

A Highly Thermostable Kanamycin Resistance Marker Expands the Tool Kit for Genetic Manipulation of *Caldicellulosiruptor bescii*

Gina L. Lipscomb,^a Jonathan M. Conway,^b Sara E. Blumer-Schuette,^{b*} Robert M. Kelly,^b Michael W. W. Adams^a

Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia, USA^a; Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina, USA^b

ABSTRACT

Caldicellulosiruptor bescii, an anaerobic Gram-positive bacterium with an optimal growth temperature of 78°C, is the most thermophilic cellulose degrader known. It is of great biotechnological interest, as it efficiently deconstructs nonpretreated lignocellulosic plant biomass. Currently, its genetic manipulation relies on a mutant uracil auxotrophic background strain that contains a random deletion in the *pyrF* genome region. The *pyrF* gene serves as a genetic marker to select for uracil prototrophy, and it can also be counterselected for loss via resistance to the compound 5-fluoroorotic acid (5-FOA). To expand the *C. bescii* genetic tool kit, kanamycin resistance was developed as a selection for genetic manipulation. A codon-optimized version of the highly thermostable kanamycin resistance gene (named *Cbhtk*) allowed the use of kanamycin selection to obtain transformants of either replicating or integrating vector constructs in *C. bescii*. These strains showed resistance to kanamycin at concentrations > 50 μg · ml⁻¹, whereas wild-type *C. bescii* was sensitive to kanamycin at 10 μg · ml⁻¹. In addition, placement of the *Cbhtk* marker between homologous recombination regions in an integrating vector allowed direct selection of a chromosomal mutation using both kanamycin and 5-FOA. Furthermore, the use of kanamycin selection enabled the targeted deletion of the *pyrE* gene in wild-type *C. bescii*, generating a uracil auxotrophic genetic background strain resistant to 5-FOA. The *pyrE* gene functioned as a counterselectable marker, like *pyrF*, and was used together with *Cbhtk* in the Δ*pyrE* background strain to delete genes encoding lactate dehydrogenase and the *CbeI* restriction enzyme.

IMPORTANCE

Caldicellulosiruptor bescii is a thermophilic anaerobic bacterium with an optimal growth temperature of 78°C, and it has the ability to efficiently deconstruct nonpretreated lignocellulosic plant biomass. It is, therefore, of biotechnological interest for genetic engineering applications geared toward biofuel production. The current genetic system used with *C. bescii* is based upon only a single selection strategy, and this uses the gene involved in a primary biosynthetic pathway. There are many advantages with an additional genetic selection using an antibiotic. This presents a challenge for thermophilic microorganisms, as only a limited number of antibiotics are stable above 50°C, and a thermostable version of the enzyme conferring antibiotic resistance must be obtained. In this work, we have developed a selection system for *C. bescii* using the antibiotic kanamycin and have shown that, in combination with the biosynthetic gene marker, it can be used to efficiently delete genes in this organism.

Caldicellulosiruptor bescii is a thermophilic anaerobic Gram-positive bacterium with an optimal growth temperature of 78°C, and it has the ability to efficiently deconstruct nonpretreated lignocellulosic plant biomass (1, 2). It is, therefore, of biotechnological interest for genetic engineering applications geared toward biofuel production (3, 4). Such organisms require a tractable genetic system for metabolic engineering applications, and such a system has been established for *C. bescii* in recent years (5, 6). A major step in developing a genetic system for *C. bescii* was the discovery that it has a restriction-modification system that severely limits its transformability (7). This barrier to transformation was overcome through the use of a methylated donor plasmid with a methylation pattern that protected the transformed DNA from restriction digestion (5). The system was further improved by deleting the gene encoding the restriction enzyme, *CbeI*, making it possible to use nonmethylated donor DNA in transformation (6).

The current *C. bescii* genetic system is based upon use of the uracil biosynthetic pathway gene *pyrF* as a selectable marker for uracil prototrophy in a uracil auxotrophic mutant strain (5, 6). The *pyrF* gene encodes orotidine 5'-monophosphate (OMP) decarboxylase, which is required for uracil biosynthesis. This en-

zyme is also able to convert the pyrimidine analog 5-fluoroorotic acid (5-FOA) to the toxic product fluorodeoxyuridine (8). Strains deficient in OMP decarboxylase activity are, therefore, uracil auxotrophs and resistant to 5-FOA. In the development of *pyrF* as a counterselectable marker for *C. bescii*, mutant uracil auxotrophic strains of *C. bescii* were isolated using 5-FOA to select for cells with random mutations in the *pyrF* gene. The first strain isolated

Received 20 February 2016 Accepted 10 May 2016

Accepted manuscript posted online 13 May 2016

Citation Lipscomb GL, Conway JM, Blumer-Schuette SE, Kelly RM, Adams MWW. 2016. A highly thermostable kanamycin resistance marker expands the tool kit for genetic manipulation of *Caldicellulosiruptor bescii*. *Appl Environ Microbiol* 82:4421–4428. doi:10.1128/AEM.00570-16.

Editor: V. Müller, Goethe University Frankfurt am Main

Address correspondence to Michael W. W. Adams, adamsm@uga.edu.

* Present address: Sara E. Blumer-Schuette, Department of Biological Sciences, Oakland University, Rochester, Michigan, USA.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00570-16>.

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contained a deletion within *pyrB*, *pyrC*, and *pyrF* and required complementation with all three of these genes (5). A second uracil auxotrophic mutant was isolated that had a random deletion within *pyrF* and *pyrA*, and this strain, designated JWCB005, required complementation with only the *pyrF* gene (9).

The JWCB005 strain has become the basis for all genetically modified strains of *C. bescii* to date and has led to the development of genetic strategies and engineering efforts in this and related organisms. For example, it has been used in developing a replicating shuttle vector for *C. bescii* and other *Caldicellulosiruptor* species (9), and this vector has been used for homologous overexpression of the genes *celA*, encoding a bifunctional glycoside hydrolase (10), and *xor*, encoding a tungsten-dependent oxidoreductase (11). Numerous gene deletions in addition to *cbel* have also been constructed, including the *ldh* gene encoding lactate dehydrogenase (12), a gene cluster encoding pectin-degrading enzymes (13), the *celA* gene (14), and a gene cluster encoding [Ni-Fe] hydrogenase maturation enzymes (15). The JWCB005 genetic background has also been used in genetic engineering efforts to insert foreign gene expression constructs into the chromosome for production of ethanol (16, 17), for detoxification of furan aldehydes (18), and for enhanced capability to deconstruct crystalline cellulose (19) and xylan (20).

While the counterselectable *pyrF* marker is versatile and has been used in genetic systems developed for various thermophilic bacteria as well as for archaea (21, 22), there are certain advantages that can be gained with the additional option of genetic selection with an antibiotic. Antibiotic selection does not preclude the use of defined medium and can be used in conjunction with nutritional markers to increase selective pressure. For thermophilic organisms, the use of antibiotics presents somewhat of a challenge, as the number of antibiotics that have stability at temperatures $>50^{\circ}\text{C}$ is limited (23). Furthermore, to employ these antibiotics, there must also be a corresponding thermostable version of the enzyme conferring antibiotic resistance.

Once such antibiotic that has been demonstrated as sufficiently thermostable is kanamycin (23), and thermostable versions of the corresponding resistance marker are also available. The *Staphylococcus aureus* kanamycin resistance gene *knt*, encoding kanamycin nucleotidyltransferase (KNT), was passaged through an *Escherichia coli* mutator strain, and thermostable variants were selected in *Bacillus stearothermophilus* at temperatures up to 70°C (24). This KNT variant, which contained two amino acid substitutions, was further enhanced for thermostability at temperatures up to 80°C by directed evolution in *Thermus thermophilus*. The resulting protein contained 19 amino acid substitutions and was named HTK for highly thermostable kanamycin nucleotidyltransferase (25). These thermostable kanamycin resistance markers have been used in development of genetic systems for a number of thermophilic organisms, including *Moorella thermoacetica* at 55°C (26), *Thermoanaerobacter tengcongensis* at 60°C (27), *Geobacillus thermoglucosidasius* at 68°C (28), *Thermus thermophilus* at 70°C (29), and *Thermotoga* spp. at 77°C (30).

We show that kanamycin selection can also be applied in *C. bescii*, thereby expanding the genetic tool kit available for this organism. *Cbhtk*, a codon-optimized version of the *htk* gene, conferred kanamycin resistance to *C. bescii* and was used to construct an alternative genetic background strain based on a clean deletion of the *pyrE* gene in wild-type *C. bescii*. Deletion of *pyrE* resulted in a strain auxotrophic for uracil and resistant to 5-FOA. The *pyrE*

gene was applied as a marker, in addition to *Cbhtk*, and together these were used to construct additional deletions in this strain using kanamycin for selection of plasmid integration and 5-FOA for counterselection of plasmid loss, establishing this system as another method for making iterative chromosomal modifications in *C. bescii*.

MATERIALS AND METHODS

Growth of *C. bescii*. *C. bescii* was routinely grown under strict anaerobic conditions at 70 to 75°C . For genetic applications, *C. bescii* was cultured in low-osmolarity defined (LOD) medium (31) with argon used in the headspace. For growth of uracil auxotrophic strains, uracil was added at a concentration of 20 to $40\ \mu\text{M}$. When required, kanamycin was used at a concentration of $50\ \mu\text{g}\cdot\text{ml}^{-1}$, unless otherwise noted. Solid medium was prepared by mixing preheated $2\times$ LOD medium with an equal volume of 6% (wt/vol) agar. A volume of $100\ \mu\text{l}$ fresh culture or culture dilution was pipetted into an empty petri dish, and medium was poured into the plate while swirling to distribute the cells within the medium. Plates were allowed to solidify and cool $20\ \text{min}$, after which the plates were inverted, placed into anaerobic canisters flushed with argon, and incubated at 65 to 75°C for 4 days. Colonies were picked by stabbing through the agar with a toothpick and inoculated into $4\ \text{ml}$ medium in screw-cap Hungate tubes. Plating and colony picking were done either on the benchtop (aerobically) or in an anaerobic Coy chamber (for increased cell viability).

Growth experiments were performed in modified DSMZ 516 medium having the following composition per liter: $1\times$ salt solution, $1\times$ vitamin solution, $1\times$ trace element solution, $0.16\ \mu\text{M}$ sodium tungstate, $0.25\ \text{mg}$ resazurin, $5\ \text{g}$ cellobiose, $0.5\ \text{g}$ yeast extract, $1\ \text{g}$ cysteine hydrochloride, $1\ \text{g}$ sodium bicarbonate, and $1\ \text{mM}$ potassium phosphate buffer (pH 7.2). The $50\times$ stock salt solution contained the following per liter: $16.5\ \text{g}$ NH_4Cl , $16.5\ \text{g}$ KCl , $16.5\ \text{g}$ $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ and $7\ \text{g}$ $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$. Stock solutions of $200\times$ vitamins and $1,000\times$ trace elements were prepared as previously described (2). This complex medium containing cellobiose as the carbon source is referred to as CC516. A defined version of this medium was prepared by excluding the yeast extract and supplementing with uracil when necessary, and this medium is referred to as DC516. Culture headspace used with CC516 and DC516 media was composed of 80% N_2 and 20% CO_2 .

Vector construction. The *htk* gene sequence (from pUB110; GenBank accession no. AB121443) was codon optimized for use in *C. bescii* and is referred to as *Cbhtk*. Codons were changed to those that occur most frequently in *C. bescii* genes, thereby eliminating any potential rare codons (see Fig. S1 in the supplemental material). The codon-optimized *Cbhtk* gene was synthesized by GeneArt gene synthesis (Thermo Fisher Scientific, Inc.). The pSBS4 replicating shuttle vector employing the *Cbhtk* gene (Fig. 1) was constructed by replacing the *pyrF* gene with *Cbhtk* in pDCW89 (9) via Gibson assembly (32). The *Cbhtk* cassette, unless otherwise noted, employs the promoter region of *Athe_2105* (6), a hypothetical conserved protein annotated as sigma 54 modulation protein/ribosomal protein S30EA; this promoter is referred to as P_{S30} .

The pJMC010 plasmid (see Fig. S2 in the supplemental material) for insertion of P_{slp} *Cbhtk* at the Δ *cbel* locus was constructed via Gibson assembly (32) of the following PCR products: the plasmid backbone, including the *pyrF* cassette and *E. coli* elements (amplified from pDCW121 [12]), the 200 -bp promoter region immediately upstream of the *slp* gene (*Athe_2303*), the *Cbhtk* gene, and ~ 1 -kb flanking regions to *cbel*. The *Cbhtk* gene was placed under the control of the P_{slp} promoter, and the P_{slp} *Cbhtk* cassette was placed between the *cbel* flanking regions. The plasmid was found to contain a mutation in the *htk* gene (L227I) which did not appear to affect the kanamycin resistance of the strain generated using this plasmid.

For deletion of *pyrE* (*Athe_1382*), pGL090 (see Fig. S2 in the supplemental material) was constructed via Gibson assembly (New England BioLabs) of the following PCR products: ~ 1 -kb upstream and downstream flanking regions to *pyrE* and the *Cbhtk* cassette and *E. coli*

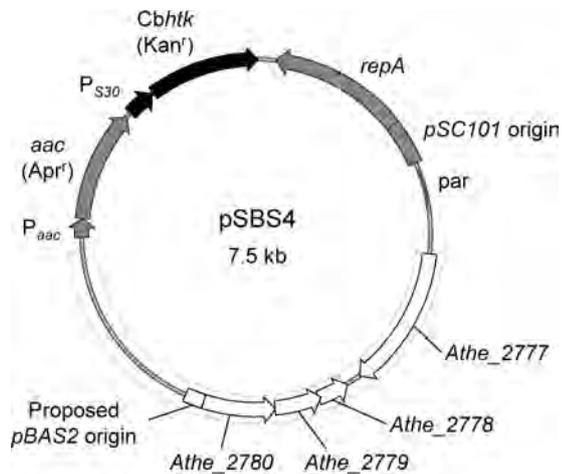


FIG 1 Replicating shuttle vector with *Cbh tk* cassette for selection of transformants via kanamycin resistance. Plasmid map of pSBS4 with *Cbh tk* cassette (black), *E. coli* elements (gray), and *C. bescii* elements (white) from native plasmid pBAS2/pATHE02 (delineated by gray line).

elements amplified from pSBS4. For deletion of *cbel* (Athe_2438), pGL100 (see Fig. S2) was constructed via Gibson assembly (New England BioLabs) of the following PCR products: ~0.5-kb upstream and downstream flanking regions to *cbel*, the *Apr^r* (apramycin resistance) and *Kan^r* (kanamycin resistance) markers (amplified from pGL090), the *E. coli* ColE1 replication origin (amplified from pSET152), and the *pyrE* gene. The *pyrE* gene was placed immediately downstream from the *Cbh tk* gene in a synthetic operon, referred to as the *Cbh tk-pyrE* cassette. A 20-bp ribosomal binding site region (5'-aggaggttggtagtagt), taken from immediately upstream of the *slp* gene (Athe_2303), was placed between *Cbh tk* and *pyrE*. A terminator region was placed at the end of the operon and consisted of 122 bp from the 3' untranslated region of the *slp* gene, wherein lies a predicted rho-independent terminator (33). The general-purpose cloning plasmid, pGL104 (see Fig. S2), was constructed via Gibson assembly (New England BioLabs) of the following PCR products: the *Cbh tk-pyrE* cassette, a mini multiple cloning site containing restriction sites that are rare in *C. bescii* (SphI-AscI-PmeI-NotI), and *E. coli* plasmid elements (*Apr^r* cassette and pSC101 origin). For deletion of *ldh* (Athe_1918), flanking regions of ~1 kb on either side of the *ldh* gene were combined via splice-overlap extension PCR (34) and cloned into pGL104 via the SphI and AscI restriction sites to generate pGL108 (see Fig. S2). All plasmids were sequence verified.

Plasmid DNA methylation. For transformation of *C. bescii* wild type (DSM 6725) and the strain MACB1018, plasmid DNA was methylated with recombinant CbeI methyltransferase (M.CbeI). The *E. coli* strain JW284, harboring the M.CbeI expression plasmid (pDCW73), was used for heterologous expression of tagged M.CbeI, as previously described (5). M.CbeI was purified from cell lysate using the His-Spin protein miniprep kit (Zymo Research). Plasmid DNA was subjected to methylation by M.CbeI followed by purification, essentially as previously described (5).

***C. bescii* transformation and strain construction.** For competent cell preparation and transformation, *C. bescii* strains were inoculated to a cell density of 6×10^5 to 8×10^5 cells \cdot ml⁻¹ into 500 ml LOD medium containing amino acids (LOD-AA) (31) and grown at 70°C to an optical density at 680 nm (OD₆₈₀) of 0.06 to 0.07. Cultures were cooled for 10 min in a bath of running cool water. Cells from a 500-ml culture were harvested at $6,000 \times g$ for 10 min. Cell pellets were washed twice consecutively in 250 ml of 10% (wt/vol) sucrose, with or without gentle suspension, and centrifugation was performed at $6,000 \times g$ for 10 min each time. Cells were washed a third time with 50 ml of 10% (wt/vol) sucrose, after which cells were suspended in 1 ml of 10% (wt/vol) sucrose, transferred to a microcentrifuge tube, and centrifuged at 14,000 rpm for 30 s. Most of the

supernatant was removed, and competent cells were gently suspended to a final volume of 100 to 125 μ l in 10% (wt/vol) sucrose. Plasmid DNA (0.5 to 1 μ g) was added in a volume of up to 5 μ l to a 50- μ l aliquot of competent cells, and this mixture was then transferred to a 1 mM gap electro- poration cuvette. Cells were electroporated in a Bio-Rad gene pulser using the following conditions: 1.8 kV, 400 Ω , and 25 μ F. Immediately after electroporation, cells were transferred to 20 ml of LOC medium (31) preheated to 70°C, and these recovery cultures were incubated at 70°C for 1 to 4 h. For selection of transformants, aliquots from the recovery cultures were periodically removed and subcultured into low-osmolarity complex (LOC) medium (31) containing 50 μ g \cdot ml⁻¹ kanamycin, using a 10% inoculum. All centrifugation, wash, and electroporation steps were performed under aerobic conditions at room temperature. Transformants were colony purified at least once on solid LOD medium containing 50 μ g \cdot ml⁻¹ kanamycin.

C. bescii strains used and constructed in this study are listed in Table 1. *C. bescii* JWCB018 (Δ *pyrFA* Δ *cbel*) and the wild type (using methylated plasmid) were transformed with the replicating shuttle vector pSBS4 to generate strains resistant to kanamycin. The wild-type transformation isolate was purified once on solid LOD medium containing 50 μ g \cdot ml⁻¹ kanamycin and designated MACB1015. The presence of the replicating pSBS4 plasmid was verified using total DNA isolated from MACB1015 to transform *E. coli* so that isolated plasmids could be restriction digested and compared to the original transformation plasmid. JWCB018 was transformed with pJMC010 for insertion of *Cbh tk* at the Δ *cbel* locus. Counterselection for plasmid loss was performed on LOD solid medium containing 4 mM 5-FOA, 40 μ M uracil, and 10 μ g \cdot ml⁻¹ kanamycin. The isolate containing *Cbh tk* inserted at the Δ *cbel* locus was purified twice on solid LOD medium containing 40 μ M uracil and 25 μ g \cdot ml⁻¹ kanamycin, and the strain was designated RKCB106. Wild-type *C. bescii* was transformed with methylated pGL090, which targeted integration at the *pyrE* locus. Counterselection on LOD solid medium containing 4 mM 5-FOA and 40 μ M uracil resulted in plasmid loss and *pyrE* deletion. Isolates were further purified once on solid LOD medium containing 40 μ M uracil. The deleted *pyrE* gene was confirmed by sequencing the locus in five isolates, and one isolate was designated MACB1018. The Δ *pyrE* strain MACB1018 was transformed with methylated pGL100 (for deletion of *cbel*) or pGL108 (for deletion of *ldh*), which each contained the *Cbh tk* marker for transformation selection using kanamycin and the *pyrE* marker for counterselection using 5-FOA. Counterselection and purification were performed as described above for MACB1018. Strains containing deletions of *cbel* and *ldh* were designated MACB1032 and MACB1034, respectively. For routine screening of cultures, genomic DNA was isolated using the ZymoBead genomic DNA kit (Zymo Research), with an added brief sonication step to improve cell lysis. All chromosomal mutations were verified by PCR screening and sequencing.

RESULTS AND DISCUSSION

Sensitivity of *C. bescii* to kanamycin. The antibiotic kanamycin is widely used in bacterial genetic systems and is well suited for application in thermophilic bacteria due to its high stability

TABLE 1 *C. bescii* strains used and constructed in this study

Strain designation	Parent	Transforming plasmid	Description	Reference
DSM 6725			Wild type	2
JWCB018	JWCB005	pDCW88	Δ <i>pyrFA</i> Δ <i>cbel</i>	6
MACB1015	DSM 6725	pSBS4	pSBS4	This study
RKCB106	JWCB018	pJMC010	Δ <i>pyrFA</i> Δ <i>cbel</i> ::P _{<i>slp</i>} <i>Cbh tk</i>	This study
MACB1018	DSM 6725	pGL090	Δ <i>pyrE</i>	This study
MACB1032	MACB1018	pGL100	Δ <i>pyrE</i> Δ <i>cbel</i>	This study
MACB1034	MACB1018	pGL108	Δ <i>pyrE</i> Δ <i>ldh</i>	This study

over a range of temperatures and pH values (23). Thermostable versions of the *S. aureus* kanamycin nucleotidyltransferase gene (*knt*) have been developed (24, 25), and these have been used in a number of thermophilic bacteria, e.g., species of *Thermoanaerobacter* (27), *Geobacillus* (28), *Thermus* (29), and *Thermotoga* (30). To determine if kanamycin would be an option for selection in *C. bescii*, the sensitivity of *C. bescii* to kanamycin was determined. Wild-type and JWCB018 ($\Delta pyrFA \Delta cbeI$) (6) strains of *C. bescii* were inoculated into medium containing kanamycin at concentrations ranging from 10 to 200 $\mu\text{g} \cdot \text{ml}^{-1}$, and these cultures were incubated at 75°C to late stationary phase (20 h). Growth of both wild type and JWCB018 was inhibited by 10 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin (see Fig. S3 in the supplemental material), making kanamycin selection a viable option for further development in *C. bescii*.

Use of the thermostable *htk* gene in transformation of *C. bescii*. To test the possibility of employing kanamycin as a selection in *C. bescii*, the highly thermostable kanamycin nucleotidyltransferase (HTK), having increased thermostability up to 80°C (25), was chosen to test as a selectable marker. The sequence of the *htk* gene was optimized to use only the most frequently occurring codons in *C. bescii* (see Fig. S1 in the supplemental material). A replicating shuttle vector employing the *Cbhtk* gene was constructed by replacing the *pyrF* gene with *Cbhtk* in pDCW89 (9), thereby generating pSBS4 (Fig. 1). *C. bescii* contains a restriction modification system that severely affects its transformability, such that methylation of transforming DNA using the corresponding methylase is necessary (5). This barrier to transformation was overcome by deletion of the gene encoding the restriction enzyme *CbeI*, generating the JWCB018 strain (6). We selected this strain to use for transformation trials with pSBS4. As a positive control for transformation, the pDCW89 replicating shuttle vector (9) was used with uracil prototrophic selection. After electroporation, samples from the recovery cultures were inoculated into LOD medium either lacking uracil, for selection of pDCW89 transformants, or containing uracil and 50 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin, for selection of pSBS4 transformants. Growth was routinely observed in these selective outgrowth cultures for both pDCW89 and pSBS4 electroporation trials, indicating that the *Cbhtk* gene was functional in conferring kanamycin resistance to *C. bescii*. Accordingly, wild-type *C. bescii* competent cells were prepared and electroporated with pSBS4 that had been methylated with heterologously expressed and purified *M.CbeI* (5), and similar results were observed. A purified pSBS4 transformation isolate was designated MACB1015. To confirm the presence of the replicating plasmid, total DNA from MACB1015 was transformed into *E. coli*, and 12 plasmid isolates were screened by restriction digestion. All isolates had restriction digestion patterns that were identical to pSBS4, indicating that the intact pSBS4 plasmid was present in the strain and that no plasmid rearrangement had taken place (see Fig. S4 in the supplemental material).

A major advantage of antibiotic selection versus uracil prototrophic selection is that there is no concern about nutrient carryover from the postelectroporation, rich recovery medium into defined selective medium. For uracil prototrophic selection, only ~1 to 2% inoculum can be used from the recovery culture into defined medium lacking uracil to prevent the carryover of too much uracil that would negate the selection. For kanamycin selection, up to 10% inoculum can be used, allowing 100% of the recovery culture to be subcultured into kanamycin-containing

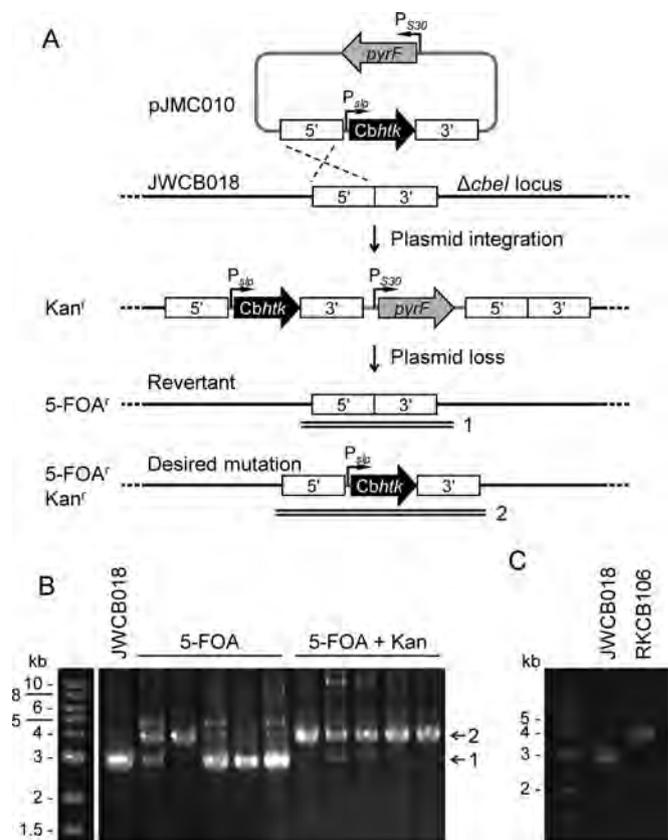


FIG 2 Direct selection of chromosomal mutations using *Cbhtk*. (A) Scheme showing selection method for targeted insertion of the P_{slp} *Cbhtk* expression cassette (black) by homologous recombination (dashed lines) into the $\Delta cbeI$ locus in *C. bescii* strain JWCB018 ($\Delta pyrFA$). Kanamycin selected for plasmid integration. To resolve the second crossover event, 5-FOA selected only for plasmid loss, and 5-FOA with kanamycin selected for plasmid loss while maintaining the inserted *Cbhtk* cassette. Numbered double lines indicate the locations of confirmation PCR products shown in B. (B) PCR screening of isolates counterselected for a second crossover event using solid LOD medium containing 4 mM 5-FOA with or without 10 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin. Colonies that resulted from 5-FOA selection were grown in LOD medium with 40 μM uracil, while colonies that resulted from 5-FOA⁺ and Kan^r selection were grown in LOD medium with 40 μM uracil and 25 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin. The *Cbhtk* knock-in had an expected amplicon size of 3.9 kb (2) compared to 2.9 kb (1), which was the expected size for the JWCB018 strain. (C) PCR confirmation of P_{slp} *Cbhtk* insertion at the $\Delta cbeI$ locus in RKC106 compared to parent strain JWCB018. PCR products shown in B and C were amplified with primers outside the *cbeI* flanking regions used for homologous recombination.

liquid medium without the experiment becoming too cumbersome.

Use of *Cbhtk* for direct selection of chromosomal mutations. Due to the low efficiency of transformation and recombination, introduction of a chromosomal mutation in *C. bescii* requires the use of a circular plasmid as opposed to linear DNA. A selection is used for plasmid integration into the chromosome, and a counterselection is used for resolving a second crossover event to achieve the desired mutation. Once integration of a plasmid containing the *pyrF* marker is obtained, counterselection for loss of *pyrF* using 5-FOA can yield the desired second crossover event or can result in reversion to the original state.

Placement of the *Cbhtk* marker between the flanking regions for homologous recombination allows for direct selection of only

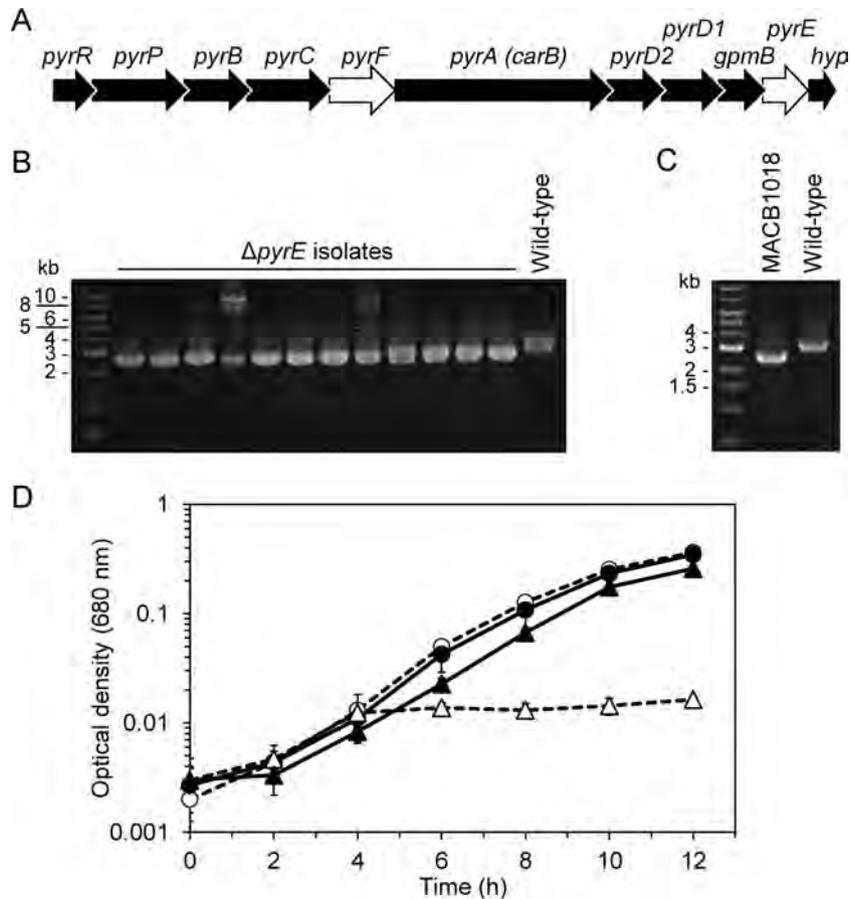


FIG 3 Deletion of *pyrE* in *C. bescii* confers uracil auxotrophy. (A) Uracil biosynthetic operon in *C. bescii* (Athe_1373 to Athe_1383). Gene symbols stand for the following enzymes: *pyrR*, uracil phosphoribosyltransferase; *pyrP*, uracil-xanthine permease; *pyrB*, aspartate carbamoyltransferase catalytic subunit; *pyrC*, dihydroorotase; *pyrF*, OMP decarboxylase; *pyrA* (also known as *carB*), carbamoyl phosphate synthase large subunit; *pyrD2*, dihydroorotate dehydrogenase electron transfer subunit; *pyrD1*, dihydroorotate dehydrogenase (NAD⁺) catalytic subunit; *gpmB*, phosphoglycerate mutase (glycolysis); *pyrE*, orotate phosphoribosyltransferase; and *hyp*, hypothetical protein. Targets for uracil auxotrophic selection and 5-FOA counterselection are indicated in white. (B) PCR screen of the *pyrE* locus in 5-FOA-resistant isolates, confirming successful *pyrE* deletion (2.5 kb) compared to wild type (3.0 kb). In two isolates, there are bands indicating that a single crossover of the pGL090 *pyrE* deletion plasmid is still present (8.9 kb). (C) PCR confirmation of *pyrE* gene deletion in MACB1018 (2.5 kb) versus wild type (3.0 kb). PCR products shown in B and C were amplified with primers outside the *pyrE* flanking regions used for homologous recombination. (D) Growth curve of wild-type strain (circles) and Δ *pyrE* strain MACB1018 (triangles) on DC516 medium either containing (closed symbols, solid lines) or lacking (open symbols, broken lines) 20 μ M uracil. Error bars represent standard deviation; $n = 3$.

the desired second crossover event, resulting in gene insertion and/or deletion. This direct selection method was demonstrated by using pJMC010 to insert *Cbhtk* at the Δ *cbeI* locus (Athe_2438) in *C. bescii* strain JWCB018 (Δ *pyrFA* Δ *cbeI*). The pJMC010 plasmid contained the P_{slp} *Cbhtk* cassette between flanking regions to the *cbeI* locus and also contained the *pyrF* cassette on the plasmid backbone. Chromosomal integration of the plasmid was selected via kanamycin resistance, and the second crossover event was resolved by using both 5-FOA and kanamycin to select for loss of the *pyrF* marker while retaining the *Cbhtk* marker (Fig. 2A). When counterselection for plasmid loss was performed in the presence of 5-FOA and kanamycin, all five screened isolates contained the desired mutation, although two also contained faint bands for the full plasmid as well as the wild-type allele. When selection with only 5-FOA was performed, four out of five screened isolates had predominant bands indicating revertants (Fig. 2B). One Δ *cbeI*:: P_{slp} *Cbhtk* isolate was further colony purified on medium containing kanamycin, and this strain was designated RKC106 (Fig. 2C). For *C. bescii*, it is not uncommon for the initial screening of trans-

formants or 5-FOA isolates to display bands indicating the presence of two alleles (the wild type and the desired allele) or the integrated plasmid. Typically, the purest isolates are chosen for moving forward, and colony purification with routine PCR screening results in final strains that are pure.

Kanamycin resistance of *Cbhtk*-containing strains. Since *C. bescii* is highly sensitive to kanamycin, it was of interest to test the kanamycin resistance level in strains containing the *Cbhtk* marker. Two strains were used: the MACB1015 strain containing the *Cbhtk* cassette expressed on a replicating shuttle vector and the RKC106 strain containing a genome-integrated copy of the P_{slp} *Cbhtk* cassette. When cultivated in medium containing various concentrations of kanamycin up to 800 μ g \cdot ml⁻¹ for 20 h, the RKC106 strain reached the same cell density as the control culture lacking kanamycin. The MACB1015 strain showed decreased cell density at concentrations ≥ 200 μ g \cdot ml⁻¹ kanamycin (see Fig. S5 in the supplemental material). The difference in the levels of kanamycin resistance between the two strains may be due to the location of *Cbhtk* expression (replicating plasmid versus

chromosome). The copy number of the pSBS4 shuttle vector is 1:1 with the chromosome, assuming it is the same as that previously published for pDCW89, upon which it is based (9); however, little is known about the replication origin of this plasmid and how plasmid partitioning to daughter cells is controlled. Differing expression levels of the *Cbhtk* marker (P_{S30} versus P_{slp}) is also a possible contributing factor. The native genes downstream of both promoters are expressed at or above the expression level of the gene encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, with the native gene for P_{slp} being expressed about 2-fold higher than that of P_{S30} (see Fig. S6 in the supplemental material).

Use of kanamycin selection for deletion of *pyrE*. Uracil auxotrophic strains can be generated by deletion of a number of genes encoding enzymes within the uracil biosynthetic pathway. Two of these can also serve as counterselectable markers: the *pyrF* and *pyrE* genes, encoding OMP decarboxylase and orotate phosphoribosyltransferase, respectively. It has been shown in *Saccharomyces cerevisiae* and in various bacteria and archaea that deletion of the genes encoding these enzymes can confer resistance to 5-FOA, an analog of a uracil biosynthetic intermediate (8, 21, 22, 35).

In *C. bescii*, the genes encoding enzymes in the uracil biosynthetic pathway are arranged in an 11-gene putative operon (Fig. 3A). The *pyrF* gene lies in the middle of the putative operon, while the *pyrE* gene is located near the end of the operon, with only one gene encoding a small hypothetical protein lying immediately downstream from it. We therefore selected *pyrE* as a target for deletion due to the lower possibility of detrimental polar effects arising during strain construction (due to integration of the entire deletion plasmid into the middle of the operon). The *Cbhtk* cassette was used in a vector designed with ~1-kb flanking regions to the *pyrE* gene (see Fig. S2 in the supplemental material). Wild-type *C. bescii* was transformed with methylated plasmid DNA. After electroporation, cells were transferred to recovery medium and incubated at 72°C for 2 h, during which time samples were withdrawn and inoculated into medium containing 50 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin for outgrowth. Genomic DNA was extracted from cultures that were able to grow in kanamycin-containing medium, and PCR screening confirmed the presence of the *Cbhtk* construct at the *pyrE* locus. These single-crossover isolates were counterselected for plasmid loss and *pyrE* deletion using solid medium containing 5-FOA and uracil. Colonies were subcultured into liquid medium containing uracil, and all 12 screened isolates contained a deletion of *pyrE* (Fig. 3B), indicating that loss of *pyrE* in *C. bescii* confers resistance to 5-FOA. After further colony purification and sequence verification, one $\Delta pyrE$ isolate was designated MACB1018 (Fig. 3C). Growth of wild-type *C. bescii* and MACB1018 in defined medium with and without supplemental uracil showed that MACB1018 exhibits uracil auxotrophy, which can be attributed to loss of the *pyrE* gene (Fig. 3D). The initial minimal growth of MACB1018 (to an OD_{680} of 0.016) can be attributed to uracil carryover from the inoculum culture.

Use of *pyrE* as a selectable marker in a $\Delta pyrE$ background strain. In order to demonstrate that the *pyrE* gene can be used as a selectable marker in a *pyrE*-deficient background strain, we chose two gene targets for deletion, *cbeI* (Athe_2438) and *ldh* (Athe_1918), both of which have previously been successfully deleted in the JWCB005 background (6, 12). Although *pyrE* should function in uracil prototrophic selection similar to *pyrF*, we chose to use *Cbhtk* in conjunction with kanamycin as the selection for

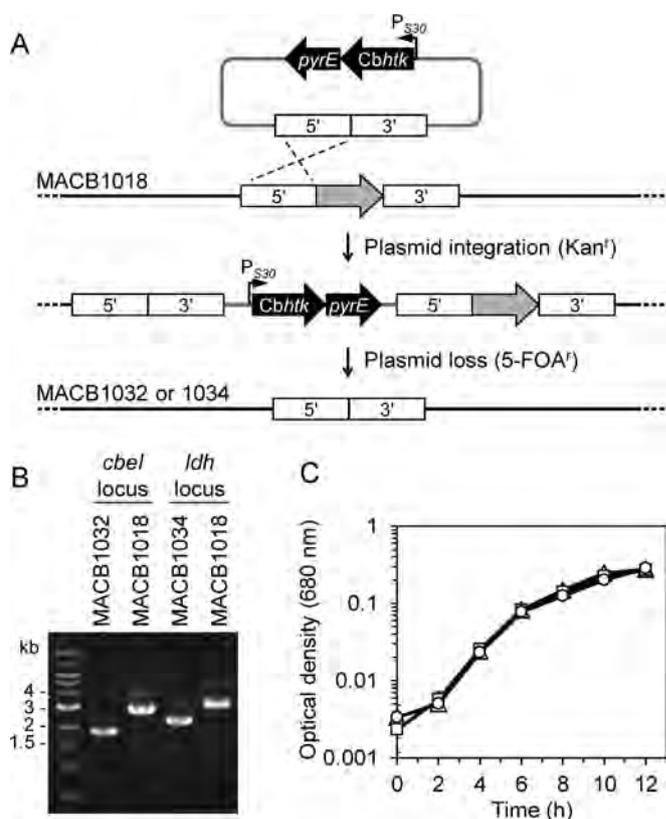


FIG 4 Use of *Cbhtk* and *pyrE* markers for genetic selection of mutations in $\Delta pyrE$ strain MACB1018. (A) Scheme showing selection method for targeted deletion of *ldh* or *cbeI* (gray) using the *Cbhtk*-*pyrE* cassette (black) by homologous recombination (dashed lines) into the chromosome of *C. bescii* strain MACB1018 ($\Delta pyrE$). Kanamycin selected for plasmid integration. To resolve the second crossover event, 5-FOA selected for plasmid loss, resulting in gene deletion or reversion to wild type (not shown). (B) PCR confirmation of *cbeI* deletion (1.9 kb) and *ldh* deletion (2.3 kb) in MACB1032 and MACB1034, respectively, compared to the expected sizes for wild-type *cbeI* (2.8 kb) and *ldh* (3.2 kb) in the MACB1018 background strain. PCR products were amplified with primers outside the flanking regions used to delete the genes. (C) Growth curve of $\Delta pyrE$ strain MACB1018 (triangles), $\Delta pyrE \Delta cbeI$ strain MACB1032 (squares), and $\Delta pyrE \Delta ldh$ strain MACB1034 (circles) in DC516 medium containing 20 μM uracil. Error bars represent standard deviation; $n = 3$.

transformation. This selection is more robust, since the use of kanamycin eliminates issues with uracil carryover from the electroporation recovery medium when transformants are selected via uracil prototrophy. The *pyrE* marker was used for 5-FOA counterselection of plasmid loss (Fig. 4A). Therefore, the *cbeI* and *ldh* deletion plasmids contained both markers combined into a synthetic operon, the *Cbhtk*-*pyrE* cassette. This cassette utilized the P_{S30} promoter, a ribosomal binding site between the genes, and a rho-independent terminator sequence at the end of the operon.

The *cbeI* and *ldh* deletion plasmids were methylated and transformed into the $\Delta pyrE$ background strain MACB1018 using kanamycin to select for transformants. Postelectroporation selective outgrowth cultures were screened by PCR and verified to contain integration of the pGL100 and pGL108 plasmids at the *cbeI* and *ldh* genome regions, respectively. After colony purification, these isolates were counterselected for plasmid loss using solid medium containing uracil and 5-FOA. In this case, 5-FOA does not directly select for deletion of the genes but selects only for plasmid loss,

which can lead to either gene deletion or reversion to the parent strain; therefore, resulting colonies were screened by PCR to identify $\Delta cbeI$ and Δldh isolates. These isolates were further colony purified, sequence verified, and designated MACB1032 ($\Delta pyrE \Delta cbeI$) and MACB1034 ($\Delta pyrE \Delta ldh$) (Fig. 4B). Growth characteristics of these strains on complex medium were similar to the parent MACB1018 (Fig. 4C). These strains will be useful in future genetic engineering efforts, since loss of CbeI function allows plasmid transformation without prior methylation of the DNA (6), and deletion of lactate dehydrogenase abolishes lactate production and can increase ethanol yield in engineered strains (12, 16).

We have demonstrated that kanamycin selection can be used in wild-type *C. bescii* to construct a uracil prototrophic strain based on deletion of the *pyrE* gene, and this strain can then be used as a genetic background to make other mutations ($\Delta cbeI$ and Δldh). Use of *Cbhtk* with kanamycin has proven to be a more robust method for selecting transformants in *C. bescii* than uracil prototrophic selection, since there are no issues with uracil contamination which can lead to background growth and false positives. When used in combination with a counterselectable marker, such as *pyrF* or *pyrE*, *Cbhtk* can be used iteratively in the same strain to make multiple mutations at multiple genome locations. Furthermore, kanamycin selection can be performed in a wild-type strain using a complex medium. It is therefore an ideal selectable marker for the initial development of nutritional auxotrophs in wild-type strains, allowing for targeted gene deletion as opposed to selection of random auxotrophic mutants that can potentially also contain mutations elsewhere in the genome. The *Cbhtk* marker should aid in the development of genetic systems in other members of the *Caldicellulosiruptor* genus as well as in related species.

ACKNOWLEDGMENTS

We thank Daehwan Chung and Janet Westpheling for many helpful discussions as well as for supplying *Caldicellulosiruptor bescii* strain JWCB018, *Escherichia coli* strain JW284, and plasmids pDCW73, pDCW89, and pDCW121.

FUNDING INFORMATION

This work, including the efforts of Gina L. Lipscomb, Jonathan M. Conway, Sara E. Blumer-Schuette, Robert M. Kelly, and Michael W. W. Adams, was funded by U.S. Department of Energy (DOE) (DE-PS02-06ER64304).

This research was supported by a grant (DE-PS02-06ER64304) from the Bioenergy Science Center (BESC), Oak Ridge National Laboratory, a U.S. Department of Energy (DOE) Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

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