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Genetics of Solvent-Producing Clostridia

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Final Technical Report

I. Development of Genetic Systems for *C. acetobutylicum*

a) *Clostridium acetobutylicum* Viruslike particle

Isolation and Characterization of a Filamentous Viruslike Particle from *Clostridium acetobutylicum* NCIB 6444 (Kim and Blaschek, J. Bacteriol. 1991. 173:530-535)

A single-stranded 6.6 kb DNA molecule complexed with protein was recovered from the supernatant of *C. acetobutylicum* NCIB 6444. Electron microscopic examination of the DNA-protein complex revealed the presence of a filamentous viruslike particle, which was designated CAK1. The double-stranded plasmidlike replicative form and the single-stranded prophage were also recovered from the cell culture following alkaline lysis. CAK1 was released from the *C. acetobutylicum* cell culture in the absence of cell lysis. Polyethylene glycol-NaCl coprecipitation of the DNA-protein complex revealed the presence of single-stranded DNA complexed with protein in a manner rendering the DNA resistant to Bal31 exonuclease. Proteinase treatment of CsCl density gradient-purified CAK1 resulted in recovery of DNase-sensitive single-stranded DNA. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of CAK1 demonstrated the presence of a 5kDa major coat protein. Hybridization data indicated that the single-stranded DNA from CAK1 has homology with the M13 phage of *Escherichia coli*. An examination of various physical properties of CAK1 suggests that it is similar to the filamentous phage recovered from gram-negative microorganisms. Although infectivity or inducibility of CAK1 could not be demonstrated, to our knowledge this represents the first report of a nonlytic filamentous viruslike particle containing single-stranded DNA being recovered from a gram-positive bacterium. The observation that vectors based on filamentous phage DNA can be stably maintained without selection pressure is of great importance for the genetic manipulation of industrially significant microorganisms.

b) Phagemid Construction and Characterization

Construction and Characterization of a Phage-Plasmid Hybrid (Phagemid), pCAK1, containing the Replicative Form of Viruslike Particle CAK1 Isolated from *Clostridium acetobutylicum* NCIB 6444 (Kim and Blaschek, J. Bacteriol. 1993. 175:3838-3843.).

A bacteriophage-plasmid hybrid (phagemid) designated pCAK1 was constructed by ligating the 5 Kbp pAK102 *Escherichia coli* plasmid (Amp^r and Em^r) and the 6.6 Kbp HaeIII linearized replicative form (RF) of the CAK1 viruslike particle from *C. acetobutylicum* NCIB 6444. Phagemid pCAK1 (11.6 Kbp) replicated via the ColE1 replication origin derived from pAK102 in *E. coli*. Single-stranded DNA molecules (ssDNA) complexed with protein in a manner which protected ssDNA from nucleases were recovered from the supernatant of *E. coli* DH11S transformants containing pCAK1 in the absence of cell lysis. This suggests that the viral strand (VS) DNA synthesis replication origin of CAK1 and associated gene expression are functional in *E. coli* DH11S. The single-stranded form of pCAK1 isolated from *E. coli* supernatant was transformed into *E. coli* DH5a' or DH11S by electroporation. The isolation of ampicillin resistant *E. coli* transformants following transformation suggests that the complementary strand (CS) DNA synthesis replication origin of CAK1 is also functional in *E. coli*. The coat proteins associated with ssDNA of pCAK1 demonstrated sensitivity to proteinase K and various solvents (ie., phenol and chloroform), similar to the results obtained previously with CAK1 (Kim and Blaschek, 1991).

Following phagemid construction in *E. coli*, pCAK1 was transformed into both *C. acetobutylicum* ATCC 824 and *C. perfringens* strain 13 by intact cell electroporation at an average transformation efficiency of 10^3 transformants /ug of DNA. Restriction enzyme analysis of pCAK1 isolated from erythromycin resistant transformants of both *C. acetobutylicum* and *C. perfringens* suggested that it was identical to that present in *E. coli* transformants.

c). Electroporation-based Transformation of the pCAK1 phagemid

Protocols for the Transformation of bacteria by Electroporation (Dower et al., *In*: Handbook of Electroporation and Electrofusion. 1992. p. 485-500). Electrotransformation of bacteria by plasmid DNA (Trevors et al., *In*: Handbook of Electroporation and Electrofusion. 1992. p. 265-290). Also - Kim and Blaschek, J. Bacteriol. 1993. 175:3838-3843.; Kim et al., Appl. Environ. Microbiol. 1994. 60:337-340.

Another interesting result of our work was the development of simple electroporation protocols for *C. acetobutylicum* using a 10% PEG solution (Kim and Blaschek, 1993; Kim et al., 1994). We suggested earlier (Kim and Blaschek, 1989) that the cell wall structure represents an additional barrier to DNA uptake in *C. perfringens*. The presence of 10% PEG and the low ionic strength of the electroporation solution result in a significant increase of the pulse duration time (30 to 40 msec), which may overcome the cell wall barrier. During electroporation-induced trans-formation, PEG may limit cytoplasmic leakage and, via exclusion of the aqueous phase, help to push the plasmid DNA into the cell and consequently increase cell viability and transformation efficiency. The difference in expression periods following electroporation of *C. acetobutylicum* and *C. perfringens* (7 vs. 1h) suggests that recovery of cell viability is related to the specific growth rate.

II. Introduction and Expression of a Heterologous Cellulase Gene in *C. acetobutylicum*

Expression of endo-b-1,4-D-glucanase from *Clostridium cellulovorans* in *Clostridium acetobutylicum* ATCC 824 following transformation of the *eng*B gene (Kim et al., Appl. Environ. Microbiol. 1994. 60:337-340.; Attwood et al., Abstracts of the 1993 Meeting of the American Society for Microbiology; Attwood et al. Submitted to Appl. Environ. Microbiol.)

A phage lambda genomic DNA library from *C. cellulovorans* ATCC 35296 was generated in lambdaZAP II and screened for cellulase components. Six *E. coli* clones exhibiting activity against Ostazin Brilliant Red-Hydroxyethyl cellulose (OBR-HEC) were isolated, and all contained an identical 3.4 kb EcoRI fragment from *C. cellulovorans*. Plaques were amplified and *in vivo* excision of the pBluescript plasmid from the lambdaZap II vector was performed with one clone. This clone was capable of hydrolyzing carboxymethylcellulose (CMC) as well as OBR-HEC. Extensive restriction enzyme analysis and partial sequence analysis of the *C. cellulovorans* gene contained in pBAW40 indicated that this gene was identical to the previously cloned and sequenced *eng*B gene of *C. cellulovorans* (Laboratory of Roy Doi). A 3.4 Kb EcoRI fragment containing the endo-ß-1,4-glucanase gene, *eng*B, from *C. cellulovorans* was subcloned into the lacZ region of the *E. coli-Clostridium acetobutylicum* shuttle vector pMTL500E to produce a 9.3 kb plasmid, designated pAK301. Plasmid pAK301 was prepared from *E. coli* and was transformed (efficiency 10^3 transformants/ug DNA) into *C. acetobutylicum* ATCC 824 using 10% PEG-based intact cell electroporation. Restriction enzyme digestion analysis of plasmid DNA recovered from the *C. acetobutylicum* transformant designated BKW-1, verified the presence of pAK301. Heterologous expression of the *C. cellulovorans eng*B gene by *C. acetobutylicum* BKW-1 was detected as zones of hydrolysis on carboxymethylcellulose (CMC) TGY plates stained with Congo Red. The extracellular cellulase preparation from *C. acetobutylicum* BKW-1 has a specific activity towards CMC which is more than four-fold that present in *C. acetobutylicum* ATCC 824 (Kim et al.,

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1994). Furthermore, this extracellular cellulase preparation from *C. acetobutylicum* BKW-1 was active against acid-swollen cellulose and Sigmacell, but not filter paper, whereas the extracellular cellulase preparation from *C. acetobutylicum* ATCC 824 did not show activity against these substrates. Verification of heterologous expression of *C. cellulovorans engB* gene in *C. acetobutylicum* was recently carried out using Western Blot Analysis. The antibodies to the *C. cellulovorans engB* gene was graciously provided by Dr. Roy Doi at the University of California. Results of this work confirms heterologous expression of the *engB* gene in *C. acetobutylicum* BKW-1. Because of the lack of transcriptional information on *engB*, we examined its expression in *C. cellulovorans* and in the heterologous hosts *E. coli* and *C. acetobutylicum* following transformation of *engB* by electroporation. Northern analysis suggested that both *E. coli* and *C. acetobutylicum* produced several transcripts of various sizes. *C. cellulovorans* produced a single transcript of 1600 b with the relative amount of *engB* mRNA from cellulose-grown cells being much greater than that from cellobiose-grown cells. Primer extensions showed that *engB* was transcribed from a single transcription initiation site in *C. cellulovorans* preceded by sequences similar to promoter sequences found in gram positive bacteria. Primer extensions from both *E. coli* and *C. acetobutylicum* strains containing the *engB* gene showed multiple transcription initiation sites, none of which corresponded to the site determined in *C. cellulovorans.* We conclude that transcriptional control of the *engB* gene is less stringent in heterologous backgrounds and postulate that expression of the *engB* gene in *C. cellulovorans* is increased in the presence of cellulose. This represents the first report of heterologous gene expression in *C. acetobutylicum.*

Another interesting aspect of the above study is the successful introduction into *C. acetobutylicum* of plasmid DNA which contains *E. coli* derived DNA. It has been suggested that the *E. coli* DNA portion of shuttle vectors may be problematic for transformation and maintainence in *C. acetobutylicum* due to the presence of a Cac824I restriction endonuclease which recognizes the sequence 5'-GCNGC-3'. The existence of a Cac824I restriction system in *C. acetobutylicum* ATCC 824 was suggested as interfering with DNA transformation, and causing severe deletion of introduced *E. coli* DNA in this microroganism. In light of the low G+C content of *C. acetobutylicum* DNA, a Cac824I site would be expected to occur only on the order of once every 2.6 kb. However, in plasmids pUC19 and pBR322, such a sequence reportedly occurs on the average of every 110 bp. Both pMTL500E and pAK301 contain at least 20 Cac824I restriction enzyme recognition sites, and yet these constructs are readily transformed and stably maintained in *C. acetobutylicum* ATCC 824 in the absence of DNA rearrangement. The only exception to this was increased dimer formation of pAK301 in *C. acetobutylicum.*

III. Isolation of *C. acetobutylicum* mutants

Isolation and Characterization of *Clostridium acetobutylicum* mutants with enhanced amylolytic activity (Annous and Blaschek, Appl. Environ. Microbiol. 1991. 57:2544-2548)

C. acetobutylicum mutants BA101 (hyperamylolytic) and BA105 (catabolite derepressed) were isolated by using N-methyl-N'-nitrosoguanidine together with selective enrichment on the glucose analog 2-deoxyglucose (Annous and Blaschek, 1991). Amylolytic enzyme production by *C. acetobutylicum* BA101 was 1.8- and 2.5-fold higher than that of the ATCC 824 strain grown in starch and glucose, respectively. *C. acetobutylicum* BA105 produced 6.5-fold more amylolytic activity on glucose relative to that of the wild-type strain.

Because of the lack of transcriptional information on *engB*, we examined its expression in *C. cellulovorans* and in the heterologous hosts *E. coli* and *C. acetobutylicum* following transformation of *engB* by electroporation. Northern analysis suggested that both *E. coli* and *C. acetobutylicum* produced several transcripts of various sizes. *C. cellulovorans* produced a single transcript of 1600 b with the relative amount of *engB* mRNA from cellulose-grown cells being much greater than that from cellobiose-grown cells. Primer extensions showed that *engB* was transcribed from a single transcription initiation site in *C. cellulovorans* preceded by sequences similar to promoter

sequences found in gram positive bacteria. Primer extensions from both *E. coli* and *C. acetobutylicum* strains containing the *eng*B gene showed multiple transcription initiation sites, none of which corresponded to the site determined in *C. cellulovorans.* We conclude that transcriptional control of the *eng*B gene is less stringent in heterologous backgrounds and postulate that expression of the *eng*B gene in *C. cellulovorans* is increased in the presence of cellulose. Portions of this work were presented at the the 1993 Meeting of the ASM (Attwood et al., 1993).

Construction of a Macrorestriction Map for *C. acetobutylicum*

Construction of a Macrorestriction Map for *Clostridium acetobutylicum* ATCC 824 using Two Dimensional Transverse Alternating Field Electrophoresis (Broussard and Blaschek, Abstracts of the 1993 ASM Meeting)

A macrorestriction map of *C. acetobutylicum* ATCC 824 chromosome was generated using a combination of the restriction enzymes MluI, SalI and SmaI. The primary structure of the chromosomal map was generated using two-dimensional reciprocal digestion with SalI and MluI. Identical spots on two-dimensional gels were identified by molecular mass and assigned to the respective fragment in the first dimension. Analysis of SmaI/SalI and SmaI/MluI two-dimensional PFGE gels allowed for alignment of internal DNA fragments and further characterization of the macrorestriction map. Chromosomal DNA embedded in agar blocks was digested with SalI, SmaI, or MluI, and was subsequently separated by TAFE using three different run conditions. The lane containing the digested DNA separated by TAFE was removed from the gel, digested with 200 U of SalI or MluI for 4 hours, and then separated by TAFE using three run conditions. The combination of digestions and TAFE separations resulted in a 5.7 Mb macrorestriction map consisting of more than 100 restriction enzyme recognition sites. The location of 12 previously cloned *C. acetobutylicum* genes was determined by Southern hybridization. The cloned DNA fragments appeared to be randomly distributed throughout the genome.

Genetic Characterization of the CAK1 replication origin.

An examination of the replication origin of the CAK1 filamentous virus-like particle recovered from *Clostridium beijerinckii* NCIMB 6444 was conducted using deletion analysis of the pCAK1 phagemid. Four deletion derivatives, pCKE, pCKP, pCEP and pDT5, were isolated and transformed into various clostridial *spp.* All the derivatives demonstrated the ability to continuously secrete single stranded DNA in *E. coli* DH11s. Overlapping of these deletion derivatives suggested the ss and ds DNA replication origins of the CAK1 filamentous virus-like particle were located on a 1.1kb region of pDT5. The 1.1kb fragment was sequenced and analyzed. The sequencing data indicated the CAK1 replication origin contained no ORF's or secondary structures which had similarity to any known filamentous viruses such as M13 *ori* or coding proteins. This finding suggests a possible unique replication mechanism for the filamentous virus-like CAK1 particle in *C. beijerinckii* .

Integrating Summary Statement

Specific Aims 1 and 2 of the original project proposal were specifically addressed during this project period. This involved the development of the pCAK1 phagemid delivery vector, refinement of the *C. acetobutylicum* electroporation protocol, selection and characterization of the *eng*B cellulase gene from *C. cellulovorans* and the introduction and successful expression of this heterologous *eng*B gene from *C. cellulovorans* in *C. acetobutylicum.* The successful expression of a heterologous *eng*B gene from *C. cellulovorans* in *C. acetobutylicum* ATCC 824 has important industrial significance for the utilization of cellulose by this ABE fermentation microorganism. Conversion efficiency testing of the developed recombinant strains in batch and continuous culture (Specific Aim 3) will be carried out once suitable strains have been developed which can utilize cellulose as sole carbon source (see below).

The functionality of pCAK1 in the *E. coli* host system, especially in generating ssDNA, in the absence of impairing *E. coli* cell viability, together with successful introduction of pCAK1 into *C. acetobutylicum* and *C. perfringens* is the basis for the construction of a M13-like genetic system for the genus *Clostridium* and is expected to allow for more sophisticated molecular genetic analysis of this genus.

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