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The identification of four histidine kinases that influence sporulation in *Clostridium thermocellum*

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ABSTRACT

In this study, we sought to identify genes involved in the onset of spore formation in *Clostridium thermocellum* via targeted gene deletions, gene over-expression, and transcriptional analysis. We determined that three putative histidine kinases, *clo1313_0286, clo1313_2735* and *clo1313_1942* were positive regulators of sporulation, while a fourth kinase, *clo1313_1973*, acted as a negative regulator. Unlike *Bacillus* or other *Clostridium* species, the deletion of a single positively regulating kinase was sufficient to abolish sporulation in this organism. Sporulation could be restored in these asporogenous strains via over-expression of any one of the positive regulators, indicating a high level of redundancy between these kinases. In addition to having a sporulation defect, deletion of *clo1313_2735* produced L-forms. Thus, this kinase may play an additional role in repressing L-form formation. This work suggests that *C. thermocellum* enters non-growth states based on the sensory input from multiple histidine kinases. The ability to control the development of non-growth states at the genetic level has the potential to inform strategies for improved strain development, as well as provide valuable insight into *C. thermocellum* biology.

1. Introduction

Clostridium thermocellum is an anaerobic soil bacterium that is able to rapidly solubilize cellulose and produces ethanol as an end product of its metabolism [1]. As such, it is an organism of interest for the production of bioethanol from cellulosic biomass. Like other members of the genus *Clostridium*, *C. thermocellum* has the ability to sporulate when subjected to unfavorable growth environments, leading to a non-growth state known as a spore [2,3]. While sporulation is extremely advantageous in environmental situations, this form is potentially undesirable in an industrial process.

In addition to spores, *C. thermocellum* forms another non-growing cell type known as the L-form [3]. L-forms occur in a variety of bacterial species and are generally defined as cell-wall-less or cell-wall-deficient bacteria [4,5]. They can be identified by their pleomorphic cell morphology, which differs significantly from the normal morphology of the species [4,5]. L-forms can arise spontaneously after prolonged cultivation or starvation, but the L-form morphology has also been connected with a variety of gene mutations including those involved in cell division [6,7], peptidoglycan biosynthesis [8], and bacteriocin production [9,10]. While L-forms are viable and able to reproduce, they appear to have lower metabolic activity than actively growing cells [11–13]. Previously, we reported on L-form formation in *C. thermocellum* strain ATCC 27405 after substrate exhaustion [3]. The genetic mechanism of L-form formation in *C. thermocellum* has not been studied to date, but is also of interest when considering the formation of non-growing cell types.

Studies of sporulation mechanisms have primarily focused on *Bacillus* species, and particularly *Bacillus subtilis.* In *B. subtilis*, the genetic program that results in spore formation begins when the master regulator protein, Spo0A, is activated through phosphorylation [14,15]. Five orphan histidine kinases, KinA [16,17], KinB [18], KinC [19,20], KinD [21], and KinE [21], and two phosphorelay proteins, Spo0F and Spo0B [15], are responsible for the phosphorylation of Spo0A [22]. The sporulation pathway is initiated when an environmental stimulus, such as nutrient limitation, triggers auto-phosphorylation of one or more of the histidine kinases [23–25]. The two domain structures necessary for auto-phosphorylation, and therefore activation, of the kinase include the catalytic

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1075-9964/

ATPase domain (referred to as "CA", also called HATPase_c) and the dimerization and histidine phosphotransfer domain (referred to as "DHp", also called HisKA) (see Refs. [26,27] for reviews). The CA domain catalyzes the phosphorylation of the conserved histidine residue, located in the DHp domain of the kinase. Subsequently, the phosphorylated DHp domain transfers the phosphoryl group from the kinase onto the appropriate regulator protein. In the *B. subtilis* sporulation cascade, activated kinases first phosphorylate the phosphotransferase, Spo0F. Phosphorylated Spo0F (Spo0F~P) in turn phosphorylates Spo0B, and Spo0B~P then phosphorylates the master regulator of sporulation, Spo0A [15,28]. Phosphorylated Spo0A (Spo0A~P) regulates the expression of its target genes by binding to DNA sequences known as "0A" boxes located within the regulatory regions of these genes [22,29]. In addition to having an "0A" box, sporulation genes regulated by Spo0A~P have weak affinities for Spo0A~P, and thus require high levels of the master regulator in order to be differentially expressed [30]. Therefore, when Spo0A~P reaches sufficiently high levels, it initiates the onset of sporulation [30] by either enhancing expression of sporulation specific genes such *spoIIA* [18], *spoIIE* [31], and *spoIIG* [32,33], or repressing the expression of genes involved in other cellular processes [17,34,35]. Through this mechanism, initiation of the sporulation program is under the direct control of Spo0A, which is subject to input from the phosphorelay and histidine kinases.

Recent evidence supports the existence of an alternative pathway for Spo0A phosphorylation in clostridia. In *Clostridium difficile* [36] and *Clostridium acetobutylicum* [37] sporulation kinases have been shown to directly phosphorylate Spo0A in the absence of Spo0F and Spo0B homologs [38–41]. The kinases identified in these studies were also orphan histidine kinases, a characteristic conserved with *B. subtilis* sporulation kinases.

We therefore hypothesized that orphan histidine kinases are also responsible for initiating sporulation in *C. thermocellum* through a similar mechanism. In this study, we aimed to identify and characterize sporulation kinases in *C. thermocellum* in order to better understand and control the sporulation process in this organism.

2. Methods

2.1. Bacterial strains and culturing conditions

Yeast and bacterial strains are listed in Table 1. *Saccharomyces cerevisiae* InvSc1was grown in YPD media and plated on SD-ura medium (Sunrise Science Products, San Diego, CA, USA) for the selection of Ura3+ plasmids. *Escherichia coli* strains were grown on LB medium supplied with chloramphenicol (15 μg/ml) for plasmid maintenance. *C. thermocellum* was cultured anaerobically in

Table 1
Bacterial strains used in this study.

Strain name	Official name	Description and or relevant characteristics	Reference or source
Microbial strains			
S. cerevisiae InvSci	N/A	Uracil auxotroph	Invitrogen
E. coli Top10	N/A	Used for plasmid maintenance	Invitrogen
E. coli BL21 (DE3)	N/A	Used for plasmid maintenance and appropriate DNA methylation	New England Biolabs
C. thermocellum			
1313	DSMZ 1313	WT	DSMZ (Germany)
1354	M1354	*Δhpt*	Argyros et al. AEM, 2011
Δspo0A	M1726	*ΔhptΔspo0A*	Gift of Mascoma Corporation
Δ1973	LL1079	*ΔhptΔclo1313_1973*	This study
Δ2735	LL1080	*ΔhptΔclo1313_2735*	This study
Δ1942	LL1081	*ΔhptΔclo1313_1942*	This study
Δ0268	LL1082	*ΔhptΔclo1313_0268*	This study
Δ2735 + p2735	LL1089	*ΔhptΔclo1313_2735* containing pEBM119, reintroduces expression of *clo1313_2735*	This study
Δ2735 + EV	LL1116	*ΔhptΔclo1313_2735* containing pAMG205, an empty vector	This study
Δ1942 + p1942	LL1090	*ΔhptΔclo1313_1942* containing pEBM120, reintroduces expression of *clo1313_1942*	This study
Δ1942 + EV	LL1119	*ΔhptΔclo1313_1942* containing pAMG205, an empty vector	This study
Δ0268 + p0268	LL1091	*ΔhptΔclo1313_0268* containing pEBM121, reintroduces expression of *clo1313_0268*	This study
Δ0268 + EV	LL1121	*ΔhptΔclo1313_0268* containing pAMG205, an empty vector	This study
Δ1942Δ1973	LL1101	*ΔhptΔclo1313_1942Δclo1313_1973*	This study
Δ1942Δ2735	LL1102	*ΔhptΔclo1313_1942Δclo1313_2735*	This study
Δ1942Δ0268	LL1103	*ΔhptΔclo1313_1942Δclo1313_0268*	This study
Δ0268Δ1973	LL1104	*ΔhptΔclo1313_0268Δclo1313_1973*	This study
Δ0268Δ2735	LL1105	*ΔhptΔclo1313_0268Δclo1313_2735*	This study
Δ2735Δ1973	LL1106	*ΔhptΔclo1313_2735Δclo1313_1973*	This study
wt + EV	LL1092	DSMZ 1313 containing pAMG205, empty vector control	This study
wt + spo0A	LL1125	DSMZ 1313 containing pEBM136, overexpresses *spo0A*	This study
wt + p1973	LL1093	DSMZ 1313 containing pEBM118, overexpresses *clo1313_1973*	This study
wt + p2735	LL1094	DSMZ 1313 containing pEBM119, overexpresses *clo1313_2735*	This study
wt + p1942	LL1095	DSMZ 1313 containing pEBM120, overexpresses *clo1313_1942*	This study
wt + p0268	LL1096	DSMZ 1313 containing pEBM121, overexpresses *clo1313_0268*	This study
wt + p 1711	LL1097	DSMZ 1313 containing pEBM122, overexpresses *clo1313_1711*	This study
wt + p0495	LL1098	DSMZ 1313 containing pEBM123, overexpresses *clo1313_0495*	This study
Δ2735 + p1942	LL1117	*ΔhptΔclo1313_2735* containing pEBM120, overexpresses *clo1313_1942*	This study
Δ2735 + p0268	LL1118	*ΔhptΔclo1313_2735* containing pEBM121, overexpresses *clo1313_0268*	This study
Δ1942 + p2735	LL1120	*ΔhptΔclo1313_1942* containing pEBM119, overexpresses *clo1313_2735*	This study
Δ1942 + p0268	LL1121	*ΔhptΔclo1313_1942* containing pEBM121, overexpresses *clo1313_0268*	This study
Δ0268 + p2735	LL1123	*ΔhptΔclo1313_0268* containing pEBM119, overexpresses *clo1313_2735*	This study
Δ0268 + 1942	LL1124	*ΔhptΔclo1313_0268* containing pEBM121, overexpresses *clo1313_1942*	This study

modified DSM122 medium [42] supplemented with 50 mM MOPS and 10 mM sodium citrate. Plasmids were maintained with the addition of 12 μg/ml thiamphenicol. *C. thermocellum* growth and mutant selection took place in either closed serum bottles purged with nitrogen or in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). All *C. thermocellum* strains were derived from the type strain DSMZ 1313 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany), and all mutants were constructed in the M1354 background strain (gift of Mascoma Corporation), in which the gene encoding hypoxanthine phosphoribosyltransferase (*hpt, clo1313_2927*) is deleted [43] providing a means for negative selection. The *C. thermocellum spo0A* deletion strain, M1726, was a gift from Mascoma Corporation and was constructed in *C. thermocellum* strain DSMZ 1313 [44] following the procedures as outlined by Argyros and coworkers [43]. Briefly, the plasmid pMU1810 (GenBank Accession # KC779546) was used to delete the gene encoding the ortholog of the *B. subtilis* response regulator of sporulation, *spo0A* (*clo1313_1409)* in a *C. thermocellum Δhpt* background (M1354) (1). This yielded the *Δhpt Δspo0A* genotype henceforth referred to as M1726.

2.2. Identification of putative sporulation kinases

The genome sequences of *C. thermocellum* DSMZ 1313 and ATCC 27405 were searched for histidine kinase genes based on the available annotation(s). By comparing the annotated kinase sequences in each genome, a mis-annotation was identified wherein clo1313_0268 was incorrectly annotated as a hypothetical protein fragment in the DSMZ1313 genome, but was the equivalent gene, cthe_2677 was annotated correctly in the ATCC 27405 genome. To resolve such discrepancies, a secondary analysis of gene sequences was undertaken using Uniprot prediction software (http://www.uniprot.org/) [45] to confirm that all of the identified genes were predicted kinases. In DSMZ 1313, 41 histidine kinases genes were identified, whereas 42 were identified in ATCC 27405 (Table S1). Of the 42 histidine kinases identified in DSMZ 1313, 19 appeared to be orphan histidine kinases. Identification of phosphodonor (HisKA) domains was performed by Simple Modular Architecture Tool (SMART), (smart.embl-heidelberg.de/) [46] and verified by Pfam prediction software [47] (http://pfam.sanger.ac.uk/). Multiple sequence alignments of the kinase HisKA domains were generated with ClustalW [48].

2.3. Plasmid construction

All plasmids were constructed via yeast gap repair cloning using a modified lazybones protocol [49,50], in which the amplified fragments and plasmid backbone were assembled into a plasmid via yeast homologous recombination. *E. coli* was used as a host strain for plasmid propagation. In order to generate the plasmids needed for gene deletions, pAMG258 (Gift from Dr. Adam Guss) was digested with BamHI and EcoRI restriction endonucleases (New England Biolabs (NEB), Ipswich, MA, USA) and all fragments were gel purified. Next, a region encompassing the respective *C. thermocellum* target gene was then amplified from genomic DNA with primers that contained an additional 40 bp of sequence homologous to either side of the EcoRI cut site of the recipient plasmid (pAMG258). The target gene was then inserted into the recipient plasmid at the EcoRI cut site by yeast-mediated recombination. After this, it was necessary to construct a fragment containing approximately 1 kb of the up and 1 kb of the downstream regions adjacent to the target gene on the chromosome. To do this, DNA regions located directly upstream and directly downstream of the respective target gene were amplified via PCR with oligomers (Integrated DNA Technologies (IDT), http://www.idtdna.com/) that contained 40 bp of homology to the recipient plasmid BamHI site on the external flanks and 40 bp of homology to each other on the internal flanks. This allowed for the target's upstream and downstream regions to be cloned adjacently. These fragments were subsequently integrated into the BamHI site of the recipient plasmid via yeast-mediated recombination. Plasmid construction primers are listed in Table S2. Prior to cloning, a small sample of each amplified product was run on a 1% agarose gel to verify the size, and if correct the product was used in the cloning reaction.

In order to construct plasmids for the expression of each kinase gene via the *gapDH* promoter, pAMG205 was digested with ZraI and XhoI restriction endonucleases (NEB), releasing the *pyrF* auxotrophic marker from the construct. The plasmid backbone was then isolated by gel electrophoresis, gel extracted and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA). Each respective gene was then amplified with DNA oligomers (IDT) designed to overlap with the cut ends of pAMG205, and was cloned in via yeast gap repair cloning. Plasmids were isolated from yeast using Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research) and were introduced via electroporation into *E. coli*

Table 2
Plasmids used in this study.

Name	Description and or relevant characteristics	Reference, source, or construction
Cloning vectors		
pAMG205	Cloning vector, contains oriColE1, CEN6, ARSH4, URA3, pGapD-cat, pNW33N replication origin	Guss et al., 2011 Biotech for Biofuels
pAMG258	Cloning vector, contains oriColE1, CEN6, ARSH4, URA3, pGapD-cat, pNW33N replication origin	Gift of Dr. Adam Guss
Deletion vectors		
pEBM100	Used to delete *clo1313_1973*. Contains1kb homology to the up and downstream regions of *clo1313_1973* cloned into the BamHI site and the entire gene region of *clo1313_1973* cloned into EcoRI of pAMG258	This study; Genbank# KC879306
pEBM101	Used to delete *clo1313_2735*. Contains1kb homology to the up and downstream regions of *clo1313_2735* cloned into the BamHI site and the entire gene region of *clo1313_2735* cloned into EcoRI of pAMG258	This study; Genbank# KC879307
pEBM102	Used to delete *clo1313_1942*. Contains1kb homology to the up and downstream regions of *clo1313_1942* cloned into the BamHI site and the entire gene region of *clo1313_1942* cloned into EcoRI of pAMG258	This study; Genbank# KC879308
pEBM103	Used to delete *clo1313_0268*. Contains1kb homology to the up and downstream regions of *clo1313_0268* cloned into the BamHI site and the entire gene region of *clo1313_0268* cloned into EcoRI of pAMG258	This study; Genbank# KC879309
Complementation/overexpression vectors		
pEBM118	Contains *clo1313_1973* driven by the *gapD* promoter in pAMG205	This study; Genbank# KC879310
pEBM119	Contains *clo1313_2735* driven by the *gapD* promoter in pAMG205	This study; Genbank# KC879311
pEBM120	Contains *clo1313_1942* driven by the *gapD* promoter in pAMG205	This study; Genbank# KC879312
pEBM121	Contains *clo1313_0268* driven by the *gapD* promoter in pAMG205	This study; Genbank# KC879313
pEBM122	Contains *clo1313_0495* driven by the *gapD* promoter in pAMG205	This study; Genbank# KC879314
pEBM123	Contains *clo1313_1711* driven by the *gapD* promoter in pAMG205	This study; Genbank# KC879315
pEBM136	Contains *clo1313_1409* (*spo0A*) driven by the *gapD* promoter in pAMG205	This study; Genbank# KF724861

Top10 (*dam* + *dcm* + *E. coli* K12 derivative from Invitrogen, Carlsbad, CA). Subsequently, plasmids were transformed via chemical competence into *E. coli* BL21 (Cat# C2523, *dam*⁻*dcm*⁻*E. coli* K12 derivative; New England Biolabs) before *C. thermocellum* transformation. Plasmids were isolated from strains using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). The DNA sequences of all plasmids used for complementation or overexpression analysis have been uploaded to GenBank and each is also listed in Table 2 along with their relevant characteristics.

2.4. Construction of strains and phenotypic analysis

C. thermocellum transformation and mutant selection was performed using methods that have been previously described [51,52]. All deletion strains were constructed in the M1354 background and single colony purified. Initial confirmation was achieved by screening for the appropriate deletion via PCR by using specific DNA oligomers (Integrated DNA Technologies, IDT) that amplified both internal and external DNA regions. Confirmation primers are listed Table S3. DNA fragments obtained with external screening primers were then sequenced by the Dartmouth Molecular Biology Core Facility to verify the absence of each gene and the construction of a clean deletion.

Kinases were re-introduced into deletion strains by transforming each deletion strain with a vector harboring the respective kinase gene or an alternative kinase gene. Overexpression strains were constructed in the same manner in the DSMZ 1313 background. In all cases, the *C. thermocellum* glyceraldehyde 3-phosphate dehydrogenase (*gapD*) promoter was used to drive expression of the target gene.

Sporulation was evaluated for all strains under standard laboratory conditions; all strains were grown in modified DSM122 media and endospore formation was evaluated after 24 h. This method produced the most consistent number of mature spores in each strain and reduced the possibility that certain kinases were more responsive to a given sporulation treatment (i.e. carbon changes or oxygen exposure, as was determined previously, see Ref. [3] for details) than others. For all single kinase deletion strains, viable cells were quantified via serial dilution plating before heat treatment (Figure S1). For all strains, spores were quantified via serial dilution after heat treatment for 30 min at 100 °C. Cells and spores were plated in modified DSM 122 media containing 8 g/L agar (Acros Organics, New Jersey, USA), and quantification is presented as colony forming units/ml (CFUs/ml). The presence of L-forms in *Δ*2735 was determined by microscopy. Strain *Δ*2735 was constructed on three independent occasions in order to verify the phenotype.

2.5. RNA isolation and gene expression measurements

RNA was isolated from all samples using RNeasy Mini Kit (Qiagen). An on-column DNase (Qiagen) treatment was used to eliminate DNA contamination followed by an additional DNase treatment and secondary clean-up procedure.

All DNA oligos used for RT-PCR are listed in Table S4. The targets selected included *spo0A* (*clo1313_1409*) and two putative sporulation sigma factors, annotated as RNA polymerase sigma 28-subunits SigF *(clo1313_2112)* and SigG *(clo1313_1772)*. Expression levels for each gene and each sample were compared against the housekeeping gene, *recA* (*clo1313_1163*). The *recA* gene was chosen as an internal reference gene based on its consistent expression across a wide range of growth conditions in both transcriptomic and proteomic profiles [53–55]. Consistent expression of *recA* was verified via qRT-PCR count threshold values when RNA concentrations were set to 30 ng/ul in samples taken throughout growth and sporulation (data not shown). DNA oligos designed to target a 70-200 bp region of each target gene were created and searched against the *C. thermocellum* chromosome for uniqueness using the BLAST algorithm. No oligo had an expectation value greater than 0.58. Melt peak analysis was determined using Bio-Rad CFX manager software, version 2.1, and no secondary peaks were detected. Primer amplification efficiency was determined by using 9 10-fold dilutions of genomic DNA to generate a standard curve for all primer pairs. The cDNA library and subsequent qPCR reactions were performed in a one-step reaction using iScript cDNA Synthesis Kit (Biorad) on CFX96 qPCR machine (Bio-Rad Corporation) with an annealing temperature of 50 °C and other cycling parameters as suggested by the iScript datasheet. Cq values were determined using Bio-Rad CFX manager software, and normalized gene expression levels were calculated using the ΔΔCq method with *recA* serving as the reference gene. All samples were run in duplicate, and at least three replicates were obtained for each sample. Statistical significance was calculated using a Student's *t*-test, with a threshold of $p = 0.05$.

3. Results

3.1. Identification of sporulation kinases

We identified 41 histidine kinases in *C. thermocellum* DSM 1313 (Table S1) including *clo1313_0268*, which had been annotated as a hypothetical protein fragment. Of the 41 total histidine kinase genes, 19 were unassociated with a response regulator gene suggesting that they operate as orphan kinases. To determine which of these orphan kinases are most likely to play a role in sporulation,

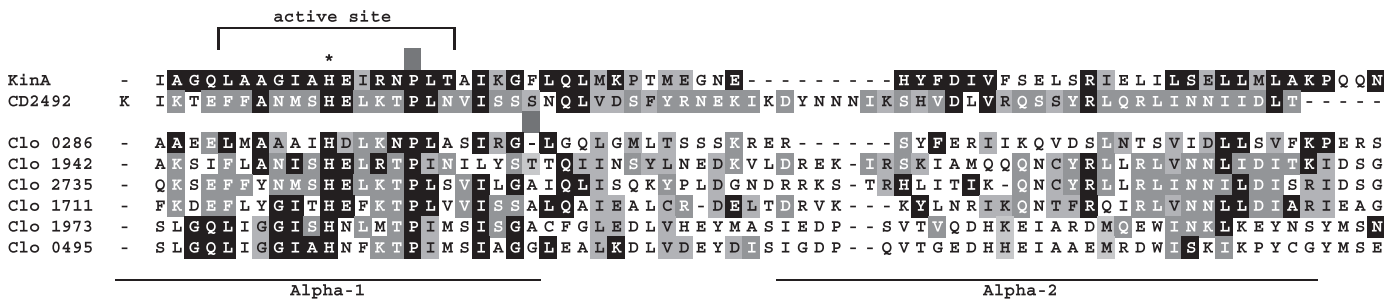


Fig. 1. Comparison of histidine kinase phosphodonor active site domains. The DHp/HisKA domains of several putative sporulation histidine kinases in *C. thermocellum* were compared to known sporulation kinases CD2492 (*C. difficile*) and KinA (*B. subtilis*). Identical residues conserved with KinA are shaded black, identical residues conserved with CD2492 are shaded dark gray, and residues that are similar but not identical to either reference sequence are shaded light gray. The phosphorylated histidine residue is denoted by an asterisk. The solid line indicates the two alpha helices of the domain, which are involved in dimerization. Alignment was constructed based on the ClustalW2 algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

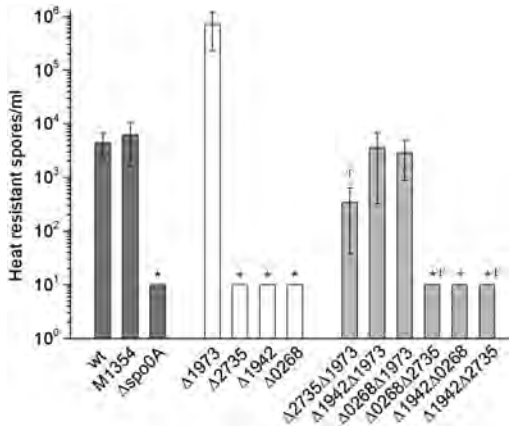


Fig. 2. Phenotypic consequences of histidine kinase deletions. Deletions were made for four putative sporulation kinases, clo1313_1973, clo1313_2735, clo1313_1942, and clo1313_0268 in the background strain M1354, resulting in the strains Δ1973, Δ2735, Δ1942 and Δ0268 respectively (white bars). Combined deletion strains were also constructed (light gray bars). The ability of each deletion strain to form spores was quantified after 24 h of growth via heat treatment and viable count plating and compared to several reference strains (gray bars). The values shown are the means ± the SEM for no fewer than 3, no more than 8 biological replicates. Values below the limit of detection (10 CFUs/ml) are indicated with an asterisk. Ψ Denotes that the L-forms phenotype of the parent strain was lost. Statistical significance is reported as follows: The background strain, M1354, was not statistically different from wild type ($p = 0.70$). Strains that gave an asporogenous phenotype (Δspo0A, Δ2735, Δ1942, Δ0268 and double mutants containing a combination of two of these mutations) were statistically different from wild type (p-values below 0.05). Strain Δ1973 was statistically different from wild type at $p = 0.1$. Double knock out strains Δ1942Δ1973 and Δ0268Δ1973 were not statistically significant from wild type (p value = 0.37 and 0.32 respectively), but sporulation frequency was statistically lower in strain Δ2735Δ1973 (p-value lower than 0.1).

each was evaluated for the presence of two vital domains; an ATPase and a phosphodonor/phosphoacceptor domain (HisKA) using SMART protein prediction software [46,56]. Only six orphan kinases contained the HisKA domain, *clo1313_1973, clo1313_2735, clo1313_1942, clo1313_0268, clo1313_0495* and *clo1313_1711*. The HisKA domains of these six kinases were compared via an alignment to other known sporulation kinases; KinA from *B. subtilis* and CD2492 from *C. difficile*. This alignment revealed that over 50% of residues contained within the active site were conserved between the *C. thermocellum* HisKA domains and the reference sequences (Fig. 1). Over 30% of all resides were conserved between the reference sequences and Clo1313_0268, Clo1313_1942, Clo1313_2735, and Clo1313_1711 domains. Clo1313_1973 and Clo1313_0495 were approximately 25% similar over the whole domain. Based on this identification process, we predicted that these six kinases were potentially involved in sporulation and they are henceforth the subjects of this study.

3.2. Phenotypic consequences of histidine kinase deletions

The six genes encoding the histidine kinases identified were targeted for deletion to determine the effect each gene had on the ability of the cells to sporulate. Deletions were made for four of the kinases including *clo1313_1973, clo1313_2735*, *clo1313_1942*, and *clo1313_0268* in the background strain M1354, creating the strains Δ1973, Δ2735, Δ1942 and Δ0268 respectively. We were unable to delete *clo1313_0495* and *clo1313_1711*. The ability of each deletion strain to form spores was determined by viable count plating after heat treatment, as has been described for *C. thermocellum* previously [3]. Results were dually verified by microscopy and compared to three reference strains including the wild-type, an *hpt* deletion strain (M1354), and a *spo0A* deletion strain (M1726) (data not shown). Like M1726, spores were undetectable in Δ2735, Δ1942, and Δ0268, but Δ1973 demonstrated enhanced sporulation compared to the wild-type and M1354 (Fig. 2). Unexpectedly, Δ2735 had a secondary phenotype in which the strain produced cells with an L-form like morphology during standard growth (Fig. 3). This phenotype was relatively unstable and the strain had to be constructed on repeated occasions in order to verify the phenotype and work with it.

To gain further insight as to how each kinase contributed to sporulation initiation, we created several double knock out strains.

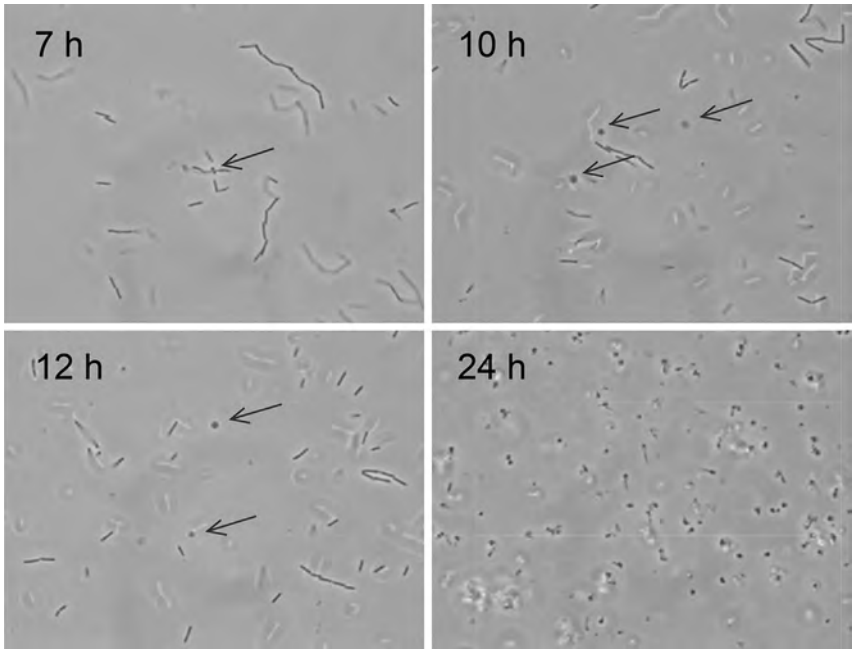
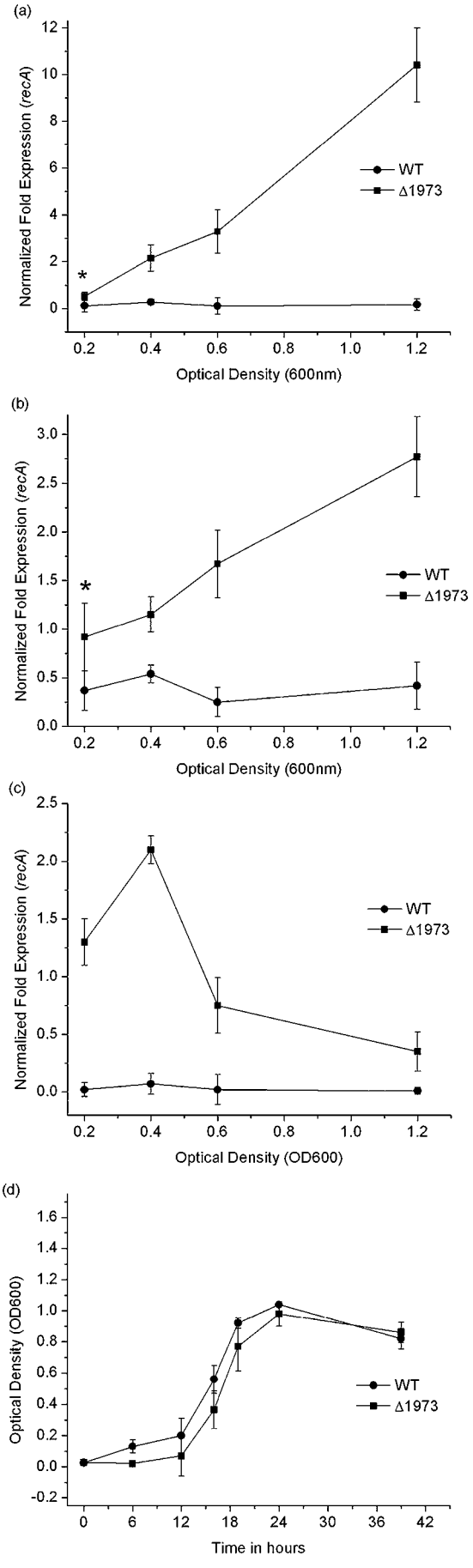


Fig. 3. L-form formation is triggered by the *clo1313_2735* deletion. In Δ2735, L-forms appear within 7 h after inoculation, and increase in number throughout a 24-h time course. Images were taken at 400× magnification. Early L-forms are denoted by arrows.


(a)
Normalized Fold Expression (recA)
Optical Density (600nm)
WT
Δ1973
*
(b)
Normalized Fold Expression (recA)
Optical Density (600nm)
WT
Δ1973
*
(c)
Normalized Fold Expression (recA)
Optical Density (OD600)
WT
Δ1973
(d)
Optical Density (OD600)
Time in hours
WT
Δ1973

First, each asporogenous strain was combined with a *clo1313_1973* deletion. In each case, when Δ1973 combined with one of the other kinase deletions sporulation was restored to approximately WT levels (Fig. 2). If an asporogenous single deletion was combined with another asporogenous deletion, the double knock out was also asporogenous. Surprisingly, when the *clo1313_2735* deletion was introduced into any other kinase deletion strain, the L-form phenotype was lost. This may have been caused by the relative instability of the L-form phenotype caused by the *clo1313_2735* mutation (discussed later).

3.3. Impact of Δ1973 on downstream sporulation genes

In other sporulating bacteria, completion of the sporulation process is dependent on the activation of Spo0A and of several sporulation specific sigma factors [57,58]. To determine what effect the *clo1313_1973* deletion had on the downstream sporulation pathway of *C. thermocellum*, gene expression was measured over time for *spo0A* (*clo1313_1409*) and two putative sporulation sigma factor genes encoding SigF (*clo1313_2111*) and SigG (*clo1313_1772*) in Δ1973 and the wild type. In *B. subtilis*, Spo0A~P directly regulates *spo0A* and *sigF* expression [29] in a positive manner. *SigG* expression also essential for the completion of spore formation, however, it is not under the direct control of Spo0A~P [29,59]. We noted upregulation of all three genes in Δ1973 compared to the wild type strain (Fig. 4). Levels of *spo0A* (Fig. 4(a)) and *sigF* (Fig. 4(b)) transcripts increased over time and peaked in stationary phase, whereas *sigG* (Fig. 4(c)) peaked during mid-log phase and decreased over time. Growth rates for Δ1973 and the wild type were found to be comparable (Fig. 4(d)). The maximum number of mature spores is reached between 18 and 24 h (data not shown).

As *C. thermocellum* sporulation is asynchronous, it is important to note that the optical density value at which the expression of *spo0A*, *sigF* and *sigG* peak (or wane) cannot be correlated with defined stages of sporulation. However, the elevated expression of these genes does suggest hyperactivity of the sporulation pathway in the absence of *clo1313_1973* and higher expression levels of sporulation-associated genes in Δ1973 are in agreement with the observed hypersporulating phenotype of this strain.

We also measured the expression of *spo0A*, *sigF* and *sigG* in the other kinase deletion strains and the *spo0A* deletion strain. None of the sporulation deficient strains had significantly different expression profiles when compared to the wild type (data not shown). We suspect this is largely due to the low sporulation frequency of wild type *C. thermocellum*, which is approximately 0.002% under standard laboratory conditions.

3.4. Phenotypic consequences of kinase gene expression in deletion and wild-type backgrounds

Each kinase gene was re-introduced into its respective deletion strain via a replicating plasmid (with the exception of Δ1973, which could not be transformed despite many attempts). The native *C. thermocellum* glyceraldehyde 3-phosphate dehydrogenase (*gapD*) promoter was used to drive expression of each gene. Re-introduction of clo1313_2735, clo1313_0268, and clo1313)_1942 into their respective deletion backgrounds recovered sporulation to detectable levels (Fig. 5).

We were able to demonstrate pronounced effects on sporulation when specific histidine kinases were targeted for deletion. However, it was not possible to make deletions of two of the kinases predicted to be involved in sporulation, and one of the deletion strains, Δ2735, had a phenotype previously never associated with spore formation. As an alternative approach, we expressed each kinase under the *gapD* promoter on a replicative plasmid in WT *C. thermocellum* to observe the resulting effects on sporulation. When this strategy was employed we observed a significant increase in sporulation for strains overexpressing *clo1313_1942, clo1313_0268* and *clo1313_1711* (Fig. 5). A slight, but still significant, increase in sporulation was also observed in the strain overexpressing *clo1313_2735*. The strain overexpressing *clo1313_1973* did not have detectable spores and overexpression of *clo1313_0495* produced roughly wild type levels of sporulation (Fig. 5). All strains were compared to DSMZ 1313 harboring an empty vector, and a *spo0A* overexpression strain, which also demonstrated a roughly 10-fold increase in sporulation frequencies compared to the control strain.

***C. thermocellum* sporulation kinases functionally complement each other** Our previous results indicated that *clo1313_1942, clo1313_0268,* and *clo1313_2735,* were all positive regulators of sporulation and that expression of each of these was essential in order for sporulation to occur in *C. thermocellum*. To better understand the specific contribution of each kinase and determine whether or not they share functionality, each of these kinases was expressed in an alternative kinase deletion background and sporulation frequencies were assessed. In all cases, each kinase was able to complement an alternative kinase deletion by restoring sporulation to detectible levels (Fig. 6). This data, combined with our phenotypic analysis of kinase deletion, reintroduction, and overexpression strains supports the idea that these kinases act independently to both positively and negatively regulate sporulation (Fig. 7).

4. Discussion

In this study, we took a genetic approach to determine the roles of several orphan histidine kinases in *C. thermocellum*. These kinases were predicted to have a role in spore formation based on their orphan distinction and similarity within their phosphodonor active site to other kinases known to be involved in sporulation initiation, a strategy that has been successfully employed elsewhere [36,37]. Through targeted gene deletions and subsequent reintroduction, as well as through over-expression in the wild type background, we showed that *clo1313_1973, clo1313_1942, clo1313_2735* and *clo1313_0268* influence sporulation in *C. thermocellum*. We also observed an unexpected L-forms phenotype when *clo1313_2735* was deleted, which may suggest that in *C. thermocellum* the mechanisms that regulate sporulation and L-form formation are both controlled by sensory input from histidine kinases.

Here we demonstrated that *clo1313_1942* and *clo1313_0268* appear to act as positive regulators of sporulation in *C. thermocellum*. Deletion of either of these two kinases reduced the sporulation frequency to undetectable levels, reintroduction reinstated sporulation, and overexpression increased sporulation levels by about 10–100-fold. The abolishment of sporulation in Δ1942

Fig. 4. Increased expression of sporulation associated genes in Δ1973. The expression of sporulation genes *spo0A* (a), *sigF* (b), and *sigG* (c) was monitored in wild type (circles) and Δ1973 (squares) throughout growth via qRT-PCR. Error bars are indicative of the standard deviation for $n = 3$ biological replicates. All results are statistically significant ($p = 0.05$) unless denoted by an asterisk. Each sample is normalized to the housekeeping gene, *recA*. (d) Growth curves for WT and Δ1973: optical density correlates with growth phase as follows: Early exponential phase occurs between 0 and 0.4; Mid-exponential phase occurs between 0.4 and 0.8; and late exponential/transition to stationary phase occurs between 0.8 and 1.2. Maximum number of mature heat resistant spores is reached between 18 and 24 h (data not shown).

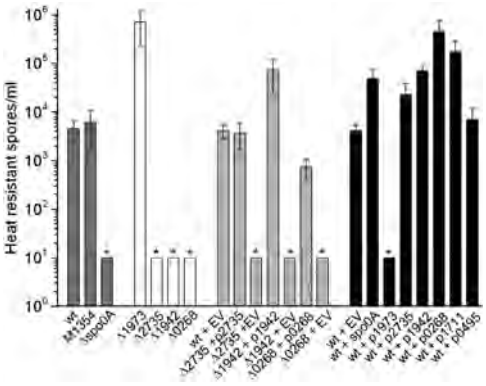


Fig. 5. Phenotypic consequence of kinase reintroduction and over-expression. Each kinase was re-introduced into either its respective deletion background (light gray bars) or the wild type background (black bars) to create kinase reintroduction and over-expression strains respectively. A *spo0A* over-expression line was also created for comparison. Spores were quantified via heat treatment and viable count plating. The values shown are the means ± the SEM for no fewer than 3, no more than 9 biological replicates. Values below the limit of detection (10 CFUs/ml) are indicated with an asterisk. EV = empty vector control. Statistical significance of background strains and single kinase deletion strains is reported in Fig. 2 legend. Statistical significance of kinase reintroduction and over-expression strains is reported as follows: All asporogenous strains (denoted by an asterisk) were statistically different from wild type (p = less than 0.05). Strains Δ0268+p0268, and wt+1942 were statistically significant at p = 0.05. Strains Δ1942+p1942, wt+p2735, wt+p0268, wt+p1711 and wt+spo0A are statistically significant at p = 0.1. Strains Δ2735+p2735, wt+EV, and wt+p0495 were not statistically different from wild type (p values = 0.42, 0.43, and 0.21 respectively).

and Δ0268 was an unexpected phenotype, as single kinase deletions typically produce a reduced sporulation frequency in *B. subtilis,* [19,23,60], *C. difficile* [36] and *C. acetobutylicum* [37], but do not produce asporogenous strains. This unexpected phenotype may be caused by the lower sporulation frequency of *C. thermocellum* compared to other sporulating organisms. *C. thermocellum* typically sporulates at a frequency of less than 10% during sporulation inducing conditions [3], and at a rate of less than 0.002% under standard lab conditions, which were employed in this study. When compared to *B. subtilis*, *C. acetobutylicum*, and *C. difficile*, in which sporulation frequencies upwards of 70% [61], 90% [62], and 60% [63] have been observed respectively, it may be that *C. thermocellum* has a genetic mechanism in place that reflects this lower sporulation frequency.

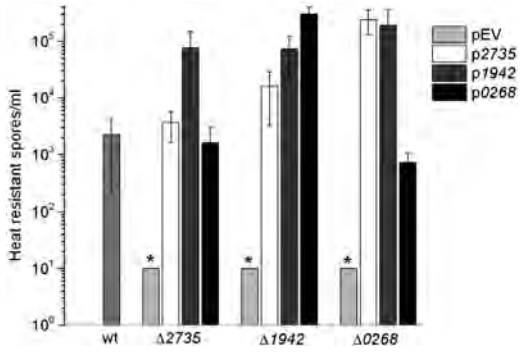


Fig. 6. Three of the *C. thermocellum* sporulation kinases functionally complement each other. Kinases involved in positively regulating *C. thermocellum* sporulation were cloned onto a replicating plasmid and expressed in an alternative kinase deletion background. Spores were quantified via heat treatment and viable count plating, and values shown are representative of n = 5 biological replicates, with error bars indicative of the S.E.M.

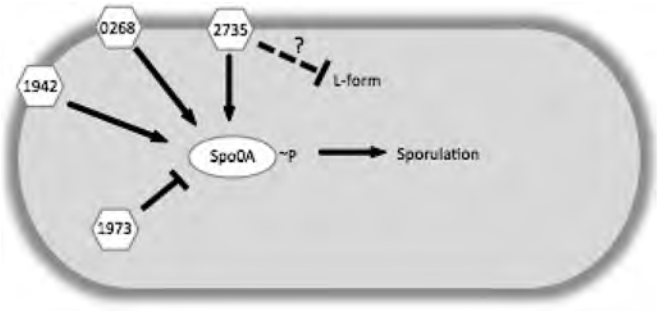


Fig. 7. Model: Histidine kinases act independently to activate or inhibit sporulation in *C. thermocellum*. The cumulative effects of kinase activity on Spo0A determine whether sporulation will be initiated. Activation of Spo0A by a respective kinase (hexagons) is depicted with an arrow; a blocked line depicts inhibition. Clo1313_2735 has an apparent role in both spore and L-form formation (dashed line). The mechanism for L-form formation in *C. thermocellum* is as of yet, unknown.

While *clo1313_1942* and *clo1313_0268* promote sporulation in *C. thermocellum*, *clo1313_1973* has a negative influence on sporulation. This is in contrast to the *B. subtilis* sporulation pathway, where the histidine kinases do not typically play a role in negative regulation. Rather, negative regulation in *B. subtilis* is controlled by the action of several phosphatases, including RapA and Spo0E, which act to dephosphorylate Spo0F~P and Spo0A~P respectively [64]. However, in *C. acetobutylicum*, negative regulation of spore formation is performed by a histidine kinase, Cac0437. This kinase dephosphorylates Spo0A~P and increases the expression levels of downstream sporulation genes, including *spo0A, sigG* and an anti-anti-sigma factor that promotes *sigF* transcription, *spoIIAA* [37]. In their study, Steiner at al. show that disruption of *cac0437* leads to a hypersporulation phenotype, while overexpression causes a decrease in sporulation frequencies. In a similar manner, deletion of *clo1313_1973* displayed a roughly 100-fold increase in spore formation relative to the wild type, while overexpression of this gene caused an asporogenous phenotype. Δ1973 also exhibits an increase in *spo0A*, *sigF* and *sigG* expression, suggesting that this gene has an impact on the expression levels of downstream sporulation genes. While the expression of the sporulation specific sigma factors of *B. subtilis* are subject to very precise timing [65], it appears that the sporulation sigma factors of many *Clostridium* species follow a different expression pattern, which may vary considerably depending on the species [66–68]. In *C. thermocellum*, *spo0A* and *sigF* appear to accumulate to their highest levels in early stationary phase, whereas *sigG* peaks in mid-exponential phase. This is pattern does not resemble the expression profiles observed in *B. subtilis*, wherein *sigF* and *sigG* are expressed sequentially for relatively short periods of time. Given the expression profiles of *spo0A* and *sigF*, the observed decrease in *sigG* expression at later time points was surprising. We hypothesized that this may have been due to insufficient lysis of forespores and lower yields of forespore RNA. We attempted to rectify this by using several methods for RNA extraction. However, different methods did not significantly alter what was observed. Resolving the precise timing of downstream sporulation gene expression was beyond the scope of this work, but given the differences already discovered in *Clostridium* sporulation pathways, it would be valuable to study this pathway in *C. thermocellum* in more detail.

The phenotype of Δ2735 was also apparently asporogenous and re-introduction of the gene restored sporulation to roughly wild-type levels. However, overexpression of this gene resulted in only a marginal increase in sporulation frequency. As this strain also produced an unexpected L-form phenotype, it seemed possible that this gene was involved in regulating some other cellular process,

which also compromised sporulation. To test this possibility, we constructed a double-knock out strain with deletions of both *clo1313_2735* and *clo1313_1973*. As the double knock-out was able to sporulate efficiently (albeit at a significantly lower rate than wild type), we conclude that Clo1313_2735 was not regulating another cell process essential for sporulation. Rather, this result suggests Clo1313_2735 and Clo1313_1973 are both regulating sporulation through opposite functions, presumably by modifying the same target. Based on the literature, this target is most likely Spo0A [36,37].

We examined other combined deletions and showed that sporulation was also restored when *clo1313_1942* and *clo1313_0268* mutations were combined with the *clo1313_1973* mutation. We also found that the positively regulating kinases were able to functionally complement each other. Taken together, these results suggest that Clo1313_2735, Clo1313_1942, and Clo1313_0268 all have the same functional activity, and that the combined activities of each of these kinases is required to counter-act the activity of the negative regulator, Clo1313_1973.

As the L-form phenotype associated with Δ2735 was relatively unstable, it was difficult to study in detail. Subcultivation of the strain, as well as repeated freeze–thaw cycles, lead to loss of the phenotype. We have reported previously on the formation of L-forms in *C. thermocellum*, which form in strain ATCC 27405 in response to starvation conditions [3]. Re-introduction of clo1313_2735 into Δ2735 restored the normal rod-shaped morphology, however it is possible that the transformation procedure itself selected for natural L-form revertants, which appeared readily. Similarly, double knock-outs containing the Δ2735 mutation did not exhibit in the L-form phenotype, suggesting that transformation or selection procedure promoted reversion.

Due to the many different genetic mechanisms that result in L-form formation in different organisms, it is difficult to speculate on a mechanism by which *C. thermocellum* regulates L-form formation via *clo1313_2735*. While two component systems (comprised of a histidine kinase and response regulator protein) are up-regulated in *E. coli* L-forms [74], the role of two component systems in L-form formation has not been explored in detail for any organism. To our knowledge, there are two accounts of an L-form-like phenotype connected with the deletion of a kinase gene. In *Streptococcus mutans*, deletion of *pknB*, which encodes a serine/threonine kinase, produces cells with a more rounded shape and increased lysis [9]. This strain also has other phenotypic differences, including defects in genetic competence [75], biofilm formation [75], acid tolerance [75], and bacteriocin production [9], suggesting that the deletion of one kinase gene can cause many changes in a diverse set of cellular processes. In *Caulobacter crescentus,* deleting the gene encoding the histidine kinase CenK compromises cell envelope integrity and triggers morphological changes that resembled membrane "blebbing" [76]. These data show that kinases can regulate diverse processes, including cellular morphology. Further work is needed in order to better understand the role of Clo1313_2735 as it relates to L-form formation, which was beyond the scope of this work.

It is unclear whether or not there are other kinases required for sporulation initiation in *C. thermocellum*. *Clo1313_1711*, another kinase identified in this study, appeared to increase sporulation levels relative to WT when overexpressed, but a clean deletion strain was not obtained. We were also unable to isolate a *clo1313_0495* knock out, but over-expression of this gene did not alter the sporulation phenotype, suggesting that this kinase either does not play a role in sporulation, or that other, undetermined factors, must be present for an effect to be seen.

There is growing evidence that sporulation in *Clostridium* species is directed by histidine kinases and proceeds in the absence of an integrative phosphorelay component. Here we identify four histidine kinases with apparent roles in *C. thermocellum* sporulation, and also observe a potential link between sporulation and L-form formation. In *C. thermocellum*, both spores and L-forms constitute non-growing cell types, which appear to confer different advantages to the organism [3]. The mechanism by which *C. thermocellum* undergoes either sporulation or L-form formation is still unclear. Yet, based on this work it is certain to include some input from sensory histidine kinases. Further analysis of this pathway has the potential to provide insight into metabolic arrest, sporulation, and L-form formation, and inform strategies for industrial biofuel production using *C. thermocellum*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2014.06.004.

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