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Deletion of the *Clostridium thermocellum recA* gene reveals that it is required for thermophilic plasmid replication but not plasmid integration at homologous DNA sequences

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Abstract

A limitation to the engineering of cellulolytic thermophiles is the availability of functional, thermostable (≥ 60 °C) replicating plasmid vectors for rapid expression and testing of genes that provide improved or novel fuel molecule production pathways. A series of plasmid vectors for genetic manipulation of the cellulolytic thermophile *Caldicellulosiruptor bescii* has recently been extended to *Clostridium thermocellum*, another cellulolytic thermophile that very efficiently solubilizes plant biomass and produces ethanol. While the *C. bescii* pBAS2 replicon on these plasmids is thermostable, the use of homologous promoters, signal sequences and genes led to undesired integration into the bacterial chromosome, a result also observed with less thermostable replicating vectors. In an attempt to overcome undesired plasmid integration in *C. thermocellum*, a deletion of *recA* was constructed. As expected, *C. thermocellum* Δ*recA* showed impaired growth in chemically defined medium and an increased susceptibility to UV damage. Interestingly, we also found that *recA* is required for replication of the *C. bescii* thermophilic plasmid pBAS2 in *C. thermocellum*, but it is not required for replication of plasmid pNW33N. In addition, the *C. thermocellum recA* mutant retained the ability to integrate homologous DNA into the *C. thermocellum* chromosome. These data indicate that *recA* can be required for replication of certain plasmids, and that a *recA*-independent mechanism exists for the integration of homologous DNA into the *C. thermocellum* chromosome. Understanding thermophilic plasmid replication is not only important for engineering of these cellulolytic thermophiles, but also for developing genetic systems in similar new potentially useful non-model organisms.

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Keywords

Plasmid; Thermophile; Genetics; Consolidated bioprocessing; RecA

Introduction

The interactions between lignocellulose-degrading microbes and the plant cell wall have spurred advances towards a renewable energy source. Complex cell wall polysaccharides are recalcitrant to degradation, and this degradation is the major bottleneck in the conversion of plant biomass to biofuels and chemicals [22]. A variety of schemes has been proposed to break down the plant cell wall and convert the released sugars into fuel molecules and bioproducts. One such scheme, Consolidated Bioprocessing (CBP), consists of one microbe performing both tasks [39]. The cellulolytic thermophile *Clostridium thermocellum* has been the subject of study for many years because of its cellulosome, an interesting extracellular protein supercomplex for degrading the plant cell wall [2, 27]. The natural ethanologenic capabilities of *C. thermocellum* suggest its application as a biofuel-producing microbe, and much work has recently been done to engineer this strain to produce more ethanol from plant biomass [1, 4, 16, 40, 42, 47].

A reliable thermostable plasmid-based expression system has important uses in metabolic engineering including the rapid screening of enzymes and regulatory signals to improve carbon metabolism, production of fuels and chemicals, and plant cell wall deconstruction. We recently developed a series of replicating plasmid vectors for the cellulolytic thermophile *Caldicellulosiruptor bescii* and used them for the expression of homologous and heterologous genes [9, 11, 12, 24]. These vectors have proven useful to extend genetic methods developed for *C. bescii* to another member of this genus, *Caldicellulosiruptor hydrothermalis*, a naïve host for the study of cellulolytic enzymes [20] and surprisingly, they have also proven to be useful for the more distantly related *Clostridium ther-mocellum.* Vectors derived from *C. bescii* plasmid pBAS2 transform *C. thermocellum* DSM 1313 at 60 °C, the optimal growth temperature of this bacterium, with a transformation efficiency greater than 3000 colony forming units per μg of transformed DNA and a copy number ~ 10 per bacterial chromosome [19]. pBAS2 has recently been used to express a thermostable butanol dehydrogenase gene in *C. thermocellum* that provides resistance to 5-hydroxym-ethylfurfural, a plant biomass-derived microbial inhibitor [25].

While pBAS2 has been shown to replicate autonomously in *C. thermocellum*, the use of homologous sequences as small as a 300-bp promoter region can result in plasmid integration into the chromosome using either pBAS2 [25] or the less thermostable replicon pNW33N [30]. To avoid this integration and to preserve the integrity of the background strain, we have relied on the use of only heterologous sequences on the plasmid [19]. Several promoters from *Caldicellulosiruptor bescii* have been shown to function in *C. thermocellum* [19, 25], but many of the tools developed for *C. thermocellum* are encoded by endogenous (i.e. homologous) sequences [38]. Preventing homologous recombination between sequences on plasmids and homologous sequences in the chromosome would thus allow the use of the best promoters, signal peptides and enzymes to meet strain development goals.

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The canonical RecA protein in the model organisms *Escherichia coli* and *Bacillus subtilis* is a single-stranded DNA (ssDNA)-binding protein that initiates homologous recombination [3]. RecA monomers join together to form a helical filament on ssDNA, protecting and preparing the DNA for strand invasion into duplex DNA. Once assembled, the RecA filament forms a second DNA-binding site that allows strand invasion. This complex scans the invaded duplex and freezes when sequence homology is detected so that replication, subsequent branch migration and recombination can occur [29]. In addition to its function in homologous recombination, RecA protein recognizes DNA damage and triggers the SOS response. It does this by promoting auto-cleavage of the global transcriptional repressor LexA, thereby alleviating repression on a variety of genes involved in DNA repair [3]. Clo1313_1163 is the only RecA homolog in *Clostridium thermocellum*, with 93% coverage and 74% amino acid identity to *B. subtilis* RecA. The likely *C. ther-mocellum* LexA homolog, Clo1313_1449, exhibits 56% amino acid identity to *B. subtilis* LexA, and contains both the characteristic Ala–Gly autocleavage site and the Ser–Lys catalytic dyad [5]. RecA and other ssDNA-binding proteins are also required for mutagenic translesion synthesis by DNA polymerase V in cases of DNA damage [3, 29]. It is not clear whether there is a homolog to DNA Pol V in *C. thermocellum*. Rather, homology searches with known DNA Pol V sequences identify a protein annotated as DNA Pol I.

The RecA protein is required for homologous recombination and although the RecA protein has been shown to have multiple functions in model organisms such as *E. coli* [3], viable *recA* mutants deficient in homologous recombination have been selected for many decades [13] and are routinely used to clone repetitive DNA sequences. A variety of phe-notypes have been observed with *recA* mutants in different bacteria. *recA* mutants in *Thermus thermophilus* [7], *E. coli* [6], and *B. subtilis* [43] all exhibit decreased cell viability. The thermophile *T. thermophilus recA* mutant has extremely low viability (only 1 colony forming unit in 10^3–10^5 cells) while more moderate decreases are observed in the other organisms (50–90% decrease in colony formation). The *T. thermophilus* strain also has a hypermutable phenotype that may be attributed to a higher mutation rate at high temperatures [7]. In *Streptomyces lividans,* a Gram-positive actinomycete, *recA* mutants are inviable in the absence of suppressor mutations [49]. Nucleoid morphologies often change as well; ~ 10% of *E. coli* mutants become anucleate, likely because ssDNA that is normally protected by intact RecA is degraded by exonucleases [44, 50]. Many organisms exhibit increased susceptibility to DNA damage such as UV rays or mitomycin C, since the RecA protein is not available to initiate recombination-mediated repair at DNA lesions [15]. However, *recA* mutants in some organisms such as *Thiobacillus ferrooxidans* are less sensitive to radiation than mutants in *E. coli* and *B. subtilis* [31].

Clostridium thermocellum Δ*recA* could be a valuable tool for increasing plasmid stability and accelerating metabolic engineering advances. Therefore, in an attempt to create a better strain for phenotypic screening via plasmid-based het-erologous gene expression, we constructed a deletion of *recA* in *C. thermocellum* and characterized its impact on strain viability and plasmid stability.

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Results and discussion

The *C. bescii pyrF* gene functions efciently for marker replacement of *recA* in *C. thermocellum*

To generate a deletion of *recA* in *C. thermocellum,* the *pyrF* gene from *C. bescii* was placed between 5′ and 3′ fanking regions of the *C. thermocellum recA* gene and transformed into *C. thermocellum* Δ*pyrF*. This parent strain is a uracil auxotroph, and transformants containing integrated plasmid DNA were selected for uracil prototrophy (Fig. 1a). The plasmid contains the pNW33N origin of replication widely used in *C. thermocellum* for engineering and the thymidine kinase *(tdk)* gene from *Thermoanaerobacterium saccharo-lyticum,* allowing for counter selection against the plasmid with 5-fuoro-2′-deoxyuridine (FUDR) and replacement of the *recA* gene with *pyrF* in the *C. thermocellum* genome. The pNW33N replicon cannot be maintained as an autonomously replicating plasmid at 60 °C [36], and under selection integrates into the *C. thermocellum* chromosome exclu-sively at that temperature. The initial uracil-prototrophic, FUDR-resistant colonies were merodiploids that required a further round of purification on solid medium. Of the isolated colonies screened, 5% sustained a clean deletion of the *recA* gene, which is a standard result for gene deletions in this organism and in other cellulolytic thermophiles in our hands.

PCR using genomic primers outside of the 5′ and 3′ flanking regions was used to verify the gene replacement, differentiated by the larger size of the 1123-bp *pyrF* cassette compared to the 996-bp wild-type sequence (Fig. 1b). Additionally, failure to amplify fragments by PCR with multiple sets of internal primers demonstrated the absence of the *recA* gene in the genome of the new strain (Fig. S1), JWCT10 (*C. thermocellum* DSM 1313 Δ*pyrF* Δ*recA::pyrF*, Table 1). *recA* deletions were also verified with similar PCR reactions in all Δ*recA* strains used in subsequent experiments to ensure that strains were not contaminated with the wild type (Fig. S2 and S3).

Deletion of *recA* resulted in UV sensitivity and slower growth rates

In comparison with the parent strain LL1005 or to the *C. thermocellum* DSM 1313 wild-type strain, the *recA* deletion strain exhibited a greater than twofold increase in the doubling time and reached somewhat lower cell densities in defined medium (Fig. 2a, Table 2). These effects were less severe in rich medium (Fig. 2b) in which the growth rate of the deletion strain was comparable to its parent strain (Table 2). In addition to growth phenotypes, Δ*recA* strains in other bacteria often exhibit sensitivity to muta-gens such as UV light. This is attributed to a deficiency in recombination-mediated double-strand break repair [3, 13]. As shown by counts of colony forming units in Fig. 2c, the *recA* deletion strain was extremely sensitive to very low doses of UV light. The effect was pronounced at UV intensities below 1000 μJ/cm^2; we observed ~ 3% cell survival at intensities as low as 100 μJ/cm^2, compared to ~ 60% cell survival in the wild type. The results with the wild-type strain are comparable to previous studies of UV sensitivity in *C. thermocellum* [17].

Deletion of *recA* resulted in loss of transformation by pBAS2 but not pNW33N

No thiamphenicol-resistant transformants resulted from 15 attempts to transform the JWCT10 Δ*recA::pyrF* dele-tion strain with pJGW37, a pBAS2 replicon containing the *cat*

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gene. This plasmid has no sequences in common with the chromosome and while we previously showed that transformation of the wild-type strain with pBAS2 replicons was efficient and resulted in stable autonomous replication of the plasmid at copy numbers ranging from 10 to 20 per chromosome [19], we have never observed plasmid integration in the absence of homologous sequences. To confirm that the *recA* deletion was responsible for the failure of pBAS2-based plasmids to transform, a plasmid was constructed to complement the deletion. Plasmid pJGW92 (Fig. 3a) contains a wild-type *recA* allele and transformants using this plasmid based on the pBAS2 replicon were readily detected in the Δ*recA* strain. DNA from these transformants back-transformed into *E. coli* confirmed the presence of autonomously replicating plasmid DNA (Fig. 3b). PCR performed on the transformants confirmed that the transformed strains still had the *C. bescii pyrF* gene in place of the *recA* gene (Fig. 3c, d).

The pBAS2 replicon has a conserved Firmicute origin of replication for rolling-circle plasmids, but the single-stranded intermediates characteristic of rolling-circle origins have not been observed [14]. While it is not an inherent feature of rolling-circle plasmids to require recombination, there is a Xer-like recombinase encoded on pBAS2 [9, 19]. The presence of this recombinase suggests that it may be required for replication as well as RecA. The canonical XerCD site specific recombinases from *E. coli* act in a *recA*-independent manner on the *dif* site in the chromosome [26]. XerCD also works on the *cer* and *psi* sites on colE1 and pSC101 plasmid replicons [8]. Because the Xer-like recombinase contained in pBAS2 is a single recombinase rather than a double recombinase like XerCD, it might behave more similarity to the single recombinase XerS found in *Strepto-cocci* and *Lactococci* [28]. In fact, we found two similar sequences ($p < 0.0001$) to the streptococcal dif_{SL} site 5′-ATT TTTCCGAAAAACTATAATTTTCTTGAAA-3′ in pBAS2 by FIMO motif discovery software [18]. One sequence is 5′-GTTTTGCAGAAATACGAGAGAGAGGTTGAGA-3′ at bases 1725–1755 on the coding strand for the pBAS2 ORFs. The other is 5′-AGTCTTCTGGAAAACAATCACAATGAA GATA-3′ at bases 482–512 on the reverse strand. The latter sequence is located less than 100 bp from the previously identified predicted ArgR-binding site [19], a site involved in XerCD-mediated recombination in other plasmids [46]. Motif discovery software could not recognize *dif*, *cer*, or *psi* sites associated with other XerCD-associated plasmids [19]. This dif_{SL}-like sequence could allow for intramolecular recombination and dimer resolution by the plasmid-encoded Xer-like recombinase.

Although *recA* has not been observed to be required for recombination with Xer site-specific recombinases in other organisms [26], the unsuccessful transformation attempts indicate that pBAS2 requires RecA function for faithful replication. Similar results have been observed in *Strepto-coccus pneumoniae.* Establishment of plasmid multi-copy rolling-circle plasmid pMV158 was blocked but not completely abolished by a *recA* mutation [34]. RecA protein in *C. thermocellum* is required for replication of plasmids based on the thermophilic replicon pBAS2, but not the mesophilic replicon pNW33N even though both plasmids apparently replicate by a rolling-circle mechanism. A pNW33N-based plasmid named pJGW93NR (Fig. 4a) successfully transformed the Δ*recA::pyrF* deletion strain to thiamphenicol resistance, and could be back-transformed into *E. coli* (Fig. 4 c). Taken together with the results for pBAS2, these data suggest that recA is required for stable

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pBAS2 replication specifically, and that replication, not DNA entry into the cell, is affected by the deletion. Nearly all plasmids so far described for thermophilic Gram-positive bacteria replicate by a rolling-circle mechanism [41, 45], although the moderate thermophile *Bacillus methanolicus* was recently shown to maintain a theta-replicating plasmid at a lower temperature [23]. It is noteworthy that ssDNA intermediates are usually formed during rolling-circle replication, and that RecA protein may be required for protection of the DNA [44] or for replication restart mechanisms [32] necessary for faithful replication of the plasmid. The reason one plasmid replicon (pBAS2) requires RecA and not another (pNW33N) is unclear.

Deletion of *recA* does not eliminate plasmid integration at homologous sequences

To test whether deletion of *recA* affected homology-dependent plasmid integration, identical plasmids based on the pNW33N replicon were constructed with (pSKW79) and without (pJGW93NR) sequences homologous to the chromosome (Fig. 4a, b). While the pJGW93NR plasmid was stably maintained outside of the chromosome (Fig. 4c), this was not the case for pSKW79. This plasmid DNA containing homologous sequences readily transformed the deletion strain, but PCR reactions with one primer specific to the plasmid and one primer specific to genomic DNA upstream of the *C. thermocellum* enolase promoter showed that the plasmid integrated into the chromosome using the 300 bp of homologous sequence present on the plasmid (Fig. 4d). Back-transformation of plasmid pSKW79 into *E. coli* was not observed.

While it is possible that an uncharacterized homologous recombination system is present in *C. thermocellum* that accounts for this plasmid integration, an alternative explanation is that single-stranded DNA formed during rolling-circle replication can act as pseudo-Okazaki fragments during replication of the chromosome, with normal DNA repair pathways (e.g., DNA polymerase, ligase, helicase) completing the plasmid integration. Additional research will be required to test this hypothesis.

Conclusion

Plasmids are fundamental tools used in microbial genetics and metabolic engineering. However, plasmids in non-standard host organisms are often poorly characterized, as are the interactions between the plasmid and host organism. Here we describe unexpected interactions between two thermophilic plasmids and the recombination machinery of *C. thermocellum*. We demonstrate that *recA* is required for transformation using the pBAS2 origin of replication, and that *recA* is not required for integration of pNW33N plasmid DNA into homologous sequences in the chromosome. These surprising results provide insight into potential interactions between the host genetic background and plasmid maintenance, and they will help inform future genetic approaches in *C. thermocellum* and related organisms.

Materials and methods

Bacterial growth and media composition

Clostridium thermocellum DSM 1313 and its derivatives were grown anaerobically in modified CTFÜD medium [35] at 60 °C, under an atmosphere of 85% N_2, 10% CO_2, and

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5% H_2. Defined medium for transformation and selec-tion was CTFUD-NY [35], that contains a vitamin mix of *p*-aminobenzoic acid, vitamin B12, biotin, and pyridoxamine in place of yeast extract. Cells were grown without shaking for transformation and for growth curves. CTFUD-NY was supplemented with 360 μM uracil when noted. Complex medium for recovery after transformation of *C. thermocel-lum* LL1005 was similar to CTFUD but contained casein (0.2% w/v) and less yeast extract (0.1% w/v), referred to as CTFUD + C. *Escherichia coli* was grown in Luria–Bertani broth supplemented with 50 μg/mL apramycin when selecting for the presence of a plasmid. Strains and plasmids used in this study are listed in Table 1.

Construction of plasmid vectors

Q5 Polymerase (NEB, Ipswich, MA) was used for the polymerase chain reaction (PCR), according the manufacturer's instructions. Fast-link Ligase (Epicentre, Madison, WI) was used for all ligation reactions. Multiple cloning steps were used to construct *pyrF* marker replacement vector pJGW61. Primers X118 (with a PstI site) and X119 (with a BamHI site) were used to amplify the genomic region ~ 1100 bp upstream and downstream of the *Clostridium thermocellum recA* gene (Clo1313_1163). Primers X102 (with a BamHI site) and X103 (with a PstI site) amplified backbone vector pDCW140, and both fragments were subjected to a BamHI/PstI digest followed by ligation to create pCTH07. The *pyrF* gene on the backbone vector was removed with a PCR amplification, T4 polynucleotide kinase phosphorylation, and blunt end ligation to recircularize the fragment, creating pCTH08. The *recA* coding sequence was removed in a similar fashion from pCTH08 using primers X120 (with a KpnI site) and X122 (with a SphI site) to create pJGW58. Next, primers JG137 (with a BglII site) and JG138 (with a BamHI) site were used to amplify the pNW33N replicon from plasmid pMU1162 [48]. Primers JG133 (with a BglII site) and X103 (with a BamHI site) were used to amplify the pJGW58 backbone, and a BglII/BamHI restriction enzyme digest of both fragments followed by ligation created pJGW59. To insert the *C. bescii pyrF* gene between the *recA* fanking regions, the 1.123-kb *pyrF* cassette from pJGW07 [20] was amplified using primers JG163 (with a KpnI site) and JG165 (with a SphI site). A PCR fragment was amplified from pJGW59 using X120 (with a KpnI site) and X122 (with a SphI site), and a KpnI/SphI restriction digest and ligation with the *pyrF* cassette from pJGW07 was used to create pJGW59.5. To create the *tdk* cassette for negative selection, primers JG168 (with a BamHI site) and DC576 (with a PstI site) were used to amplify a fragment from backbone vector pDCW140 containing the Cbes_2105 promoter [10], and this fragment was digested and ligated to a PCR fragment of the *Thermoanaerobacterium saccharolyticum tdk* gene (Tsac_0324) amplified by primers JG166 (with a PstI site) and JG167 (with a BamHI site). The resulting vector pJGW60, containing the *tdk* gene driven by the Cbes_2105 promoter, was used as a template for PCR with primers JG169 (with a NheI site) and JG170 (with an AatII site). This *tdk* cassette was ligated to the PCR fragment ampli-fied from pJGW59.5 by primers DC176 with a NheI site and DC230 with an AatII site to create the final knockout vector pJGW61.

Plasmid pJGW91 was the precursor to plasmids pJGW92 and pJGW93. pJGW91 was created by amplifying pSKW01 [25] with primers DC576 (with a PstI site) and DC700 (with an AvrII site), and amplifying the *recA* gene from *C. thermocellum* DSM1313 genomic

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DNA with primers JG248 (with a PstI site) and JG249 (with an AvrII site), digesting these products with PstI and AvrII, and ligating them together. pSKW01 contains the entire pBAS2 plasmid sequence [14] and a *C. bescii pyrF* selectable marker, as well as an *E. coli* pSC101 replication origin and an apramycin resistance cassette for cloning in *E. coli.* To swap the selectable marker *pyrF* for chloramphenicol acetyltransferase (*cat*) in pJGW92, JG024 (with an XbaI site) and X014 (with an NdeI site) amplified pJGW91, JG099 (with an XbaI site) and JG250 (with an NdeI site) amplified the *cat* marker from pDCW196 [19], and the fragments were digested with XbaI and NdeI, and ligated. To swap the pBAS2 replication origin for pNW33N to make pJGW93, JG251 (with a BglII site) and JG252 (with a BamHI site) amplified pJGW92, JG137 (with a BglII site) and JG138 (with a BamHI site) amplified the pNW33N replicon from pMU1162 [48], and the fragments were digested with BglII and BamHI, and ligated.

pJGW93NR was created by a blunt end ligation of a PCR product amplified from pJGW93 using primers DC576 and DC700, effectively removing the recA gene sequence. pSKW79 was created by amplifying plasmid pSKW04 with primers SK026 (with an AvrII site) and JG251 (with a BglII site), amplifying the pNW33N replicon from pMU1162 with SK258 (with an AvrII site) and JG137 (with a BglII site), digesting the fragments with AvrII and BglII, and ligating them together.

Marker replacement of *C. thermocellum recA* with *pyrF* from *C. bescii*

Clostridium thermocellum LL1005 was grown to OD_{600} ~ 0.7 in 150 mL of defined CTFUD-NY medium [19, 37] supplemented with 360 μM uracil and 0.4 g/L glycine (Sigma, St. Louis, MO). Cells were harvested in three 50 mL aliquots at 7000×*g* for 8 min at 4 °C, and washed twice with a solution of ice-cold 10% glycerol (w/v) and 10% sucrose (w/v). Each of the three pellets was resuspended in 350 μL of the wash solution, and pelleted in one Eppendorf tube with a refrigerated desktop centrifuge for 5 min at 13,000 rpm at 4 °C. The pellet was homogenized with a pipet, and divided into 30 μL aliquots. 1 μg of pJGW61 plasmid isolated from *E. coli* BL21 as described previously [21] was mixed with a cell aliquot and electroporated in a 1-mm gap cuvette with an exponential pulse using a BioRad Gene Pulser (1.8 kV, 25 μF, 350 Ω). Cells were immediately recovered in 10 mL CTFUD-C at 50 °C to allow replication of the pNW33 N replicon. 50 μL subcultures were transferred every 2 h into 20 mL CTFUD-NY at 60 °C to select for uracil prototrophy. After 3–5 days, several subcultures became turbid, and these cultures were diluted again to ensure that cells were prototrophs. The fresh cultures were plated onto CTFUD medium with 4.5 g/L yeast extract and 0.8% (w/v) agar, supplemented with 10 μg/mL 5-fuoro-2′-deoxy-uridine (FUDR) to select against the plasmid backbone. Colonies formed after 2–3 days, and were picked into CTFUD-NY medium. Only 2 of 20 colonies grew in CTFUD-NY medium, both of which were shown by PCR with internal primers to be merodiploids (Figure S1). After a subsequent round of plating on solid CTFUD-NY medium, 1 in 20 colonies sustained a clean deletion of the *recA* gene. This strain was named JWCT10.

UV sensitivity assay

25 mL cultures of both LL1005 and JWCT10 were grown in CTFUD medium to early exponential phase (OD_{600} = 0.35–0.4). Cells were divided into 3 mL aliquots and chilled on

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ice for 20 min before being harvested at 4000×*g* for 6 min. The cells were then suspended in 3 mL ice-cold 0.1 M Mg_2SO_4 (JT Baker, Phillipsburg, NJ) as recommended in the literature to eliminate absorption of UV light by the rich medium [17]. Resuspended cells were then exposed on 100 × 15 mm plastic Petri dishes to varying intensities of UV light ranging from 0 to 1000 μJ/cm^2, using a Stratalinker 1800 UV crosslinker (Stratagene, La Jolla, CA). Although no apparent photolyase homologs are present in the *C. thermocellum* genome that would photoreactivate DNA in response to UV damage [33], cells were manipulated and incubated in the dark after UV exposure. Cells were diluted and plated on CTFUD medium at 60 °C for 3 days. Percent survival was calculated for each exposed sample by comparing colony forming units (CFU) to the CFU of cells not exposed to UV light.

Transformation of *C. thermocellum recA* deletion strain

Clostridium thermocellum LL1005 was grown to OD_{600} ~ 0.4 in 150 mL CTFUD medium with 1 g/L yeast extract [19, 37] supplemented with 40 μM uracil and 0.4 g/L glycine (Sigma, St. Louis, MO). Cells were harvested in three 50 mL aliquots at 7000×*g* for 8 min at 4 °C, and washed twice with a solution of ice-cold 10% glycerol (w/v) and 10% sucrose (w/v). Each of the three pellets was resuspended in 350 μL of the wash solution, and pelleted in one Eppendorf tube with a refrigerated desktop centrifuge for 5 min at 13,000 rpm at 4 °C. The pellet was homogenized with a pipet, and divided into 30 μL aliquots. 700 ng of plasmid isolated from *E. coli* BL21 as described previously [21] was mixed with each aliquot, and cells were electroporated in a 1-mm gap cuvette with an exponential pulse using a BioRad Gene Pulser (1.8 kV, 25 μF, 350 Ω). Cells were immediately recovered in 10 mL CTFUD-C at 53 °C for plasmids with the pNW33N replicon or 60 °C for plasmids with the pBAS2 replicon. 1 mL subcultures were transferred three times over the course of 24 h into 10 mL CTFUD + 6 μg/mL thiamphenicol at 53 or 60 °C to select for resistance. After 4–9 days, several subcultures became turbid, and these cultures were diluted again to ensure that cells were thiamphenicol resistant. The fresh cultures were plated onto CTFUD medium with 1 g/L yeast extract and 0.8% (w/v) agar, supplemented with 10 μg/ mL thiamphenicol. Colonies formed after 3 days and were picked into the same liquid medium. We point out that PCR using primers with homology to the wild-type *recA* sequence did not amplify DNA after 30 cycles but when the number of cycles was extended to 40, a faint band was observed in the transformed *recA* deletion strain not obvious in the figure. Control reactions containing purified plasmid pJGW92 mixed with purified *recA* genomic DNA revealed that this was a PCR artifact, likely resulting from hybridization and amplification between the plasmids and genomic DNA that is only visible with extended cycles (Fig. 4).

Analysis of plasmid autonomous replication

5 mL of *C. thermocellum* culture was harvested at 3220×*g* for 7 min, and the pellets were used for extraction of DNA with the Quick-gDNA Extraction kit (Zymo Research, Irving, CA, USA). PCR was performed with primers JG162 and JG144 to check that no wild-type sequence was present and with primers JG161 and DC232 to check for the *pyrF* marker, according to the manufacturer's instructions (94 °C duplex denaturation, 56 °C annealing temperature, 1 min per kb at 72 °C for elongation). *E. coli* DH5α was electrotransformed with ~ 180 ng of the mixture of genomic and plasmid DNA via single electric pulse (2.5 kV, 200 Ω, and 25 μF) in a pre-chilled 2-mm cuvette using a Bio-Rad Gene Pulser. The cells

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were placed into 1 mL SOC media for 1 h with shaking at 37 °C, and then plated onto LB agar supplemented with 50 μg/mL apramycin. The colonies were picked into 10 mL LB with 50 μg/mL apramycin, and the plasmid DNA was extracted using a Miniprep kit (Qiagen, Valencia, CA, USA) and screened with restriction enzymes EcoRI and AvaI for pJGW92 and NcoI and AvaI for pJGW93 (NEB).

Bioinformatic analysis

The National Center for Biotechnology Information (NCBI) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to search for homologous proteins to known RecA, LexA, and DNA Pol V in the *Clostridium thermocellum* DSM 1313 genome. Amino acid similarity was also determined with the NCBI Basic Local Alignment Search Tool (BLAST). FIMO in the MEME Suite was used for motif searches with default settings [18].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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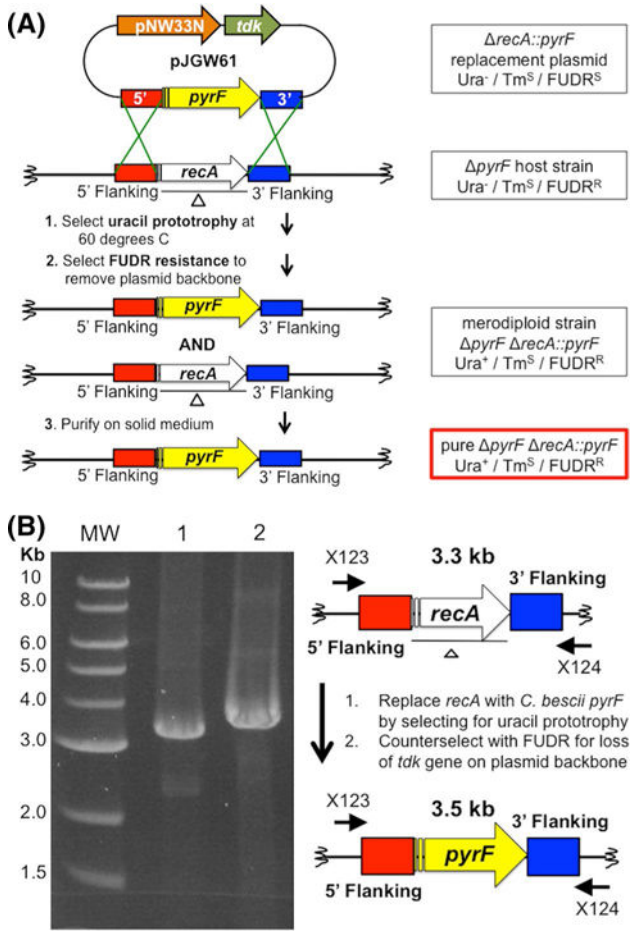

Fig. 1.

The *recA* gene was deleted by marker replacement with *C. bescii pyrF*. **a** Scheme to delete the *recA* gene. Gene replacement plasmid pJGW61 transformed *C. thermocellum* Δ*pyrF* to uracil prototrophy. Then a combination of uracil protrophic selection and FUDR exposure selected for those cells which had acquired the *pyrF* gene but lost the *tdk* gene on the plasmid backbone. Finally, the merodiploid was cured on solid medium to generate a pure Δ*recA* strain. *pyrF C. bescii* orotidine 5′-phosphate decarboxylase-encoding gene (Cbes_1377), *recA Clostridium thermocellum* DSM 1313 RecA-encoding gene (Clo1313_1163), *tdk Thermoanaerobacterium saccharolyticum* thymidine kinase-encoding gene (Tsac_0324), Tm^S thiamphenicol sensitive, *FUDR* 5-fuoro-2′-deoxy-uridine, *pNW33N* replication origin from plasmid pNW33N. **b** Verification of the *pyrF* replacement of *recA*. PCR using primers X123 and X124. Wild-type band, 3.3 kb. *pyrF* replacement band, 3.5 kb. The 5′-fanking sequence is 1126 bp and the 3′-fanking sequence is 1069 bp, The *C. bescii pyrF* cassette is 1123 bp. The deleted wild-type *recA* sequence is 996 bp. *MW* molecular weight standards. Lane 1: wild-type *C. thermocellum* genomic DNA. Lane 2: *C. thermocellum* Δ*recA::pyrF* genomic DNA

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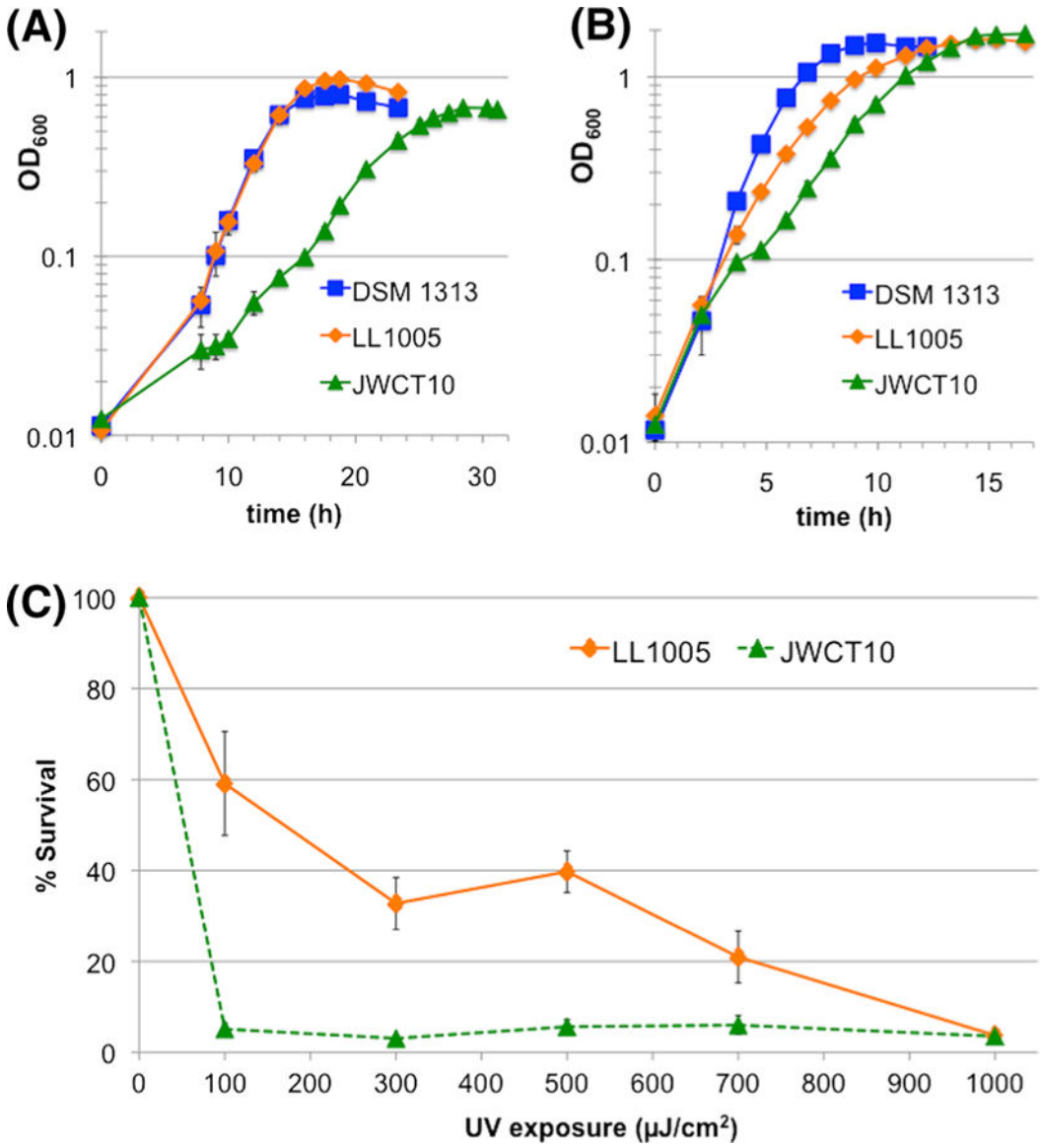

Fig. 2.

Phenotypic analyses of *C. thermocellum* Δ*recA*. **a** Growth in defined medium supplemented with uracil as measured by OD_{600}. **b** Growth in rich medium (4.5 g/L yeast extract) supplemented with uracil as measured by OD_{600}. **c** UV sensitivity of *C. thermocellum* wild type and Δ*recA*. Percent survival is calculated by colony forming units from cells treated with increasing amounts of UV compared to untreated cells. Error bars show the standard deviation resulting from two biological replicates. DSM 1313: wild-type *C. thermocellum*. LL1005: *C. thermocellum* Δ*pyrF*. JWCT10: *C. thermocellum* Δ*pyrF* Δ*recA::pyrF*

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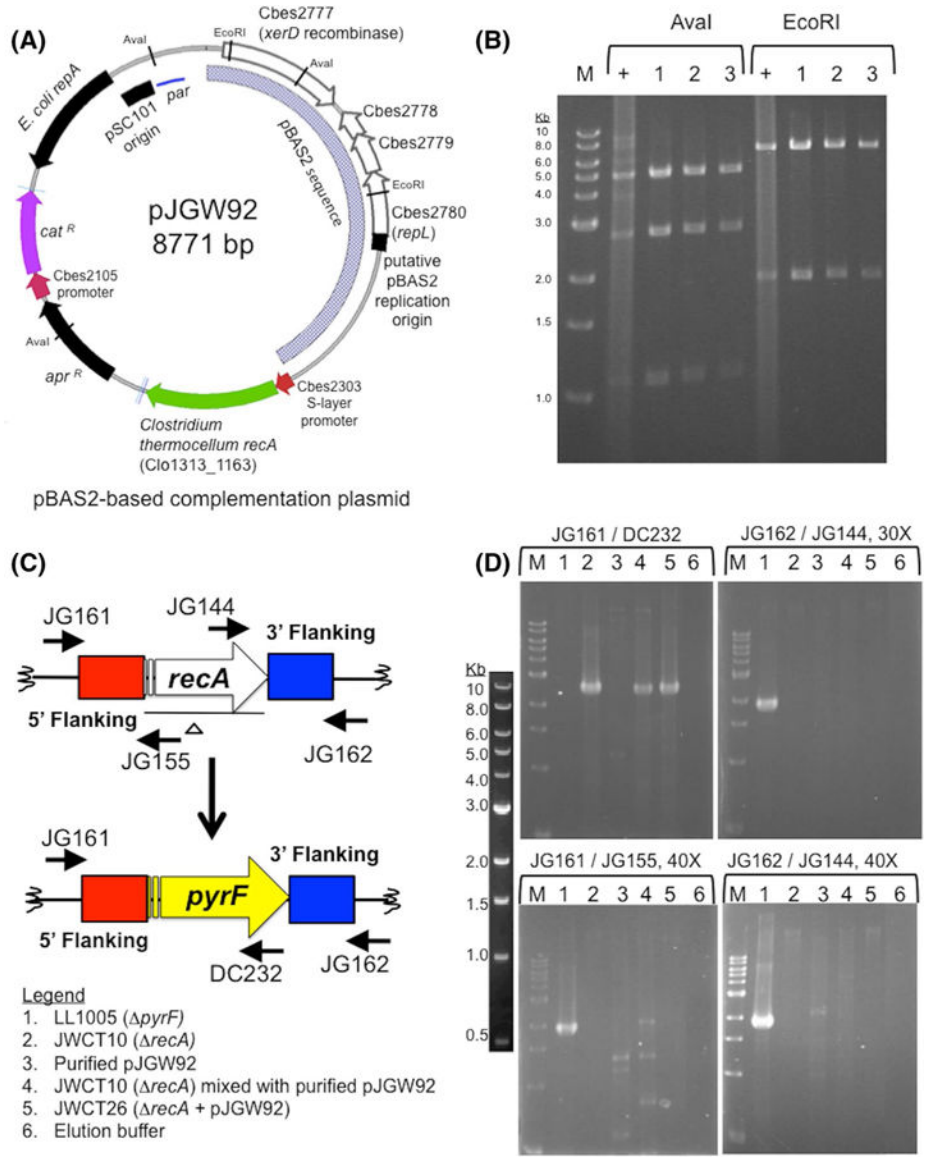

Fig. 3.

The Δ*recA* strain is transformable when it is complemented. **a** *recA* complementation plasmid pJGW92. The hatched region was derived from *C. bescii* native plasmid pBAS2. *apr*^{R} apramycin resistance casette, *cat*^{R} thiamphenicol resistance casette, *repA* replication initiation protein for the *E. coli* pSC101 replication origin, *par* partitioning locus for *E. coli*. Restriction sites for structural verification are shown on the plasmid map. **b** Restriction digests with AvaI and EcoRI. The expected bands from AvaI are 5.1, 2.6, and 1.1 kb. The expected bands from EcoRI are 6.9 and 1.9 kb. + purified pJGW92 from *E. coli*. Lanes 1–3: plasmids isolated from *E. coli* transformed with DNA isolated from JWCT26 (Δ*recA* + pJGW92). **c** Δ*recA* strains transformed with complementation plasmids retain the chromosomal deletion of *recA*. Primers indicated in the gene diagram were used to amplify DNA extracted from *C. thermocellum* strains. **d** JG161 and DC232 verified the replace-ment of *recA* by the *C. bescii pyrF* gene. Two different primer pairs (JG161/JG155 and JG162/

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JG144) were used to ensure that the transformed strain retained the *recA* deletion. PCR was performed for both 30 cycles (30X) and 40 cycles (40X) using primer pair JG162/JG144. The molecular weight ladder in kilobases for all gels is shown

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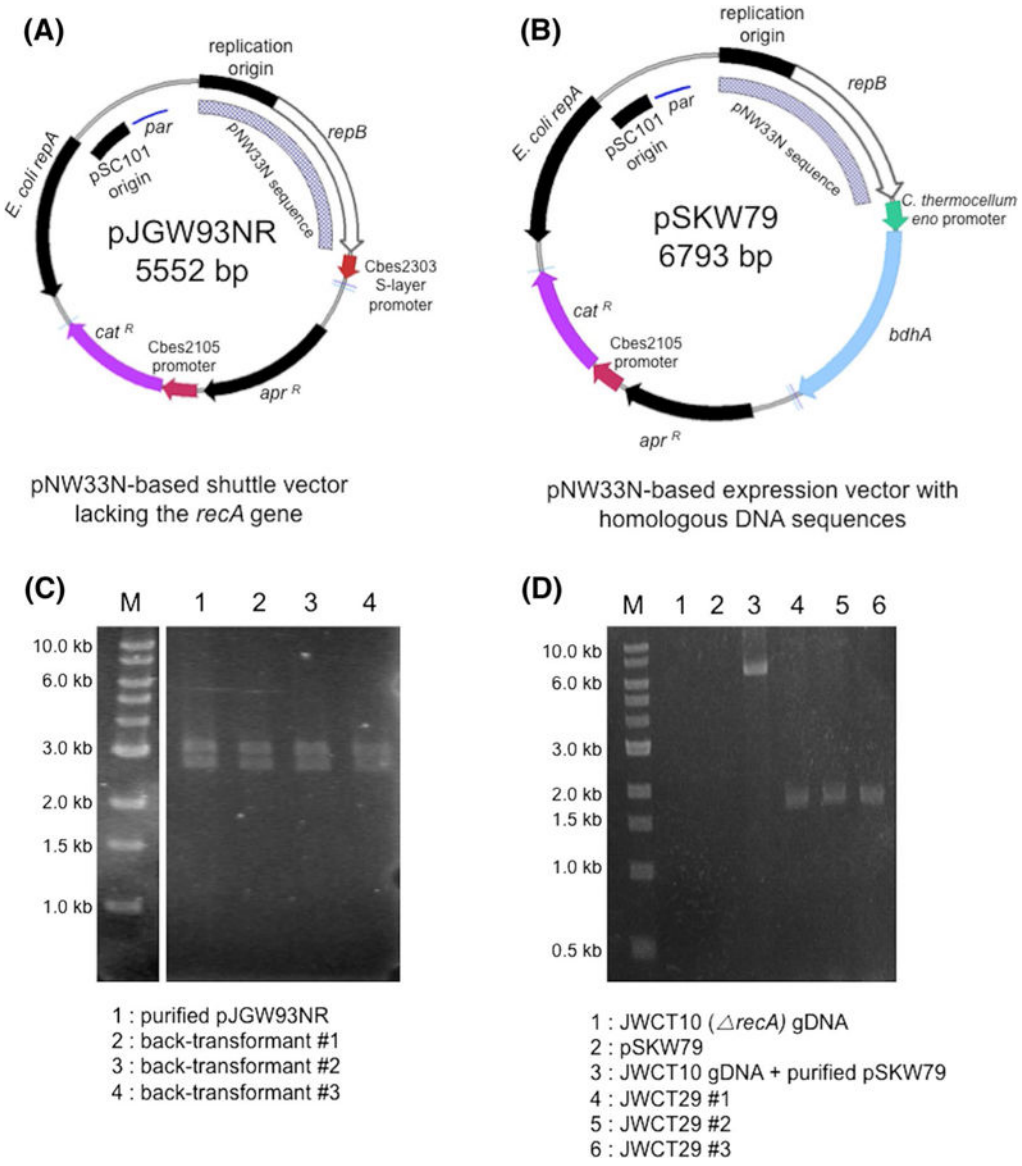

Fig. 4.

Plasmids based on the pNW33N replicon replicate and integrate in *C. thermocellum* Δ*recA*. **a** pNW33N-based shuttle vector lacking the *recA* gene. **b** pJGW93NR expressing *bdhA* from the *Clostridium thermocellum eno* promoter. In both plasmids, the hatched region is a replicon derived from plasmid pNW33N. apr^R apramycin resistance casette, cat^R thiamphenicol resistance casette, *repA* replication initiation protein for the *E. coli* pSC101 replication origin, *par* partitioning locus for *E. coli*, *eno* enolase promoter, regulatory region from Clo1313_2090. *bdhA*, butanol dehydrogenase (Teth39_1597) from *Thermoanaerobacter pseudethanolicus* 39E. **c** NcoI restriction enzyme digest of purified pNW33N-based plasmid pJGW93NR and plasmid DNA isolated from *E. coli* transformed with JWCT28 genomic DNA. Expected bands: 2.9 kb + 2.6 kb. **d** Gel showing the results of a PCR using integration-specific primers SK038 and DC461. An integration of the plasmid

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will result in a 1.7-kb PCR product amplified from the enolase promoter region in the chromosome and the pSKW79 plasmid

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Table 1

Strains and plasmids used in this study

Strain or plasmid	Genotype/phenotype	Source
C. thermocellum		
LL1005	*C. thermocellum* DSM 1313 Δ*pyrF* (*ura*$^{-}$/5-FOA^{R})	[48]
JWCT02	*C. thermocellum* Δ*pyrF* + pDCW89 (*ura*$^{+}$/5-FOA^{S})	[19]
JWCT03	*C. thermocellum* Δ*pyrF* + pDCW196 (*ura*$^{+}$/5-FOA^{S}/Tm^{R})	[19]
JWCT10	*C. thermocellum* Δ*pyrF* Δ*recA*::P$_{Cbes_2105}$-*pyrF* (*ura*$^{+}$/5-FOA^{S})	This work
JWCT26	*C. thermocellum* Δ*pyrF* Δ*recA*::P$_{Cbes_2105}$-*pyrF* + pJGW92 (*ura*$^{+}$/5-FOA^{S}/Tm^{R})	This work
JWCT28	*C. thermocellum* Δ*pyrF* Δ*recA*::P$_{Cbes_2105}$-*pyrF* + pJGW93NR (*ura*$^{+}$/5-FOA^{S}/Tm^{R})	This work
JWCT29	*C. thermocellum* Δ*pyrF* Δ*recA*::P$_{Cbes_2105}$-*pyrF* + pSKW79 (*ura*$^{+}$/5-FOA^{S}/Tm^{R})	This work
E. coli		
JW421	BL21 containing pJGW37 (Apr^{R})	[19]
JW448	DH5α containing pJGW61 (Apr^{R})	This work
JW449	BL21 containing pJGW61 (Apr^{R})	This work
JW478	DH5α containing pJGW91 Apr^{R})	This work
JW479	DH5α containing pJGW92 (Apr^{R}, Cm^{R})	This work
JW480	BL21 containing pJGW92 (Apr^{R}, Cm^{R})	This work
JW481	BL21 containing pJGW93NR (Apr^{R}, Cm^{R})	This work
JW506	BL21 containing pCTH07	This work
JW579	BL21 containing pSKW79	This work
Plasmids		
pDCW89	*E. coli/C. bescii/C. thermocellum* shuttle vector with pBAS2 replicon (P$_{Cbes2105}$–*pyrF*, Apr^{R})	[9]
pDCW196	*E. coli/C. thermocellum* shuttle vector with pBAS2 replicon (P$_{Cbes2105}$–*pyrF*/P$_{gapDH}$–*cat*, Apr^{R}, Cm^{R})	[19]
pJGW37	*E. coli/C. thermocellum* shuttle vector with pBAS2 replicon (P$_{Cbes2105}$–*cat*, Apr^{R}, Cm^{R})	[19]
pJGW61	*recA* (Clo1313_1163) marker replacement vector with pNW33N replicon (P$_{Cbes2105}$–*pyrF*, Apr^{R})	This work
pJGW91	*E. coli/C. thermocellum* shuttle vector expressing *recA* with pBAS2 replicon (P$_{Cbes2105}$–*pyrF*, Apr^{R})	This work
pJGW92	*E. coli/C. thermocellum* shuttle vector expressing *recA* with pBAS2 replicon (P$_{Cbes2105}$–*cat*, Apr^{R}, Cm^{R})	This work
pJGW93	*E. coli/C. thermocellum* shuttle vector expressing *recA* with pNW33N replicon (P$_{Cbes2105}$ – *cat*, Apr^{R}, Cm^{R})	This work

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Strain or plasmid	Genotype/phenotype	Source
pJGW93 NR	*E. coli*/*C. thermocellum* shuttle vector with pNW33N replicon and empty S-layer protein expression cassette ($P_{Cbes2105}$–*cat*, Apr^R, Cm^R)	This work
pSKW79	*E. coli*/*C. thermocellum* shuttle vector with pNW33N replicon and *bdhA* gene driven by the *C. thermocellum eno* promoter ($P_{Cbes2105}$–*cat*, Apr^R, Cm^R)	This work
pMU1162	*S. cerevisiae*–*E. coli*–*C. thermocellum* vector with pNW33N replicon (P_{cbp}-*pyrF* cassette, Amp^R, Cm^R)	[48]

5-FOA^R 5-fluoroorotic acid resistant, *5-FOA*^{S} 5-fluoroorotic acid sensitive, *Tm*^{R} thiamphenicol resistant, *Tm*^{S} thiamphenicol sensitive

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Table 2

Comparative doubling times of wild-type and mutant strains

Medium	DSM 1313 (h)	LL1005 ΔpyrF (h)	JWCT10 ΔrecA::pyrF (h)
CTFUD with 4.5 g/L yeast extract	1.9 ± 0.02	3.4 ± 0.2	3.1 ± 0.05
CTFUD without yeast extract	2.0 ± 0.04	2.0 ± 0.07	4.7 ± 0.2