Whole-genome sequence of an evolved Clostridium pasteurianum strain reveals Spo0A deficiency responsible for increased butanol production and superior growth

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Abstract

Background: Biodiesel production results in crude glycerol waste from the transesterification of fatty acids (10 % w/w). The solventogenic Clostridium pasteurianum, an anaerobic Firmicute, can produce butanol from glycerol as the sole carbon source. Coupling butanol fermentation with biodiesel production can improve the overall economic viability of biofuels. However, crude glycerol contains growth-inhibiting byproducts which reduce feedstock consumption and solvent production.

Results: To obtain a strain with improved characteristics, a random mutagenesis and directed evolution selection technique was used. A wild-type C. pasteurianum (ATCC 6013) culture was chemically mutagenized, and the resulting population underwent 10 days of selection in increasing concentrations of crude glycerol (80–150 g/L). The best-performing mutant (M150B) showed a 91 % increase in butanol production in 100 g/L crude glycerol compared to the wild-type strain, as well as increased growth rate, a higher final optical density, and less production of the side product PDO (1,3-propanediol). Wild-type and M150B strains were sequenced via Single Molecule Real-Time (SMRT) sequencing. Mutations introduced to the M150B genome were identified by sequence comparison to the wild-type and published closed sequences. A major mutation (a deletion) in the gene of the master transcriptional regulator of sporulation, Spo0A, was identified. A spo0A single gene knockout strain was constructed using a double–crossover genome-editing method. The Spo0A-deficient strain showed similar tolerance to crude glycerol as the evolved mutant strain M150B. Methylation patterns on genomic DNA identified by SMRT sequencing were used to transform plasmid DNA to overcome the native C. pasteurianum restriction endonuclease.

Conclusions: Solvent production in the absence of Spo0A shows C. pasteurianum differs in solvent-production regulation compared to other solventogenic Clostridium. Growth-associated butanol production shows C. pasteurianum to be an attractive option for further engineering as it may prove a better candidate for butanol production through continuous fermentation.

Keywords: Butanol, Clostridium pasteurianum, Spo0A, Methylome, Mutagenesis, SMRT sequencing

Background

The production of biodiesel has led to production of large quantities of waste glycerol [1]. The transesterification of plant- and animal-derived triglycerides with methanol yields 10 wt% crude glycerol. Crude glycerol can be refined, but increased production of biodiesel has flooded the market, making such processes economically unviable [2]; this economic shift has moved glycerol from coproduct to waste [3]. Conversely, the excess crude glycerol makes it an ideal feedstock for producing chemicals and biofuels. Crude glycerol has been utilized in various
ways including as a supplement of livestock diets, as a reactant for chemical catalytic processes, and as a feedstock for chemicals produced by bioconversion [4].

Clostridium pasteurianum (Cpa), a Gram+ Firmicute obligate anaerobe, is capable of making n-butanol and 1,3-propanediol (PDO) directly from glycerol as sole carbon source, a capability not shared by other well-studied Clostridium organisms [5–7]. Butanol is of particular interest as it is fungible with gasoline due to its high energy density, low vapor pressure, and low water solubility (77 g/L). As glycerol is a more reduced carbon source than glucose, the theoretical yield for butanol production from glycerol is 17% higher than from glucose on a carbon-mole basis. This reducing power is released in the initial step of glycerol degradation in the form of NADH. Cpa has been shown to produce up to 17 g/L of butanol in optimized batch culture [8]. In a more recent study, a mutant Cpa strain was shown to produce up to 17.8 g/L butanol from pure glycerol at very high rates from high-density cultures, with minimal byproduct formation [9].

The impurities of crude glycerol are inhibitory to microbial growth [1, 2, 10–12], and are thus a large obstacle to using crude glycerol for producing fuels and chemicals. Fatty acids, salts, and alcohol from biodiesel production persist in significant amounts in crude glycerol. Growth on 25 g/L crude glycerol has been shown to lead to a 40% decrease in solvent production [2]. In addition, increased lag times and decreased substrate uptake have been reported for several Clostridium organisms when grown on crude glycerol [2, 13]. The most toxic of these impurities is linoleic acid, a polyunsaturated omega-6 fatty acid [12]. The nonlinear structure caused by the two double bonds likely causes membrane depolarization [14]. Cpa solvent production has been shown to be completely abolished at 1.25 g/L linoleic acid (along with severely inhibited growth), whereas production of solvents was not greatly affected by fatty acids with lower levels of saturation [12]. In order for crude glycerol to be effectively converted to butanol, a tolerant strain of Cpa must be developed.

The Cpa genome has recently been sequenced and shown to be amenable to genome editing [11, 15], but low transformation efficiencies prohibit the use of high throughput library-based genome engineering tools. Here, we employed directed evolution of mutagen-treated Cpa aiming to select mutants tolerant to and using increasing concentrations of crude glycerol as the primary carbon and energy source. A tolerant mutant was isolated which exhibited increased tolerance to crude glycerol and improved butanol productivity. Single Molecule Real-Time (SMRT) sequencing of the mutant and WT strains identified all mutations in the tolerant mutant. Among other variants, a 24-bp deletion was identified in the key sporulation transcriptional regulator Spo0A. We confirm the that Spo0A inactivation is responsible for the tolerant and improved solvent-producing phenotype; we knocked out the spo0A gene in the WT strain and show that the engineered strain displays similar tolerance to crude glycerol as the evolved strain. In order to interpret the impact of Spo0A knockout on product formation, we used the Cpa genome sequencing to identify and map key product-formation genes, and analyzed them in a comparative analysis against other solventogenic Clostridium organisms. SMRT analysis enabled the identification of multiple DNA methylation motifs, some novel, including the confirmation of a cytosine methylation motif which enables the evasion of the endogenous type II endonuclease. We used a codon-optimized version of the native DNA methylase to enable efficient plasmid DNA transformation.

Results

Mutation, selection, and isolation of a mutant strain tolerant to crude glycerol

Industrial butanol production from crude glycerol would require a strain that can readily grow on crude glycerol with a high growth rate and short lag phase and that can produce high butanol titers with good selectivity. The negative effect of crude glycerol on the growth of the WT strain is apparent at 80 g/L (Fig. 1a), while no growth was observed at 150 g/L or higher. It has been shown that the most toxic components of crude glycerol are the residual fatty acids that persist in the glycerol layer in biodiesel production [12]. The most toxic of these was found to be those with higher degrees of unsaturation, and notably, linoleic acid, which completely abolishes growth of Cpa at 1.25 g/L [12] and has been found to negatively affect growth rates of some Gram+ bacteria at concentrations as low as 3 mg/L [16]. Here, we found growth of Cpa was almost completely abolished at concentrations of 0.075 g/L when grown on 30 g/L glycerol and significantly affected at just 0.02 g/L 9-cis,12-cis-linoleic acid (Fig. 1b).

The crude glycerol (Feed Energy, Des Moines, Iowa) used in this study was analyzed for its respective components (by New Jersey Feed Lab). 0.8–2% of the crude glycerol is fatty acids. The variation in concentration of fatty acids in the crude glycerol depends on the depth of the sample (low at the bottom, high at the top). As we attempted to uniformly sample from the top of the glycerol for each study, variations are to be expected from batch to batch and especially over time as the crude glycerol stock was depleted. The fatty acid profile of the crude glycerol was over half attributed to linoleic acid (Fig. 1c).

In order to obtain a strain of Cpa that is tolerant to crude glycerol, a mutagenesis and directed evolution...
strategy was employed. Cpa cultures were mutated twice by exposure to 50 µg/mL N-methyl-N′-nitro-N-nitrosoguanidine (NTG) for 15 min, washed thrice with PT buffer, and recovered in 2xYTG media as described [17]. The mutated Cpa population was grown on CGM-crude glycerol medium and serially transferred eight times in increasing concentrations of crude glycerol (Fig. 1d). Each batch increased in crude glycerol by 10 g/L starting with 80 g/L in batch 1. After 10 days and 28 generations of growth, the final population sustained strong growth with little lag time in 150 g/L crude glycerol, a level that completely inhibited growth of the WT strain. Single colonies from the final population were isolated from solid media and assayed for growth in 100 g/L crude glycerol. A single mutant (M150B) was identified with consistently short lag-phase times.

In simple batch cultures without pH control and feeding or medium optimization, Cpa strain M150B showed a decreased lag phase, 65 % higher glycerol consumption (40.4 g/L), and 91 % higher butanol production (final titer 7.1 g/L) compared to the WT in 100 g/L crude glycerol (Fig. 2a). The WT strain produced more PDO than the mutant (2.6 vs. 1.5 g/L). Interestingly, M150B also outperformed the WT strain when grown on pure glycerol (Fig. 2). While there was no lag phase for either the mutant or WT when grown on 60 g/L pure glycerol, M150B consumed 75 % more glycerol (43 g/L) and produced 80 % more butanol (11.7 g/L). As under the crude glycerol condition, the WT produced more PDO than the mutant from pure glycerol, but for both strains, less was produced overall (1.5 and 0.9 g/L). Acetate, a component of CGM media (~1.8 g/L), was partially consumed by both strains under both conditions. Ethanol and lactate were produced at low levels (<0.5 g/L) in both strains under both conditions.

In addition to the improved fermentation properties, we also observed that this mutant was asporogenous. We confirmed that the M150B strain did not form spores by phase-contrast microscopy after 6 days of growth in CGM media (Additional file 1: Figure S1). We observe plentiful phase-bright sporulation in the WT cells, while the M150B cells show no such spore formation. We also performed a chloroform-based sporulation assay [18] where the WT strain survived 10 min of 50 % v/v chloroform treatment, while M150B did not.
SMRT sequencing of *Cpa* ATCC 6013 and the M150B mutant strains identifies a large deletion on the *spo0A* gene as likely responsible for the tolerant and butanol-production phenotype

We desired to determine the genetic source of the improved characteristics of the evolved strain M150B. Three *Cpa* ATCC 6013 sequences have been published recently. Each successive genomic sequencing project has resolved the size of the genome and the number of contigs (from 4,285,687 bases assembled into 37 contigs [19], to 4,420,124 bp in 12 contigs [15], to finally 4,351,223 bp in a single closed circular chromosome [20]). All sequence comparisons, gene names, and genomic loci here are based on the closed circular chromosome (Genbank CP009267.1).

We sequenced both the WT and mutated strains using the Pacific Biosciences RSII. The WT genomic DNA was isolated during the transition phase, while the mutant M150B’s gDNA was isolated during mid-exponential phase. The WT sequencing resulted in 46,455 mapped reads with an average length of 8271 bp and an average 78.4× coverage. The M150B sequencing resulted in 107,963 mapped reads with average read length of 6471 and average 121.1× coverage. The coverage of the M150B genome depended on genome locus, while that of the WT did not (Additional file 1: Supplemental Results, Figure S2); the coverage for M150B was the greatest near the origin of replication. We suspect this is due to the state of growth of the culture at the time of gDNA harvesting.

We compared our sequence of the WT ATCC 6013 strain with the most recently published sequence [20] and observed 17 differences (Additional file 1: Table S1). We did not observe the mutation noted in the *Spo0A* gene by Rotta et al. [20]. In contrast to the reported sequence, position 255 of *Spo0A* is glutamic acid, not the lysine (or glutamine) reported for ATCC 6013, making it similar to the *Cpa* DSM 525 [21] sequence and other *Clostridium* strains. When comparing the sequence of M150B with the same published sequence, we found the same 17 differences contained in our WT strain along with 67 other variants (Table 1). Of these 67 variants, 66 were substitutions. Ten were in regions that were not annotated to
## Table 1  Sequence variants from sequenced wild-type Cpa 6013 vs. M150B

| Mut. locus [20] | Mut. type | CDS | Mut. | Ref. | Var. |
|----------------|-----------|-----|------|------|------|
| 135360         | Sub.      | No CDS | A267V | C    | T    |
| 177873         | Sub.      | Hypothetical cell wall-binding protein, c01710 | I257T | A    | G    |
| 256006         | Sub.      | Glycosyltransferase, c02430 | P483L | C    | T    |
| 328268         | Sub.      | Xanthine dehydrogenase, molybdenum binding; pudD1 | P187S | T    | C    |
| 511135         | Sub.      | Pyridine nucleotide-disulfide oxidoreductase; NADH dehydrogenase; coenzyme A disulfide reductase; c04890 | SS79N+ | G    | A    |
| 547010         | Sub.      | Pyruvate-flavodoxin oxidoreductase, nifU1 | P429S | G    | A    |
| 623217         | Sub.      | lysis; c05770 | A129T | G    | A    |
| 633860         | Sub.      | Hypothetical protein; c05850 | Silent | C    | T    |
| 664502         | Sub.      | Methyl-accepting chemotaxis protein; c06130 | T311A | A    | G    |
| 774540         | Sub.      | Metallophosphoesterase; c07050 | G294E | G    | A    |
| 805746         | Sub.      | Putative amidase domain-containing protein; c07320 | S1026F | C    | T    |
| 822080         | Sub.      | tRN Glu-specific nuclease WapA; wapA1 | O641tag | G    | A    |
| 849186         | Sub.      | Putative glycosyltransferase; c07760 | E95K+ | G    | A    |
| 888906         | Sub.      | Phage infection protein; c08050 | A128V | C    | T    |
| 905016         | Sub.      | putative L-ascorbate-6-phosphate lactonase UlaG; ulaG | G32D | G    | A    |
| 959280         | Sub.      | No CDS | G32D | C    | T    |
| 1010600        | Sub.      | Radical SAM domain protein; c09370 | E311K+ | G    | A    |
| 1018923        | Sub.      | No CDS | Silent | G    | A    |
| 1034853        | Sub.      | Putative AAA-ATPase; c09620 | T311A | A    | G    |
| 1047038        | Sub.      | DNA-binding response regulator; c09780 | G378E | G    | A    |
| 1052341        | Sub.      | Hypothetical protein; c09830 | S1026F | C    | T    |
| 1058795        | Sub.      | Nicotinamidase; c09830 | V311+ | G    | A    |
| 1263298        | Sub.      | No CDS | V311+ | G    | A    |
| 1384612        | Sub.      | Spore germination protein KA; genKα2 | E95K+ | G    | A    |
| 1408727        | Sub.      | hypothetical protein; c13080 | A154T | G    | A    |
| 1595471        | Sub.      | Lactate-responsive regulator LldR; c14940 | A300T | G    | A    |
| 1597713        | Sub.      | Electron transfer flavoprotein, alpha subunit; etfA1 | Silent | G    | A    |
| 1608597        | Sub.      | CAAX amino terminal protease family protein; c15040 | V81+ | G    | A    |
| 1729728        | Sub.      | Integral membrane protein; c16180 | A674V | G    | A    |
| 1796914        | Sub.      | Phenylalanyl-tRNA synthetase beta chain; pheT | E44K+ | G    | A    |
| 1973896        | Sub.      | Flagellum-specific ATP synthase FliF, FliF | A75T | G    | A    |
| 1976216        | Sub.      | Flagellar basal-body rod protein; fbg3 | Silent | C    | T    |
| 2055679        | Del.      | Stage 0 sporulation protein A, spo0A | Del. 235–242 | ATACCATAGAATAATTGAGT |
| 2822361        | Sub.      | CRISPR-associated helicase/endonuclease Cas3, cas3 | E316F | C    | T    |
| 3198907        | Sub.      | No CDS | E316F | C    | T    |
| 3208358        | Sub.      | Exonuclease SbcC, sbcC | A558T | C    | T    |
| 3222314        | Sub.      | Hypothetical protein, CF-17 family; c29890 | A558T | C    | T    |
| 3270753        | Sub.      | No CDS | E213K+ | C    | T    |
| 3272757        | Sub.      | tRNA-dihydrouridine synthase; c30410 | C16K+ | C    | T    |
| 3273913        | Sub.      | Fe-S oxidoreductase; c30430 | T87A | A    | G    |
| 3297381        | Sub.      | No CDS | T87A | A    | G    |
| 3348577        | Sub.      | Transcriptional regulator, TetR family; c31160 | S20N+ | C    | T    |
| 3399757        | Sub.      | Hydrolase (HAD superfamily); c31590 | P219S | G    | A    |
| 3439637        | Sub.      | Multiple sugar transporter, membrane-spanning permease protein MsmG, msmG2 | A236V | G    | A    |
| 3488562        | Sub.      | Potassium-transporting ATPase C chain; kdpC | T283I | G    | A    |
contain genes. Of the 56 remaining, 14 were silent mutations, 14 were conservative amino acid substitutions, one generated a premature stop codon in a putative glycosyltransferase, and 27 others were nonconservative amino acid substitutions. In addition, we found a single deletion of 24 bp in the gene encoding the master sporulation factor Spo0A [22] (Fig. 3).

We also observed three point mutations in flagella-related genes. A silent mutation was found in flgG3, coding for a flagellar protein and a conservative mutation was found in the flagellum-specific ATP synthase, fliI (E44K). A nonconservative mutation was found in whiG, coding for a sigma factor specific to a flagellar operon (Table 1). The A75T mutation in the WhiG gene is in a highly conserved region in WhiG and σ^A proteins supposed to be the promoter recognition element [23]. The combined effects of these mutations may be responsible for M150B not staying in suspension as long as its parent strain. M150B cultures consistently settle quickly and more compactly compared to the WT when not actively agitated (Additional file 1: Figure S3).

We also observed point mutations in genes related to central metabolism and solvent production. nifJ1 encoding pyruvate–flavodoxin oxidoreductase, which catalyzes pyruvate and coenzyme A to acetyl-CoA and carbon dioxide (Fig. 4) has a S579 N mutation. However, Cpa contains two paralogs, nifJ2 and nifJ3, which may compensate for the mutated nifJ1. The etfA1 gene, encoding the electron-transfer flavoprotein alpha subunit, which contains an FAD-binding domain, has an A300T mutation. The Cpa genome encodes the paralog etfA3, which contains the major domains of the EtfA (Fig. 5c). EtfA together with EtfB and butyryl-CoA dehydrogenase likely form the Etf/Bcd complex, which couples the NADH-dependent reduction of crotonyl-CoA to butanoyl-CoA with the reduction of ferredoxin [24] (Fig. 4).

The deletion of 24 bp in M150B’s single copy of the spo0A gene led to an in frame deletion of eight amino acid residues in the σA activator region (Fig. 3) [25]. The σA activator region is essential for binding to σA-dependent promoters [25]. This mutation is unique in that it is a deletion and not an A-G or T-C single nucleotide variant, commonly observed in NTG-mutated strains. We observed that the deletion of the 24 bp corresponded exactly with two identical 8 bp sequences (5′-ATACCATA-3′) surrounding the deletion (Fig. 3). This is likely due to homologous recombination of these overlapping regions, which removed the section between these sequences and one of the copies of the sequence. It is noteworthy that while sporulation was disrupted as could be predicted by such a deletion, solvent production remained. This is unexpected as it stands in contrast to what was observed in Spo0A mutants in C. beijerinckii (Cbe) [26] and C. acetobutylicum (Cac) [22]. In those strains, Spo0A regulates the expression of key solvent genes and notably of the genes in the sol locus [22], and as a result, in their Spo0A mutants, solvent production is nearly abolished.

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**Table 1 continued**

| Mut. locus [20] | Mut. type | CDS | Mut. | Ref. | Var. |
|----------------|-----------|-----|------|------|------|
| 3497476 | Sub. | Fatty-acid peroxygenase; cypC | P255S | G | A |
| 3521979 | Sub. | ATP phosphoribosyltransferase regulatory subunit; hisZ | P96L | G | A |
| 3531067 | Sub. | Cysteine desulfurase; c32790 | Silent | G | A |
| 3536341 | Sub. | Chaperone protein HtpG; htpG | Silent | G | A |
| 3593919 | Sub. | No CDS | Silent | G | A |
| 3595710 | Sub. | 2-iminoacetate synthase; thiH2 | P77S | G | A |
| 3641811 | Sub. | Possible surface protein; c33730 | T742I | G | A |
| 3666696 | Sub. | No CDS | G | A |
| 3698846 | Sub. | L-arabinose transport system permease; araQ2 | T235I | G | A |
| 3759007 | Sub. | Hypothetical protein; c34910 | E729P | C | T |
| 3776313 | Sub. | RNA-binding protein; c35130 | S523N | C | T |
| 3863797 | Sub. | DNA-3-methyladenine glycosylase II; c35950 | E119K | C | T |
| 3872706 | Sub. | Autoinducer 2 sensor kinase/phosphatase LuxQ; luxQ3 | E242F | C | T |
| 3909641 | Sub. | Signal-transduction and transcriptional-control protein; stc3 | A657V | C | T |
| 3933580 | Sub. | SOS ribosomal protein L2; rplB | Silent | C | T |
| 3936650 | Sub. | SOS ribosomal protein L3; rplC | G191D | C | T |
| 4163499 | Sub. | Signal transduction histidine kinase; c39070 | S439N | C | T |
| 4231311 | Sub. | Putative competence-damage inducible protein; cinA | Silent | C | T |

Positive substitutions are indicated by a plus symbol.
Mapping the solvent-formation capabilities of \textit{C. pasteurianum} on its sequenced genome illuminates the role of multiple gene orthologs/paralogs on its unique metabolic traits

In order to gain a better understanding of the aforementioned differences in the regulation of solvent formation between \textit{Cpa} and the two well-established model solventogenic \textit{Clostridium} organisms, we mapped the core primary metabolic reactions of \textit{Cpa} to the sequenced genome and compared this mapping to that in \textit{Cac}. This has only recently been enabled through a closed and annotated genome sequence.

Glycerol utilization by the cells leads to either entry to glycolysis at dihydroxyacetone phosphate or reduction to glycerol. The genes for the reductive pathway to PDO are also located in a likely operon (Fig. 5b). The propanediol dehydrogenase is encoded by three genes (\textit{pduE}, c22790; \textit{pduD}, c22800; and \textit{dhaB}, c22810), which precede a diol dehydrogenase-reactivating factor gene (\textit{ddrA}, c22780) and the PDO dehydrogenase (\textit{dhaT2}, c22740). The PDO dehydrogenase also has an extra copy (\textit{dhaT1}, c11040, 43 % identical by protein). The presence of these multiple gene paralogs suggests their likely importance in achieving the fast rates of glycerol utilization and growth on glycerol.

It has been well established that \textit{Cpa} ferments glycerol to form PDO and butanol (Fig. 2). Unlike the model solventogenic \textit{Clostridium} \textit{Cac}, it does not produce acetone \cite{19, 27}. It does, however, have a \textit{Cac}-like \textit{sol} locus on its chromosome containing \textit{adhE2} (a fusion protein coding for an aldehyde-alcohol dehydrogenase, c15160), followed by \textit{ctfA} and \textit{ctfB2} (c15170, c15180, encoding the two units of the CoA-transfrase, CoAT), and, next, in an opposite orientation a monocistronic putative \textit{adc} (acetooacetate decarboxylase, Adc, c15190) (Figs. 6a, 7). In \textit{Cac}, \textit{AdhE1} catalyzes the conversion of butyryl-CoA to butanol \cite{28, 29}, \textit{Coa}-transferase (\textit{Coa}AT) catalyzes the conversion of acetooacetate to acetocetyl-CoA \cite{30}, which is accompanied by acetate and/or butyrate uptake.
Acetoacetyl-CoA is converted to acetone by Adc [31]. In Cac, the sol locus, encoded on the pSOL1 megaplasmid [32], contains the adhE1, ctfA, ctfB genes in an operon organization (although recent work in our lab shows that the ctfA/B genes can be also transcribed independently of the adhE1 gene), and the monocistronic adc gene in the opposite orientation. There is exists a very similar paralog of the AdhE1 protein also encoded on the Cac pSOL1 megaplasmid, annotated as adhE (CA_P0035), but that protein, although it can catalyze butanol formation, is not involved in the in vivo formation of butanol as it is not expressed under normal solventogenic conditions [33]. The Cpa adhE2 gene is 76% similar with the Cac adhE1 on the DNA level and 82% on the amino acid level. Likewise, the ctfA, ctfB2, and adc genes are similar to their Cac orthologs (Cac ctfA 74% by DNA, 73% by protein; Cac ctfB 71% by DNA, 71% by protein; Cac adc 76% by DNA, 84% by protein, respectively). Cpa has three more annotated adhE genes (adhE1, c05730 [similarities: 73% DNA, 77% protein to Cac adhE1]; adhE3, c15660 [70%]

Fig. 5 a-c Cartoons of parts of the glycerol to butanol genomic loci: a glycerol to central metabolism, b reductive pathway of glycerol to 1,3-propanediol (c22750 codes a hypothetical protein and c22760 codes for cobyrinic acid a,c-diamide adenosyltransferase), c acetocetyl-CoA to butanoyl-CoA locus including the Etf/Bcd complex (rex encodes a redox-sensing transcriptional repressor)

Fig. 6 Cartoon of the sol loci in C.pasteurianum, C. acetobutylicum, and C. beijerinckii. Genomic positions are given at the beginning and end of the cartoon, and the relative sizes are to scale. Reported and putative 0A boxes are indicated by red triangles above the cartoon on the top strand and below if on the bottom strand.
Fig. 7 Transmission electron micrographs of *C. pasteurianum* after 5 days in culture. **a** WT cells show characteristic sporulation development including clostridial-form bulging cell shape. **b–c** M150B and ΔSpo0A and cells are rod shaped with no obvious sporulation development. **d** Isolation and enlargement of individual WT cell image from 7A shows spore core (c), cortex (cx), granulose bodies (g), and the multilayered spore coat structure (solid red arrow). **e, f** Isolation and enlargement of individual M150B and ΔSpo0A cell images from 7b, c show probable mesosomes (thick striped arrows) and globular electron dense regions (fine striped arrows).
DNA, 64% protein to Cac adhE]; and adhE4, c28930 [73% DNA, 76% protein to Cac adhE1] and two butanol dehydrogenase genes (bdh1, c04370 [71% DNA, 72% protein to Cac bdhA] and bdh2, c15980 [46% DNA, 39% protein to Cac bdhB]) located in different parts of the genome. Notably, Cpa contains an additional set of genes encoding a CoAT (atoD, c08850 [55% DNA, 46% protein to Cac ctFA] and ctFB1, c08840 [62% DNA, 55% protein to Cac ctFB]). One assumes naming c08850 'atoD' was inspired by the standard Escherichia coli nomenclature, but it might be more appropriately named ctFA1 (with the c15170 gene as ctFA2). The kdc gene (keto-acid decarboxylase) of Cpa (c04400) has 53% similarity to the pyruvate decarboxylase gene of Cac (pdc, 40% by protein), which catalyzes the conversion of pyruvate to acetaldehyde.

When comparing the soloci in Cpa and Cac, we observe relatively large gaps between the adhE2, ctF4, and ctFB2 genes in Cpa (432 bp and 162 bp, respectively), while the Cac orthologs are much closer together (only 63 and 5 bp), indicating a tighter operon structure (Fig. 6). The expression of the Cac adhE1-ctFAB operon has been shown to be controlled by an activated (phosphorylated) Spo0A(34), and a Spo0A-binding site (0A box) has been proposed [26] (Fig. 6). In addition to the Spo0A-binding site, Cac has putative repeated regulatory elements, which overlap with the 0A box [34]. While Cpa has a similar 0A box in this region, these repeated elements do not seem to be present. In addition, Cpa has 3 additional adhE genes, two of which do not have any identifiable 0A boxes upstream. This may play a role in butanol formation being unaffected by the loss of Spo0A activity as demonstrated by the inability of the strain to sporulate. Cac also has three putative 0A boxes upstream of the adc gene. Cpa has none that could be identified. This is notable since Cpa seems to contain all the genetic elements necessary for acetone production (an intact sol locus), it does not produce acetone. This would suggest that the genes for acetone formation may be expressed and used only under specific culture conditions not currently employed in standard laboratory Cpa cultures.

The Cbe sol operon differs considerably from Cac and Cpa (Fig. 6). Its sol operon does not contain a bifunctional adhE gene as in Cac and Cpa, but contains a gene coding for an aldehyde dehydrogenase (ald, Cbei_3832). A bifunctional adhE gene (Cbei_0305) is located in another locus. The adc gene (Cbei_3833) is oriented in the same orientation as ald, ctFA, and ctFB and these are all transcribed in a polycistron [35] with a putative 0A box upstream.

These differences in the regulation of solventigenic genes between Cpa and the two classical solventogenic clostridia, Cac and Cbe, are of fundamental and practical interest. The role of Spo0A in solvent production regulates results in more complex phenotypes, and, notably, strain degeneration and bistability in solvent production [18, 36, 37]. Strain degeneration refers to the simultaneous loss of the ability to sporulate and produce solvents. While bistability in solvent production refers to the different outcomes (high versus low) of solvent production depending on the state of the culture inoculum used. The two phenotypes are linked and are likely related to the complex role of the sporulation-specific sigma factor SigK, which controls both Spo0A expression as well as late sporulation events [36, 38]. Cpa appears to be shielded from such phenotypic complexities, which result in performance-limiting practical bioprocessing issues [31].

To sum, mapping of the proteins that are responsible for solvent formation on the Cpa genome, and comparative analysis against the corresponding genes in the two well-studied Clostridium solventogens, Cac and Cbe, demonstrated distinct genetic organizations and regulatory elements that would explain the observed phenotypic differences. The latter include the fact that, in contrast to Cac and Cbe, butanol formation in Cpa is not controlled by Spo0A, and is in fact growth associated (Fig. 2) rather than a stationary-phase phenomenon.

**New findings from revisiting the SMRT-sequencing-enabled Cpa-DNA methylation analysis**

Using SMRT sequencing it is possible to interrogate the native methylome of the genome as no amplification steps occur prior to sequencing. As the DNA is being sequenced, modification to bases result in a delay in the called bases, called the interpulse duration ratio (IPD) [39]. Using the PacBio RS Modification and Motif Analysis (MAMA) protocol, we observe in both the WT and M150B genomes the previously reported N5-methyladenine modification motifs associated with Type II and Type I restriction modification systems in Cpa (m-6A modifications: 5′-GAaGGTC-3′ and 5′- AAaGNNNNNCTCC-3′, respectively) [15]. The MAMA protocol also returned two previously unreported N5-methyladenine modification motifs 5′-GRTAAAaG-3′ and 5′-CAAAAAaR-3′ in both sequenced genomes (R = G or A; Table 2).

Methylcytosine modifications are more difficult to determine compared to m-6A [39]. Cpa carries a Type II restriction endonuclease identified as a barrier to plasmid transformation as it recognizes and cuts 5′-CGCG-3′ motifs [11, 40]. The MAMA protocol returned two different motifs containing 5′-CGCG-3′ for the WT and M150B (5′-CGCaGNNNNNNNTNANA-3′ and 5′-MNNNNNCGGAAAANANT-3′, respectively), but these seem to be overspecified since there are only 11 and 14 of these sequences in the Cpa genome, respectively. Furthermore, the modified base differs between the two. By
analyzing the IPD data directly, however, we observed that the average IPD for the first cytosine of 5′-CmGCG-3′ (1.25) is significantly higher than the IPD in 5′-Cm-3′ (0.86) and 5′-CmG-3′ (0.89) (two-sided heteroscedastic t test p values of 9.0E-7 and 4.5E-6, respectively), indicating this motif is likely modified. The IPD for the second cytosine in 5′-CGmG-3′ (1.11) is also significantly higher than in 5′-Cm-3′ and 5′-CmG-3′ (p values of 1.2E-3 and 4.1E-3, respectively), but it indicates lesser frequency of methylation on the second cytosine.

We identified bepIM (c05910) on the Cpa genome encoding the modification methylase Bepl. We codon optimized the Cpa bepIM for E. coli and had it synthesized and cloned into a plasmid (pCpaDcm2.0, Additional file 1: Table S2, DNA 2.0). Prior to transformation, we first methylated all shuttle plasmids by transforming them into an E. coli methylation strain carrying pCpaDcm2.0. No successful transformations were performed without this prior in vivo methylation. Plasmid transformation in Cpa has been shown previously using the heterologous M.FnuDII methyltransferase (5′-C′G′GCG-3′) to evade the endogenous restriction enzyme [11]. While methylation of plasmids with heterologous genes is effective, methylene analysis and synthetic expression of the native methyltransferase enzymes is a generalizable method to transform bacteria with an unknown endonuclease recognition sequence.

**Table 2 Methylation data and analysis**

| Motif       | Modified position | Type | % motifs detected | # of motifs detected | # of motifs in genome | Mean modification QV | Mean motif coverage | Partner motif |
|-------------|-------------------|------|-------------------|----------------------|-----------------------|----------------------|---------------------|--------------|
| GRTAAAG     | 6                 | m6A  | 99.65             | 2579                 | 2588                  | 87.0                 | 55.4                |              |
| AAGNNNNNCTCC| 2                 | m6A  | 99.61             | 51                 | 513                   | 93.1                 | 59.4                | GGAGNNNNNCTT  |
| GGAGNNNNNCTT| 3                 | m6A  | 99.03             | 508                 | 513                   | 88.1                 | 58.0                | AAGNNNNNCTCC  |
| GATC        | 2                 | m6A  | 99.14             | 13,348              | 13,464                | 85.8                 | 58.4                |              |
| CAAAAAR     | 6                 | m6A  | 97.10             | 3786                | 3899                  | 74.0                 | 56.3                | GATC         |

Construction of Cpa Spo0A knockout confirms that Spo0A inactivation is responsible for conferring tolerance to and better growth on crude glycerol, as well as enhanced growth-associated butanol production

The mutation in spo0A was the most conspicuous of all identified, so we decided to test the hypothesis that this deletion was the primary cause of the changes in phenotype we observed. We used the MazF knockout system [41] to design a 200 bp deletion at the start of the spo0A gene, including the ribosomal-binding site, start codon, and 60 codons. The design replaces this 200 bp with a chloramphenicol acetyltransferase gene (*cat*) with FRT sites on either side. The recombination plasmid (pKOmazF-Spo0A) contains regions of homology (sized 536 and 506 bp) flanking either side of the *cat* gene and FRT sites. The plasmid was constructed in the manner as previously described [41] and transformed via the previously reported protocol [11] with the exception of the methylation method (see above). Successful recombination was confirmed via size verification of the insert via PCR and agarose gel imaging from three isolated colonies that grew on solid 2xYTG supplemented with thiamphenicol and lactose. Recombination plasmid removal was performed with serial transfers in unselective media and confirmed by replica plating. The integrated *cat* gene was removed with the transformed p94-FLP containing the flippase gene as described [41] and confirmed by PCR and Sanger sequencing. Briefly, Cpa Δspo0A::ThR + p94-Flp was grown in liquid media supplemented with erythromycin at 30 °C and subsequently plated on solid 2xYTG + Em media. Replica plating confirmed thiamphenicol sensitivity. Loss of p94-Flp was confirmed by replica plating after two serial transfers in unselective media.

To confirm the sporulation deficient phenotype was due to the deletion in the spo0A gene, the WT, M150B, and ΔSpo0A strains were imaged by transmission electron microscopy (TEM). The three strains were cultured for 5 days before cells were prepared for TEM analysis. As expected, the WT strain (Fig. 7a, d) displayed the characteristic features of sporulating solventogenic *Clostridium* cells (and notably of Cac [18, 42]), showing spore development at different maturation stages, and an unusually large number of the characteristic granulose bodies for all cells compared to other *Clostridium* organisms. The granulose bodies appear as lightly electron dense in an electron translucent background, which is also somewhat unusual compared to other solventogenic *Clostridium* cells.

The M150B and ΔSpo0A strains display no granulose bodies, septum, or forespore structures, showing only vegetative cells (Fig. 7b, c) with no bulging structures characteristic of sporulating clostridial-form cells [18, 42]. All cells displayed in their core irregular electron
translucent regions, many cells contain one or two globular lightly electron dense features, all surrounded by an electron dense region that defines the cell shape (Fig. 7e, f). We also observed identifiable membranous structures (Fig. 7e, f), which may be mesosomes, an artifact of the chemical fixation process [43]. The M150B and ΔSpo0A cells look similar to the ΔsigK Cac cells [38], where sporulation has been blocked just prior to Spo0A expression and activation, and also similar to the Cac cells with an inactivated sigF gene [37]. In Clostridium organisms [36], SigK acts both very early (prior to Spo0A expression) and very late in sporulation, while SigF acts just after Spo0A activation.

We tested the growth of the knockout strain in 100 g/L crude glycerol along with the wild-type and the M150B mutant. The M150B and ΔSpo0A strains show a markedly reduced lag time compared to the WT strain (Fig. 8a). Unlike previous results (Fig. 2), the WT strain achieves similar OD to the evolved strain after 48 h. Metabolite analysis by HPLC show improved butanol
formation for the Spo0A knockout strain (Fig. 8b). The WT strain produced 50 % more PDO than the M150B and ΔSpo0A strain, which is consistent with what was seen previously. These data show that the observed phenotype of the M150B strain in tolerating higher levels of crude glycerol, faster growth and reduced lag time on glycerol and improved levels of butanol formation can be recapitulated by Spo0A inactivation.

Discussion

It has been suggested that Cpa's ability to consume glycerol as the sole carbon source is likely due to its reductive PDO pathway. As glycerol is more reduced than glucose, more reducing equivalents are generated to get to glyceraldehyde-3-phosphate, necessitating some glycerol reduction to PDO [8]. The Cac genome does not contain the PDO producing pathway, which is likely the reason it cannot use glycerol as a sole substrate [7]. Cpa contains multiple paralogs of genes in the glycerol to butanol pathway: glycerol dehydrogenase (4 annotated paralogs), aldehyde-alcohol dehydrogenase (adhE) [4], pyruvate-flavodoxin oxidoreductase [3], acetoacetate CoA-transferase [2], 1,3-propanediol dehydrogenase [2], NADH-dependent butanol dehydrogenase [2], and NADPH-dependent butanol dehydrogenase [2]. The large number of paralogs of these core genes may account for the observed high rates of growth and product formation. It has been noted that Cpa fermentation products vary under similar conditions, and was thus theorized that the regulation of fermentation related genes is not very strict [8].

Using whole-genome sequencing we are able to fully assess the differences between the parent and evolved daughter strain, M150B. We showed that inactivation of the master regulator of sporulation in C. pasteurianum, and all Clostridium organisms [36], Spo0A, confers an increased tolerance to crude glycerol stress. As M150B was superior to WT in pure glycerol as well, another selection mechanism seems to have been fast growth on glycerol. Spo0A is a highly conserved protein, which is the master regulator of sporulation in Clostridium and Bacillus [36, 44]. Spo0A comprises a phosphoacceptor domain and a transcription activation, or ‘effector’, domain. Phosphorylation of the phosphoacceptor domain affects transcription, both positively and negatively, for a large number of genes by binding to Spo0A-binding motif (the ‘OA box’, 5′-TGNCGAA-3′) [25, 26]. The OA box can be found in the promoter region of a variety of stationary-phase-related genes controlled by the activated (phosphorylated) Spo0A, including, in Clostridium organisms, the gene for the first sporulation-specific sigma factor SigF [36]. Blocking the expression of SigF blocks the expression of the all downstream sporulation-specific sigma factors (SigG, E and K), and this is the mechanism by which Spo0A inactivation abolishes sporulation [36].

The effector domain contains a DNA-binding helix-turn-helix region along with a σ54 promoter region (Fig. 3) [25]. Mutations of B. subtilis spo0A in this region have been shown to have lower or failed activation from σ54 promoters and these mutants are asporogenous [25]. The activation of Spo0A in Cac has been shown to be dependent on one of two histidine kinase mediated phosphorylation pathways: one involving a single gene Cac_0323 (having 32 % identical ortholog Cpa_c33080) and the other necessarily involving both the genes Cac_0903 and Cac_3319 (with 38 and 27 % identical orthologs Cpa_c33080 and Cpa_c19640, respectively) [45]. A Cac_3319 mutant showed significantly reduced sporulation activity as well as increased butanol production [45, 46], showing solvent production regulation to be a highly complex and relatively poorly understood process.

The presence of Spo0A has been shown to be essential for solventogenesis in Cac and C. beijerinckii [26]. Overexpression in Cac leads to early onset of sporulation and solventogenesis [22], as well as to increased butanol tolerance and prolonged metabolism as a result of large and complex changes in the transcriptional program of the cells [47]. Strong butanol production in the Cpa ΔSpo0A shows that although Cpa is closely related to Cac, solvent production is not regulated in the same manner. Beyond the present findings that demonstrate that Spo0A is not necessary for butanol formation, there are also several other distinct differences worth noting. First, butanol formation in Cpa is growth associated in contrast to Cac and Cbe, where butanol production occurs as active growth ceases and continues into the stationary phase of the culture [42]. Since little butyrate and no acetate is formed in Cpa fermentations (Fig. 2), acid and notably butyrate [48] (or butyryl-P [49]) is not obviously a trigger for solventogenesis as in Cac and other solventogenic Clostridium organisms. Related to the latter is the fact that, as extensively discussed [9], in Cpa, there is no acetone formation, which characterizes solvent formation in Cac and Cbe, where it accompanies acid re-uptake. It is still a mystery why Cpa produces no acetone despite the fact that, as discussed above, all the genes for its formation are present on the Cpa genome, some with several paralogs. In this context, it is noteworthy that Cac, when grown on glucose in the presence of glycerol in continuous culture, it forms no acetone [7]. The authors of Ref. [7] noted that this is mechanistically similar to the impact of gassing continuous culture of Cac with carbon monoxide [50], which inhibits hydrogen production. They concluded [7] that: “Acetone formation requires no reduction energy, and it appears that the cellular control
mechanisms avoid its production in order to maximize the regeneration of NAD(P) in the reduced nucleotide-consuming pathway”.

**Conclusion**

In conclusion, this present study provides a genomic understanding of the unique phenotypic Cpa characteristics, and strengthens the case for using Cpa as a butanol producer. It has the advantage of growth-associated butanol production with little concomitant acid and no acetone production. Growth-associated butanol production would in principle enable the development of continuous or continuous-like processes, which are difficult to develop with the well-known solventogens like Cac and Cbe due to the well-known degeneration phenotype [31, 36]. As was shown recently [9], Cpa strains and culture media can be readily developed to enable Cpa cultures of high cell densities producing high titers of butanol with very good productivities. The present finding would further increase the appeal of such Cpa strains, which can become even more productive with further strain engineering, such as for minimizing or eliminating PDO formation. It has been documented [51] that, as hosts for the production of chemicals and fuels, Clostridium organisms have the major advantage of using a broad spectrum of substrates deriving from biomass (hexoses, pentoses, oligosaccharides, xylans), and although not extensively studied, Cpa does not appear to be an exception to this assessment. Its genome codes the necessary genes that would make it an effective user of such a broad spectrum of substrates, on top of its powerful ability to effectively and quickly utilize crude glycerol, alone and in combination with other carbohydrates.

**Methods**

**Bacterial growth and maintenance conditions**

Strains used in this study are listed in Table 3. C. pasteurianum ATCC 6013 was grown anaerobically at 37 °C in liquid CGM (per liter of distilled water: KH2PO4, 0.75 g; K2HPO4, 0.75 g; MgSO4 [anhydrous], 0.348 g; MnSO4·H2O, 0.01 g; FeSO4·7H2O, 0.01 g; NaCl, 1.0 g; asparagin, 2.0 g; yeast extract, 5.0 g; sodium acetate, 2.46 g; (NH4)2SO4, 2.0 g; and para-aminobenzoic acid [PABA], 0.04 g; titrated to pH 6.8) with, unless otherwise stated, 80 g/L glucose as the main carbon source. C. pasteurianum ATCC 6013 was grown anaerobically at 37 °C on solid 2xYTG (pH 6.5) agar plates supplemented with the appropriate antibiotic (50 µg/mL ampicillin, 35 µg/mL chloramphenicol, 25 µg/mL kanamycin). E. coli strains were stored at −85 °C in LB medium supplemented with 15 % glycerol.

**Analytical methods**

Cell density was measured at 600 nm using a Beckman Coulter DU 730 spectrophotometer. Samples were diluted with the appropriate medium to maintain readings in the linear range (below 0.40). DNA concentrations and purities were measured at 260 and 280 nm using a NanoDrop (Wilmington, DE, USA) spectrophotometer.

Culture supernatants were analyzed for acetate, butyrate, acetone, butanol, ethanol, acetoain, 1,3-propanediol, and glycerol concentrations using an Agilent LC high-performance liquid chromatography system (with 1260 Infinity standard autosampler, isocratic pump, refractive index detector, and in-line vacuum degasser) and Agilent ChemStation software. A Bio-Rad Aminex HPX-87H anion exchange column was used with a mobile phase of 0.05 mM sulfuric acid flowing at 0.50 mL/min. No acetoin was detected in any cultures. Lactic acid concentration in the supernatant was determined by a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH, USA).

**Growth and metabolite assays**

Cpa overnight CGM cultures were inoculated with individual colonies taken from solid media. For glycerol studies, a starter culture of CGM supplemented with 60 g/L molecular biology grade glycerol (no glucose) was inoculated with overnight culture to an OD = 0.020–0.050 and allowed to grow to mid-exponential phase (OD 0.4–0.8). Experimental cultures of 20–80 mL CGM supplemented with 60 g/L molecular biology grade glycerol or 100 g/L agar plates supplemented with the appropriate antibiotic (50 µg/mL ampicillin, 35 µg/mL chloramphenicol, 25 µg/mL kanamycin). E. coli strains were stored at −85 °C in LB medium supplemented with 15 % glycerol.

Table 3 Strains and plasmids used in this study

| Strain          | Description                        | Source                  |
|-----------------|------------------------------------|-------------------------|
| Cpa ATCC 6013   | Wild-type Clostridium pasteurianum | ATCC                    |
| Cpa M150B       | Mutant isolated from crude glycerol selection | This study             |
| Cpa ΔSpo0A      | Constructed Spo0A deficient strain | This study              |
| NEB Turbo       | Cloning strain for DNA manipulation | NERB                    |
| Plasmid         |                                    |                         |
| pCpaDcm2.0      | Methylating plasmid                | DNA 2.0                 |
| pKO-mazF        | General recombination plasmid      | Al-Hinai et al. [41]    |
| pKO-mazF-spo0A  | Knockout plasmid targeting Spo0A   | This study              |
| p94-Flp         | Flippase plasmid for Thr removal   | Al-Hinai et al. [41]    |
crude glycerol were inoculated from starter cultures to an OD of 0.070. All media was stored at 37 °C anaerobically for at least 48 h prior to use. Samples were taken for turbidity and metabolite analysis simultaneously. For metabolite analysis, samples were centrifuged for 1 min at 16,000 rcf, and the supernatant was removed and stored at −20 °C until analyzed. Samples were filtered with a 0.22 µm syringe filter prior to downstream analysis.

**Whole-genome SMRT sequencing**

High molecular weight genomic DNA was isolated from *C. pasteurianum* ATCC 6013 and M150B strains using Genomic-tip 100/G (QIAGEN) according the manufacturer’s instructions. Single molecule real time sequencing (SMRT) sequencing was performed at the University of Delaware DNA Sequencing and Genotyping Center. SMRTbell DNA libraries were constructed according to the Pacbio standard protocol. Template preparation was performed using BluePippin (Sage Science) size-selection system including DNA damage and end repair steps and ligation to hairpin adapters. Both libraries were size-selected starting at 7 kb and with an average library size of 20 kb as measured by Fragment Analyzer (Advanced Analytical Technologies, Inc). Sequencing was performed on PacBio RSII (Pacific Biosciences, Menlo Park, California) instrument using P4-C2 chemistry, mag-bead loading and 3-h movie time. To identify consensus and variant sequences, quality-filtered reads were mapped against a reference sequence (accession number CP009267 [20]) using the RS Resequencing protocol within the SMRT Analysis version 2.3 through the SMRT Portal. The RS Modification and Motif Analysis protocol was used to identify base modifications and methyltransferase recognition motifs.

**Knockout of Cpa spo0A**

Plasmids used in this study are listed in Table 3. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Iowa City, IA, USA) and are listed in Additional file 1: Table S2. Two ~500 bp regions of homology designed to surround the ribosomal-binding site and first section of the spo0A gene (including the start codon) were amplified with primers 140 & 141 and 142 & 143. These were sequentially cloned into the pKO-mazF vector [41] using SpEl & Agel and MluI & NcoI to generate pKO-mazF-spo0A. Successful cloning at each stage was confirmed by PCR and Sanger sequencing. Newly constructed plasmids were transformed into *E. coli* Turbo (NEB) cells and subsequently isolated and confirmed via PCR confirmation and Sanger sequencing. Immediately prior to transformation of plasmids into *Cpa*, plasmids were electroporated with *E. coli* ER1821(pCpaDcm2.0) (NEB). For plasmid methylation, cultures were supplemented with 1 mM rhamnose at least 4 h prior to plasmid extraction via miniprep. At least 500 ng of methylated plasmids were subsequently transformed into *Cpa* as described by Pyne et al. [11].

The bepLM gene was synthetically constructed (DNA 2.0, Menlo Park, CA, USA) for optimized codon usage in *E. coli* (see Additional file 1: Table S1). The bepLM gene was cloned into plasmid pD881 (pCpaDcm2.0), which uses a rhamnose inducible promoter.

**Microscopy**

For light microscopy, samples taken from 6 day old CGM culture were pelleted and washed twice with 1 % w/v NaCl. Slides were imaged using a Leica widefield microscope with phase contrast to distinguish cell morphology. For transmission electron microscopy, *Cpa* strains cultured for 5 days in CGM were pelleted and the supernatant was removed. The bacterial pellet was treated with 16 % paraformaldehyde and 8 % glutaraldehyde to the culture medium for a final concentration of 2 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer and inverted multiple times to mix. Cultures were fixed for 2 h at room temperature, pelleted and resuspended in buffer. The cells were then pelleted and embedded with 4 % low melting point agarose and cut into 1–2 mm³ cubes. Samples were then dehydrated in increasing concentrations of acetone washes for 15 min each (25, 50, 75, 95, 100, 100 %). The samples were infiltrated with 50/50 mixture of acetone/n-BGE for 30 min, then infiltrated with 100 % n-BGE for an additional 30 min. Samples were infiltrated with Quetol resin in increasing concentrations with n-BGE as diluent: 25 % overnight, 50 % for 8 h, 75 % overnight, 100 % for 8 h, 100 % overnight, and 100 % for 8 h. Samples were embedded in BEEM capsules with fresh resin and polymerized for 48 h at 60 °C. Blocks were sectioned and imaged as described previously [42]. All microscopy imaging was performed at Delaware Biotechnology Institute Bioimaging Center.

**Additional file**

Additional file 1. Table S1: Nucleotide sequence variants from published data. Table S2: Sequence and features of pDcm2.0 with codon optimized *Cpa* bepLM. Figure S1: Phase-contrast microscopy of WT and M150B after 6 days. Phase-bright forespores observed in the WT and the asporogenous phenotype is apparent in the M150B strain. Figure S2: Whole genome SMRT sequencing coverage depth overview across reference genome. Cpa wild type ATCC 6013 and Cpa mutant M150B coverage across the reference genome. Figure S3: Cultures of the wild type, M150B, and Δspo0A Cpa strains after 5 days culturing. Cultures were allowed to rest without agitation. M150B cultures consistently settled quickly compared to wild type, while the Δspo0A consistently remained in suspension longer than the wild type. This shows the M150B phenotype is not due to the Spo0A deficiency.
Abbreviations
Cpa. Clostridium pasteurianum; PDO: 1,3-propanediol; SMRT: single molecule real-time; WT: wild type; NTG: N-methyl-N’-nitro-N-nitrosoguanidine; CGM: clostralid growth media; Cac. C. acetobutylicum; Cbe. C. beijerinckii; IPD: interpulse duration ratio, MAMA: modification and motif analysis.

Authors’ contribution
NRS designed experiments with ETP and carried out growth and metabolite experiments, whole-genome sequencing and analysis, microscopy experiments, genetic knockout construction, and wrote the manuscript with ETP. KPV performed whole-genome sequencing and analysis. TSG performed genome sequencing analysis and laboratory preparation procedures. ETP designed experiments and wrote the manuscript with NRS. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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