGGGTCCA
ACAACATTTCTTCCATCACTTACAGTTCAGGTTGCAATGGCAGGTGCAATGCTTATTGGTCTTCATCATAGAATTTGTT
ATACAACATCAGCATCAGTTCTTACAGAAGCAGTTAAACAGCCAGATCTTCCACCAGGTTATGTTCAGCTTTGTCAGCT
TGTTATGTCAGGTCAGCTTTCAGATCCAGAAAAACTTCTTGAATCACTTGAAAATTTTTGGAATGGTGTTCAGGAATGG
GCAGAAAGACATGGTTATATTGTTGATGTTTCAAAAAGAATTCCATTTTAA
```

Figure S1. Codon optimization of *htk* for use in *C. bescii*. (A) Codon usage for *C. bescii* genome, *htk* (from pUB110) and codon-optimized Cb*htk*. (B) Nucleotide sequence of codon-optimized Cb*htk*.

Figure S2

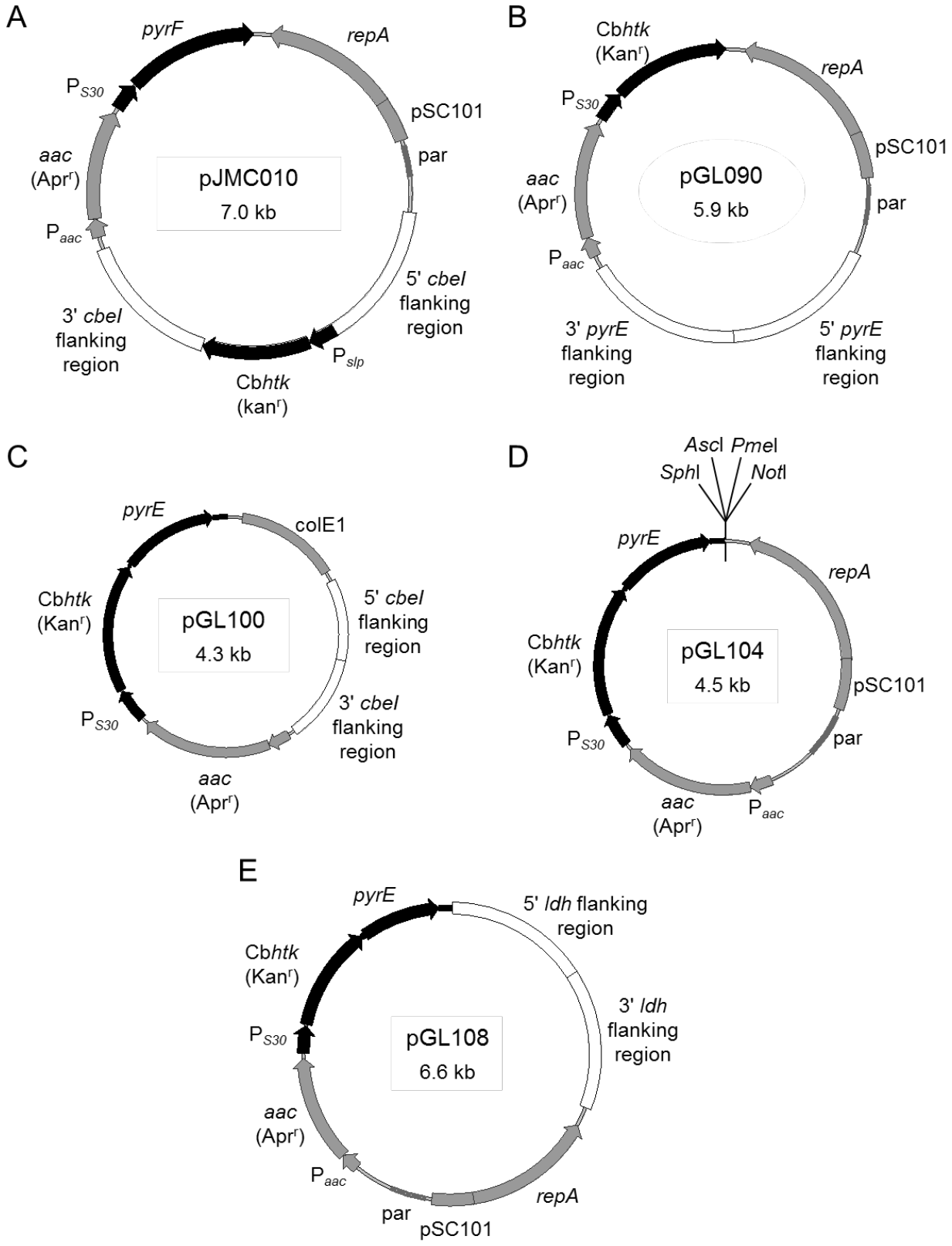

A
pyrF
repA
P S30
pSC101
par
aac
(Apr r)
pJMC010
7.0 kb
P aac
5' cbeI
flanking
region
3' cbeI
flanking
region
P slp
Cbhtk
(kan r)
B
Cbhtk
(Kan r)
repA
P S30
pSC101
aac
(Apr r)
pGL090
5.9 kb
par
P aac
3' pyrE
flanking
region
5' pyrE
flanking
region
C
pyrE
colE1
Cbhtk
(Kan r)
pGL100
4.3 kb
5' cbeI
flanking
region
3' cbeI
flanking
region
P S30
aac
(Apr r)
D
AscI PmeI
SphI
NotI
pyrE
repA
Cbhtk
(Kan r)
pGL104
4.5 kb
pSC101
P S30
par
aac
(Apr r)
P aac
E
pyrE
5' ldh flanking
region
Cbhtk
(Kan r)
3' ldh
flanking
region
P S30
pGL108
6.6 kb
aac
(Apr r)
P aac
repA
par
pSC101

Figure S2. Plasmids used in *C. bescii* strain construction. (A) Plasmid for insertion of Cb*htk* cassette into *C. bescii* JWCB018 chromosome at Δ*cbeI* locus using selections based on Cb*htk* and *pyrF*. (B) Plasmid for targeted deletion of *pyrE* from wild-type *C. bescii* using selection based on Cb*htk*. (C) Plasmid for deletion of *cbeI* in MACB1018 using selections based on Cb*htk* and *pyrE*. (D) Plasmid employing the Cb*htk-pyrE* cassette and containing a mini multiple cloning site. (E) Plasmid for deletion of *ldh* in MACB1018 using selections based on Cb*htk* and *pyrE*. For all plasmids shown, marker cassettes used for genetic selection in *C. bescii* are shown in black, *E. coli* elements are shown in grey, and flanking regions for homologous recombination are shown in white.

Figure S3

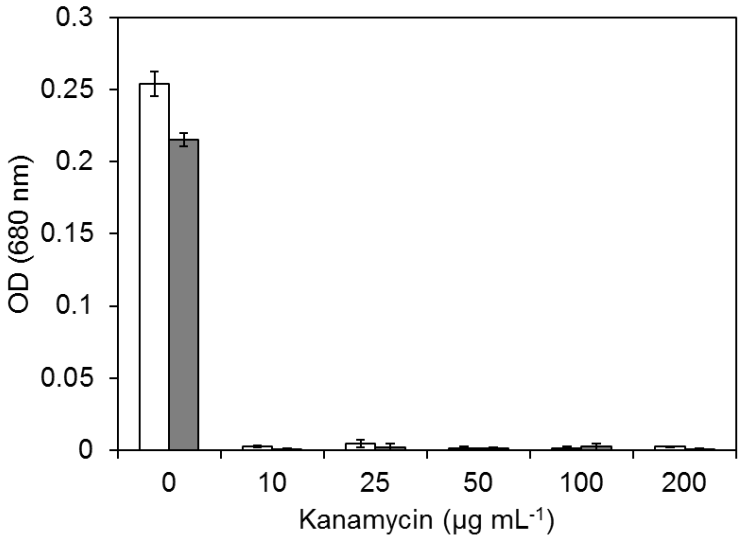


Figure S3. Kanamycin sensitivity of *C. bescii*. Wild-type (white bars) and JWCB018 (grey bars) were grown for 20 h in CC516 medium containing kanamycin at the indicated concentrations. Growth was measured by optical density at 680 nm. Error bars represent standard deviation; *n* = 3.

Figure S4

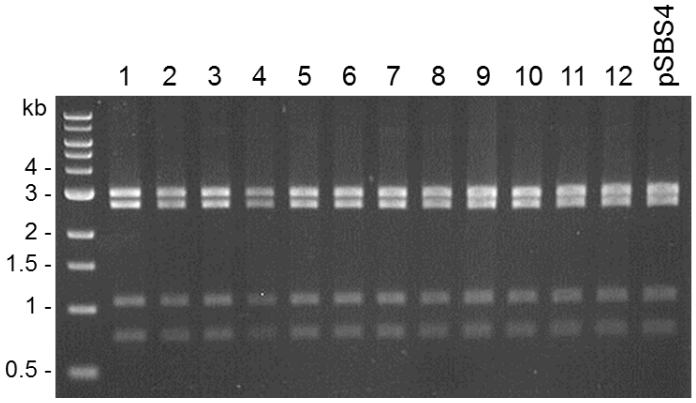


Figure S4. Confirmation of pSBS4 plasmid transformation of wild-type *C. bescii.* Total DNA from MACB1015 was transformed into *E. coli*, and plasmid isolates (1-12), along with purified pSBS4 as a control, were restriction digested with *Ava*I, resulting in expected fragment sizes of 3.0, 2.7, 1.1 and 0.8 kb.

Figure S5

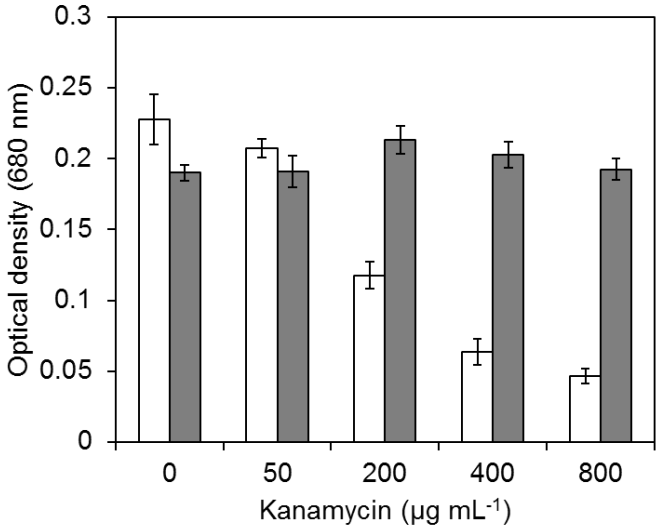


Figure S5 Kanamycin resistance of strains containing a Cb*htk* expression cassette. MACB1015 (white bars) and RKCB106 (grey bars) were grown for 20 h in CC516 medium with 20 μM uracil and containing kanamycin at the indicated concentrations. Growth was measured by optical density at 680 nm. Error bars represent standard deviation, *n* = 3.

Figure S6

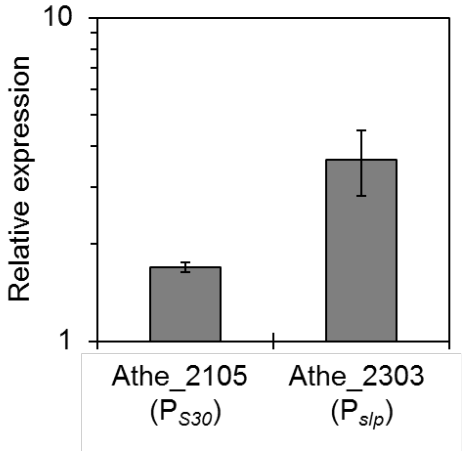


Figure S6. Expression levels of native genes downstream of P_{S30} and P_{slp} promoters. Wild-type *C. bescii* was grown to mid-log phase, RNA was extracted, and reverse-transcriptase quantitative PCR was performed using primer pairs targeting the Athe_2105 and Athe_2303 genes, downstream from the P_{S30} and P_{slp} promoters, respectively. Expression is shown relative to that of the control gene Athe_1406, encoding the glycolytic enzyme GAPDH. Error bars represent standard deviation, n = 2 technical replicates.