

## PROJECT SUMMARY

The Project Summary should include a statement of objectives, methods to be employed, and the significance of the proposed activity to the advancement of knowledge or education. Avoid use of first person to complete this summary. **DO NOT EXCEED ONE PAGE.** (Some Programs may impose more stringent limits.)

DOE/CE/50364--T1

### PATHWAY ENGINEERING TO IMPROVE ETHANOL PRODUCTION BY THERMOPHILIC BACTERIA

A proposal for renewed funding to  
the National Science Foundation, Biotechnology Program  
and the Department of Energy, Biofuels Program

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MASTER

## PROJECT SUMMARY

Continuation of a research project jointly funded by the NSF and DOE is proposed. The primary project goal is to develop and characterize strains of *C. thermocellum* and *C. thermosaccharolyticum* having ethanol selectivity similar to more conventional ethanol-producing organisms. An additional goal is to document the maximum concentration of ethanol that can be produced by thermophiles. These goals build on results from the previous project, including development of most of the genetic tools required for pathway engineering in the target organisms. As well, we demonstrated that the tolerance of *C. thermosaccharolyticum* to added ethanol is sufficiently high to allow practical utilization should similar tolerance to produced ethanol be demonstrated, and that inhibition by neutralizing agents may explain the limited concentrations of ethanol produced in studies to date. Task 1 involves optimization of electrotransformation, using either modified conditions or alternative plasmids to improve upon the low but reproducible transformation frequencies we have obtained thus far. Task 2 involves cloning target thermophilic genes (those encoding for acetate kinase, phosphotransacetylase, lactate dehydrogenase, hydrogenase, and pyruvate dehydrogenase from *C. thermocellum* and *C. thermosaccharolyticum*) in *E. coli*. Task 3 consists of preparing plasmid constructs containing deleted copies of target genes, introducing these constructs into thermophilic hosts via electroporation, and selection of strains in which non-functional deleted genes have replaced functional target genes via homologous recombination. In Task 4, fermentation studies will be carried out directed toward realizing the ethanol production potential of thermophiles and characterization of modified strains produced in Task 3. Project results are expected to be significant in the context of: 1) advancing technology for production of ethanol, a sustainable fuel and chemical feedstock of considerable promise; 2) demonstrating the efficacy of consolidated bioprocessing, a process concept generically applicable and potentially highly advantageous for low cost production of commodity products from biomass; and 3) extending pathway (or metabolic) engineering by applying it to anaerobic thermophilic bacteria, a potentially important group of microorganisms for which molecular-level tools and information are not well-developed.

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## A. RESULTS FROM PRIOR SUPPORT\*.

As this proposal is submitted jointly to the NSF and DOE, directly relevant previous work supported by both sponsors is included here. Such previous work involves molecular studies and fermentation studies, as summarized below. All molecular work was sponsored by grants BCS-9215130 and BCS-9314449, 2-year jointly-awarded grants with funding from both the NSF Biotechnology Program and the DOE Biofuels Program. Fermentation work was sponsored by these grants as well as a one-year subcontract from the National Renewable Energy Laboratory begun in February of 1995; No. XAC-5-15162-01. Sponsorship is noted in the list of publications at the end of this section.

### I. Molecular Studies

Restriction endonuclease activity has been characterized for both *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* (e.g.). *C. thermocellum* cell extracts incubated at 55°C exhibit specific endonuclease activity with very little non-specific exonuclease activity. The Dam methylation system of *Escherichia coli* offers complete protection from digestion by *C. thermocellum* ATCC 27405 cell extracts for all DNA tested (totaling >100kb, insuring that most restriction sequences are represented). Several lines of evidence suggest that *C. thermocellum* ATCC 27405 contains a single restriction system recognizing the sequence 5' GATC 3'. These include: protection by the Dam methylation system (which methylates the adenine of the GATC sequence), a very similar electrophoretic banding pattern for digests obtained with *Mbo*I (which cuts at the GATC sequence) and cell extracts, and banding patterns explainable by GATC cutting for all DNA tested (totalling > 100 kb). Cell extracts made from *C. thermosaccharolyticum* ATCC 31960 do not exhibit specific endonuclease activity under the conditions tested. Genomic DNA from *C. thermocellum* is not cut by *Mbo*I, consistent with the genome being methylated so as to protect it from autodigestion. Genomic DNA from *C. thermosaccharolyticum* is cut by *Mbo*I (which does not cut DNA methylated at GATC), consistent with it not being methylated. Restriction endonuclease activity can play a major role in impeding transformation of *Clostridium* and other organisms for which genetic systems are not well-developed. These results would appear to effectively remove this impediment for the organisms of interest.

Electrotransformation of *C. thermosaccharolyticum* ATCC 31960 was achieved (e.g.). We utilized the plasmid pCTC1 developed by Williams et al (210), which contains the Gram positive bacterial replicon pAMB1, the Gram negative bacterial R2 replicon, and carries a gene encoding for an adenine methylase originating from *Enterococcus faecalis* and conferring resistance to erythromycin. Erythromycin was found to be effective at preventing growth at a concentration of 1 µg/ml in solid media and 10 µg/ml in liquid media; thiamphenicol was also effective at 20 and 25 µg/ml in solid and liquid media respectively. Kanamycin and chloramphenicol were not effective up to 125 µg/ml. Evidence supporting transformation was provided by Southern blots, detection of the plasmid in 10 out of 10 erythromycin-resistant clones, retransformation of *E. coli* with plasmid isolated from *C. thermosaccharolyticum*, and a proportional relationship between the number of transformants and the amount of DNA added. Electroporation was carried out in a glycerol buffer under conditions that included a 2.0 kV pulse, 800 Ω resistance, 25 µF capacitance, 0.2 cm cuvette gap, and a 16 msec time constant; the mean survival frequency was 0.95. Transformation of *C. thermosaccharolyticum* was not obtained using a sucrose/phosphate buffer such as others have employed for mesophilic *Clostridium* (3,17,137,163), although positive controls were run with *Clostridium beijerinckii*. Similar results were obtained with fresh and frozen cells. Transformation efficiencies were very low for plasmid DNA prepared in *E. coli* (0.60 transformants/µg DNA for fresh and frozen cells), and 100-fold higher for plasmid DNA prepared in *C. thermosaccharolyticum* (an average of 61 transformants/µg DNA). This difference would appear to be due to one or more factors other than restriction incompatibility. The observation that pCTC1 recovered from transformed *C. thermosaccharolyticum* could be used to transform either *E. coli* or *C. thermosaccharolyticum* indicates that the plasmid is

\* Lettered references refer to the bibliography of supported work at the end of this section, numbered references refer to the main bibliography at the end of the proposal

replicated independently of the chromosome at both 45°C and 60°C growth temperatures. Restriction analysis shows no indication of plasmid rearrangement upon passage through *C. thermosaccharolyticum*. These results represent the first report of transformation of *C. thermosaccharolyticum* as well as the first demonstration of expression of a foreign gene in this organism.

We have obtained phenotypic evidence suggesting that *C. thermocellum* has also been transformed with pCTC1. Using electroporation conditions similar to those used for *C. thermosaccharolyticum*, presumptive transformants were obtained (as indicated by growth in the presence of 10 µg/ml erythromycin with no growth observed in non-transformed controls) from electroporation with pCTC1 prepared in *E. coli* ED8767 (dam<sup>+</sup>, dcm<sup>+</sup>). Retransformation of *C. thermocellum* with pCTC1 recovered from *C. thermocellum* was successfully carried out, again with no growth in non-transformed controls. When documentation of transformation of *C. thermocellum* is complete, we anticipate submission of a manuscript.

In addition to transformation, we have also investigated cloning of catabolic genes. We initially pursued a complementation-based approach using mutant strains of *E. coli* (kindly furnished by G. Ferro-Luzzi Ames) deficient in phosphotransacetylase (Pta), acetate kinase (Ack) or both, grown under conditions selective for complementation as described by LeVine et al (114). By using a pML703 plasmid (kindly provided by J. Ferry) overexpressing the *ack* gene from *Methanoscincina thermophila* as a model system, we have successfully complemented an *ack*-minus recipient, and have overexpressed the *M. thermophila* ACK activity 30 times higher than the *E. coli* wild type. Thereafter, four *C. thermocellum* genomic libraries were prepared by partial digestion of chromosomal DNA with *Alu*I, *Pst*I, *Sau*3AI, and *Xba*I respectively followed by ligation into the bluescript SK plasmid. Attempts to obtain complementation using the *C. thermocellum* libraries have not however been successful to date. Cloning via complementation was complicated by occasional mutant reversion, and by leaky growth of the *E. coli* mutants on minimal acetate plates due to an acetate assimilation pathway mediated by acetyl-CoA synthetase (96,204).

In light of the difficulties encountered with cloning by complementation, we investigated a cloning strategy using the Polymerase Chain Reaction (PCR) instead. Using available sequence information, PCR primers were synthesized designed to amplify fragments of genes coding for lactic dehydrogenase (*ldh*), acetate kinase (*ack*), phosphotransacetylase (*pta*), and hydrogenase (*hyd*). Primer design involved selection of 20 to 25 bp sequences that exhibit a high degree of conservation among different species while also minimizing degenerate codons. Results to date are presented below:

Amplification of catabolic gene fragments using PCR (J.H. Kim, D.A. Hogsett, D. Westenberg, M.L. Guerinot, L.R. Lynd, unpublished)

Gene	Fragment Size (bp)	Source of Template DNA				Organism/Ref. for sequence comparison
		<i>C. thermocellum</i>	<i>C. thermosaccharolyticum</i>	<i>C. thermocellum</i>	<i>C. thermosaccharolyticum</i>	
<i>ack</i>	320	Amino acid identity 61%	Nucleic acid identity 60%			<i>Methanoscincina thermophila</i> (106)
<i>hyd</i> <sup>1</sup>	360	53%	58%	51 to 57%	58 to 65%	<i>Clostridium acetobutylicum</i> (175)
	600			50 to 63%	62 to 71%	

<sup>1</sup> 360 bp fragments were synthesized with a common set of primers for both *C. thermocellum* and *C. thermosaccharolyticum*. Identity ranges reflect current discrepancies for sequencing with forward and reverse primers.

As presented in the preceding table, we have obtained 4 PCR products that show significant sequence identity (for both amino acids and nucleic acids) with the genes for which primers were designed (*ack*, *hyd*). In addition, we have amplified a 180 bp fragment presumptively from *ldh* in *C. thermosaccharolyticum* that shows some promise (32% amino acid identity with *ldh* from *Bacillus megaterium* (200b)); further characterization will be necessary to definitively determine if we have been successful in amplifying an *ldh* fragment. As well, we are investigating several alternative primer sets for *ldh*-targeted PCR. The *ack* fragment has been subcloned in *E. coli* using a pGEM-T vector. The *ack* fragment may be particularly useful, since the *ack* and *pta* genes are adjacent in most organisms in which they have been located (82,181,197), and thus an *ack* fragment might be used as a probe to clone *pta* as well as *ack*. Labeled probes prepared using the *ack* fragment showed strong hybridization with *C. thermocellum* genomic DNA, confirming that we have in fact amplified *Clostridium* DNA. Weak hybridization was observed between the amplified *ack* fragment from *C. thermocellum* and *Methanosarcina thermophila* genomic DNA, and no hybridization was observed when the *C. thermocellum* genome was probed with the *ack* and *pta* genes of *M. thermophila* and *E. coli* respectively. These results suggest that cloning of *ack* is not readily achieved using heterologous probing, and further support the use of a PCR-based approach. A paper is anticipated in which we present amplification of gene fragments via PCR (described above) as well as use of these fragments as homologous probes to screen gene libraries in the course of cloning catabolic genes.

In summary, results from the previous jointly funded project (BCS-9215130 and BCS-9314449) go a long way toward providing the requisite tools for pathway engineering of the targeted organisms. We believe our progress is significant in light of the nascent state of knowledge of genetic manipulation of thermophilic anaerobes, as well as the fact that our labs had very little prior experience with many of the techniques required. In the course of the previous project, we benefited significantly from the molecular biology expertise of Dr. Mary Lou Guerinot (Department of Biological Sciences at Dartmouth), who appears as co-author on a paper from the project (g). It is thus fitting that Dr. Guerinot appears as a Co-P.I. on the present proposal.

## II. Fermentation Studies

The ethanol tolerance of *C. thermosaccharolyticum* ATCC 31960 was studied in continuous culture using a new technique based on measurement of effluent substrate concentrations (a). Produced ethanol was supplemented with added ethanol to achieve various concentrations. The ethanol tolerance of *C. thermosaccharolyticum* was significantly greater than expected based on previous reports, which may be explained in part by acclimation occurring over time periods exceeding those typical of batch systems studied previously. P<sub>50</sub> values (the concentration of ethanol required to result in 50% inhibition of growth rate) were 40 g/L at 55°C, and 26 g/L at 60°C. The observed trend of increasing ethanol tolerance with decreasing temperature is consistent with both experimental data and biophysical understanding based on other microorganisms. The tolerance of *C. thermosaccharolyticum* to added ethanol is somewhat greater than that of more conventional ethanol-producing organisms (yeast, *Zymomonas*) at temperatures typically used in simultaneous saccharification and fermentation (SSF) processes, and appears to be adequate for use of thermophiles to produce ethanol in a practical context. Ester-linked phospholipid membrane analysis revealed that growth in the presence of high concentrations of ethanol (33 g/L) resulted in a membrane profile having increased fluidity and molecular diversity. Ethanol-induced changes included a significant increase in shorter chain unsaturated fatty acids (C15:0) at the expense of longer chain unsaturated fatty acids (C17:0) and a slight increase in the amount of mono-unsaturated fatty acids.

Prompted by the high tolerance of *C. thermosaccharolyticum* to added ethanol, a series of experiments involving continuous culture of *C. thermosaccharolyticum* at progressively higher feed xylose concentrations was carried out (b) in an effort to produce ethanol at concentrations commensurate with the documented ethanol tolerance. A systematic study of nutritional requirements of *C. thermosaccharolyticum* (c) contributed to this effort. In the course of increasing the feed xylose concentration, changes in the growth medium recipe were necessary at

several points in order to achieve complete xylose utilization (presumably to supply deficient nutrients). Steady-states were repeatedly achieved with a 75 g/L xylose feed, with production of 22 to 23 g/L ethanol, about 13 g/L acetate, and essentially no lactate. Efforts to further increase the feed xylose concentration from 75 g/L to 87.5 g/L were, however, unsuccessful. Washout of the culture began shortly after the step change in feed xylose concentration, as reflected by an increase in unutilized xylose and a decrease in culture optical density, ethanol, and acetate. Washout was also accompanied by production of significant amounts ( $> 3 \text{ g/L}$ ) of lactic acid, consistent with our general understanding that lactic acid production only occurs when the culture is stressed. Nearly identical results were obtained when the feed-xylose step change from 75 g/L to 87.5 g/L was repeated with elevated nutrient concentrations, indicating that nutrient limitation was not responsible for the culture's failure to reach steady-state at 87.5 g/L feed. The hypothesis that ethanol inhibition was responsible for this failure was tested by adding 4 g/L ethanol to the feed of a steady-state culture fed with 75 g/L xylose. Addition of ethanol in this way resulted in a stable steady-state and an ethanol concentration of 26 g/L (higher than was observed when the feed xylose concentration was changed from 75 g/L to 87.5 g/L), indicating that ethanol inhibition was not the limiting factor. Noting that the concentration of potassium ion was high (~19 g/L) in the steady-states obtained with 75 g/L feed xylose and that it had risen to yet higher values during the feed step-change experiment, the hypothesis was investigated that washout was due to high concentrations of potassium ion resulting from the use of KOH for pH control. When KCl was added to the feed of a steady-state culture fed with 75 g/L xylose, the results were essentially the same as observed in the initial feed step-change experiment: washout was observed accompanied by transient lactic acid production.

The above-described experiments provide strong evidence that potassium ion was the primary factor responsible for preventing production of ethanol by *C. thermosaccharolyticum* at concentrations  $> 23 \text{ g/L}$ , and that ethanol inhibition per se played little or no role in inhibiting the culture under the test conditions. The literature contains no reports of a thermophile producing more than 30 g/L ethanol, which has generally been attributed to the intolerance of these organisms to ethanol. However, our data suggests an alternative explanation: inhibition by neutralizing agents. If this hypothesis proves correct, then it may provide a means to produce higher ethanol concentrations than hitherto reported using thermophilic cultures. Inhibition by neutralizing agents may also resolve the current discrepancy between tolerance to added ethanol as compared to the maximum concentrations of ethanol produced by thermophiles. These data also allow an evaluation of the tolerance of *C. thermosaccharolyticum* to potassium ion. The observed tolerance (less than complete inhibition at 19 g/L  $\text{K}^+$ ) would appear to be rather high compared to the cation tolerance observed for a variety of pure and mixed cultures (541,51). For example, *Zymomonas mobilis* is completely inhibited by 10 g/L  $\text{K}^+$  (41).

Appropriate goals for further fermentation work include: use of less inhibitory neutralizing agents as a means to document production of ethanol at concentrations  $> 23 \text{ g/L}$ , demonstration that cocultures of *C. thermosaccharolyticum* and *C. thermocellum* can produce similarly high ethanol concentrations from cellulosic substrates, and utilization of realistic raw materials for commercial ethanol production. Progress in these directions to date has involved co-cultivation of *C. thermocellum* and *C. thermosaccharolyticum* on paper sludge (primary clarifier sludge obtained from the Crown Vantage paper mill in Berlin, New Hampshire). We have found that this material does not inhibit fermentation at concentrations up to 60 g/L cellulose. In addition, we have obtained a steady-state continuous coculture growing on xylose-supplemented paper sludge. Data presented in Section II.1 demonstrate the capacity of the thermophilic co-culture to achieve high conversion of mixed carbohydrates, although results to date are at low substrate concentrations. At a 24 hour residence time and an overall carbohydrate concentration of 16 g/L (2/3 of which was cellulose), the coculture produced all required saccharolytic enzymes and achieved  $> 90\%$  of theoretical conversion of each carbohydrate component (cellulose, xylan, and xylose). In a second set of experiments, we have demonstrated that batch co-cultures of *C. thermocellum* and *C. thermosaccharolyticum* produce 25 g/L ethanol from 100 g/L Avicel. These results indicate that the ability of thermophiles to produce high ethanol concentrations is not restricted to *C. thermosaccharolyticum*.

## PUBLICATIONS AND PRESENTATIONS\*

### Papers and manuscripts

- a. Baskaran, S., Hyung-Jun Ahn, L.R. Lynd. 1995. Ethanol tolerance of *Clostridium thermosaccharolyticum* in continuous culture. *Biotechnology Progress*, **11**: 276-281.†
- b. Baskaran, S., S. Casten, L.R. Lynd. Continuous cultivation of *C. thermosaccharolyticum* at high feed xylose concentrations: characterization and alleviation of limiting factors. In preparation for submission to *Biotechnol. Bioeng.* †, ††
- c. Baskaran, S., D.A.L. Hogsett, L.R. Lynd. 1995. Optimization of a chemically defined, minimal medium for *Clostridium thermosaccharolyticum*. *Appl. Biochem. & Biotechnol.* **51/52**:399-411. †, ††
- d. Demain, A.L., L.R. Lynd. Turning garbage into motor fuel: fanciful dream or feasible scheme? pP. 573-581 In: K. Shimada, K. Ohmiya, Y. Kobayashi, S. Hoshino, K. Sakka, S. Karita (eds.) *Genetics, Biochemistry, and Ecology of Lignocellulose Degradation*. Uni Publishers, Tokyo (1994)
- e. Demain, A.L., T.R. Klapatch, K.-H. Jung, L.R. Lynd. Recombinant DNA technology in the development of an economical biomass conversion process for liquid fuel. *Annals of the New York Academy of Sciences* (In press).
- f. Klapatch, T.R., A.L. Demain, L.R. Lynd. Restriction endonuclease activity in *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. *Appl. Microbiol. Biotechnol.* (In press)†
- g. Klapatch, T.R., M.L. Guerinot, L.R. Lynd. Electroporation of *Clostridium thermosaccharolyticum*. Under revision for *Industrial Microbiol.*†

### Presentations

Baskaran, S., D.A. Hogsett, and L.R. Lynd. Product formation by thermophilic clostridia grown on minimal, defined media. Presented at the Sixteenth Symposium on Biotechnology for Fuels & Chemicals. Gatlinburg, TN, May 1994. †, ††

Baskaran, S., L.R. Lynd. Fuel ethanol production from cellulosic biomass using *C. thermosaccharolyticum*. Presented at the Annual Meeting of the AIChE, Miami, FL., Nov, 1995. †, ††

Demain, A.L., L.R. Lynd. Turning garbage into motor fuel: fanciful dream or feasible scheme? *Genetics, Biochemistry, and Ecology of Cellulose Degradation*. Toba, Mie Prefecture, Japan, November, 1993.†

Demain, A.L., T.R. Klapatch, K.-H. Jung, L.R. Lynd. Recombinant DNA technology in the development of an economical biomass conversion process for liquid fuel. *Engineering Foundation Conference: Recombinant Biotechnology III - The Integration of Biological and Engineering Science*. Deauville, France, October 1994.†

Hogsett, D.A., T.R. Klapatch, J.H. Kim, A.L. Demain, L.R. Lynd. Fermentative pathway engineering of *Clostridium thermosaccharolyticum* and *Clostridium thermocellum* - electroporation and gene cloning. Presented at the Annual Meeting of the AIChE, Miami, FL., Nov. 1995.†

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\* † Supported by joint grants BCS-9215130 and BCS-9314449; †† Supported by subcontract No. XAC-5-15162-01 from the National Renewable Energy Laboratory.

Klapatch, T.R., D.A. Hogsett, K.H-Jung, A.L. Demain, L.R. Lynd. Pathway engineering and organism development for ethanol production by thermophilic bacteria. Presented at ACS National Meeting, April, 1995.†

Klapatch, T.R., K.H. Jung, A.L. Demain, L.R. Lynd. Pathway engineering to improve ethanol production by thermophilic clostridia. Presented at the National Meeting of the AIChE, San Francisco, November 1994.†

Lynd, L.R. Bioreactor and organism design for biomass ethanol production. Invited presentation for the Department of Chemical Engineering Seminar Series, University of Wisconsin, Madison, October 5, 1995.†

Lynd, L.R. Ethanol production from cellulosic biomass: bioreactor and biocatalyst design. Invited seminar for the Hawaii Natural Energy Institute, Manoa, HI March 20, 1995.†,††

Lynd, L.R. Thermophilic ethanol producing bacteria: Industrial microorganisms? Invited presentation at the Meeting of the New England Section of the Society for Industrial Microbiology, Cambridge, MA. October 25, 1995.†,††

Lynd, L.R., S. Baskaran, P. van Walsum, J.-H. Kim, S. Casten, W.-L. Lin, G. Ozcengiz, A.L. Demain. Cellulose ethanol production with consolidated bioprocessing using thermophilic bacteria. To be presented at 18th Symposium for Biotechnology for Fuels and Chemicals, Gatlinburg, TN. †,††

Lynd, L.R., T.R. Klapatch, K.H. Jung, A.L. Demain. Restriction system characterization and electrotransformation of thermophilic clostridia. Presented at the "Clostridium III" Workshop, June 1994.†

**Human Resource Summary for NSF Grants BCS-9215130 and BCS-9314449,  
"Pathway Engineering to Improve Ethanol Production by Thermophilic  
Bacteria"**

Dates: 6/93 to 6/95, with a no-cost extension until 4/96. Total Support: \$467,895

Name Current Position

*Visiting Professor*

Jin-Han Kim<sup>1</sup> Associate Professor, Junior College of Inchon, Korea

*Post-doctoral Associates*

David Hogsett Senior Scientist, Bioenergy Inc., Austin, MN

Kyung-Hwa Jung Research Assoc., Korean Institute Science & Technol.

Weng-Long Lin (partially supported) Continuing

*Graduate Students*

Sunitha Baskaran<sup>2</sup> (partially supported) Associate, McKinsey & Co., New York, NY

Taryn Klapatch<sup>2</sup> Senior Scientist, Merck and Co., West Point, PA

*Undergraduates*

Reenah Kim<sup>2</sup> (Women in Science Intern)

Juan Leon (Honors thesis)

Kathryn Miller<sup>2</sup> (Women in Science Intern)

Sunita Pal<sup>2</sup> (Senior Fellow)

Nicole Strauss<sup>2</sup> (Women in Science Intern)

<sup>1</sup>Supported by funds from his institution; materials and supplies furnished by grant. <sup>2</sup> Women.

## B. PROJECT DESCRIPTION

### I. Introduction

**1. Commodity products from biomass.** Production of fuels and commodity chemicals from cellulosic biomass is a good idea. The basic reasons for this are simple: cellulosic biomass ("biomass") is the most abundant renewable resource on earth (37), it can be produced indigenously, the cost of cellulosic feedstocks is lower than that of the bulk fuels and chemicals that can be produced from them provided that high yields are achieved (126), and the ratio of energy output:energy input for many biomass-based processes is decidedly favorable (124,126,215).

Ethanol produced from cellulosic biomass ("cellulosic ethanol") is a strategically-important biomass-based biocommodity product with significant potential for beneficial application as both a fuel (126,215) and a chemical feedstock (25,34,141,148,161,180). At the same time, cellulosic ethanol technology is a leading model system for biomass-derived biocommodity products, with an extensive body of information available. The existing corn ethanol industry provides a vital foundation from which to launch a much larger biomass-based industry. Factors motivating an eventual transition from corn to cellulosic feedstocks include: the potential for lower production costs, which foster the use of ethanol in forms with greater priority pollutant benefits; a much larger potential resource base; more environmentally-benign feedstock production; a much more favorable process energy balance; and much larger greenhouse benefits (see 124,126,215).

**2. Consolidated Bioprocessing.** In a recently-accepted publication (127), the P.I. and colleagues from the National Renewable Energy Laboratory (NREL) posed the question: "What are the likely features and cost of a facility producing cellulosic ethanol at a level of maturity comparable to a refinery?". Most likely and best-case mature technology scenarios were developed for production of ethanol from a cellulosic energy crop returning 14.2% on investment. For the most likely scenario, with feedstock costing \$38.6/delivered dry ton, the projected ethanol selling price is 50 cents/gallon; for the best-case scenario, with feedstock costing \$34/delivered dry ton, the projected selling price is 34 cents/gallon. These prices are markedly lower than the 118 cents per gallon selling price projected for base-case technology, with by far the largest share of cost reductions due to improved conversion technology. Consolidated bioprocessing - a defining theme of this proposal - had the greatest cost reduction impact among the many conversion technology improvements considered, with most of the remaining cost reduction coming from improved pretreatment.

Consolidated bioprocessing (CBP) refers to a processing strategy that simultaneously accomplishes cellulase production, cellulose hydrolysis, and fermentation in a single bioreactor. As depicted in Figure 1\*, CBP (also called Direct Microbial Conversion in the ethanol literature) is the logical endpoint in the evolution of biomass conversion technology. The key difference between CBP and other biomass processing strategies is that a single microbial system is employed for both cellulase production and fermentation. This difference has several significant ramifications, including no capital or operating costs for dedicated enzyme production, greatly reduced diversion of substrate for enzyme production, and compatible enzyme and fermentation systems. Dedicated cellulase production, e.g. by an aerobic fungus such as *Trichoderma reesei*, is relatively slow and expensive - some believe prohibitively expensive for biocommodity applications (194). As a result, the process is forced to operate with low cellulase loadings and hence large and expensive SSF reactors (70,126). CBP is in principle not subject to this constraint. The analysis of Lynd et al (127) illustrates the potential impact of these features. Relative to a state-of-the-art SSF base-case, this study projects a 50% reduction in the price of ethanol production exclusive of substrate, a 31% reduction in the cost of production overall, and a savings of 31¢/gallon for a hypothetical high-yielding CBP system. In short, CBP is a logical focal point for developing very low cost processes for production of ethanol and other biocommodity products from cellulosic biomass.

\* Figures and Tables follow the text beginning on page 16.

To be useful in CBP, a microbial system (either a single organism or a defined coculture) must exhibit the following properties:

- 1) synthesize active cellulase hemicellulase enzyme systems at high levels
- 2) ferment sugars arising from both cellulose and hemicellulose
- 3) produce desired product(s) at high selectivity
- 4) produce desired product(s) at sufficiently high concentration.

Although no microbial system exists that possesses all of these qualities to the desired degree, the tools of modern molecular biology make possible the creation of such a system. Realization of this possibility is the central goal of this proposal. The proposed project involves thermophilic bacteria because they possess the features listed above to an unusual extent.

## II. BACKGROUND.

**1. Thermophilic ethanol-producing bacteria.** Moderate thermophiles (optimal growth temperature ~ 60°C) have frequently been proposed for the production of ethanol, and are among the most frequently considered organisms in the context of CBP (27,70,98,105,120,123,184). Described thermophilic ethanol producing bacteria include cellulolytic species that cannot use xylose (e.g. *Clostridium thermocellum*), as well as non-cellulolytic species capable of fermenting xylose and other biomass sugars (e.g. *Clostridium thermosaccharolyticum*, *Clostridium thermohydrosulfuricum*, and *Thermoanaerobacter ethanolicus*) (184). *C. thermocellum* is a prolific producer of cellulase (128), and produces one of the most active cellulase enzyme systems known (78). A coculture of *C. thermocellum* and a xylose-utilizing thermophile exhibits both excellent cellulase production and the ability to readily ferment hemicellulose sugars (71,147,174,199). Thus thermophiles naturally exhibit properties 1) and 2) listed in the preceding paragraph. Thermophiles such as those mentioned above also produce ethanol, usually as the primary fermentation product. However production of organic acids such as acetic and lactic acid lowers the ethanol selectivity (property 3) below values acceptable for most applications. Evidence is presented subsequently that thermophiles may have the capacity to produce high ethanol concentrations (property 4).

The ability of thermophilic co-cultures to simultaneously and effectively utilize different biomass constituents is illustrated by data presented in Table 1, involving continuous fermentation of xylose-supplemented paper sludge (primary clarifier solids from a bleached Kraft mill diverted from landfill disposal) by a steady-state co-culture of *C. thermocellum* and *C. thermosaccharolyticum*. In this experiment, three distinct carbohydrates - cellulose originating from paper sludge, hemicellulose originating from paper sludge, and exogenously-added xylose - are utilized simultaneously with endogenously-produced hydrolytic enzymes. In a 24 hour residence time, > 90% theoretical carbohydrate utilization is achieved for all three carbohydrates. While underscoring the potential of thermophiles, the data in Table 1 also illustrate the limitations characteristic of studies investigating thermophilic ethanol production: low ethanol selectivity due to co-production of organic acids - in this case acetate; and rather low ethanol concentration - in this case about 5 g/L. Ethanol selectivity and producing high ethanol concentrations, the two key unresolved issues for thermophilic ethanol production, are addressed in subsequent sections.

**2. Ethanol Selectivity.** Ethanol selectivity may be defined as the molar production of ethanol divided by the molar production of all non-ethanol products, and is thus a measure of the production of ethanol relative to other products. For very large scale fuel ethanol production, at which markets for by-products will be saturated, high selectivity is an absolute requirement for a practical process in the foreseeable future. Even at smaller scales, the incentive for high selectivity is very strong. In light of these considerations, selectivity values of 10 or more would appear to be necessary to avoid putting a process alternative at a significant disadvantage. As a point of reference, the selectivity for fermentation of hexoses by yeast is about 12.5 (130). Although we have observed higher values under some conditions, reliably-achieved selectivity values in our hands are not more than 1 for *C. thermocellum* and not more than 2.5 for *C. thermosaccharolyticum*. Economic analyses have consistently shown cost sensitivity to be

greater for ethanol selectivity/yield than for any other parameter (67B,123,214B), although cellulase production by the fermenting organism has generally not been considered in such analyses.

As reviewed elsewhere (91), the literature on thermophilic ethanol selectivity for prominent ethanol-producing thermophiles such as *C. thermocellum*, *C. thermohydrosulfuricum*, *C. thermosaccharolyticum*, and *T. ethanolicus* follows a common pattern: early reports of strains with very high selectivity are followed by subsequent studies by other groups reporting much lower selectivity. In addition, there is a trend toward lower selectivity under conditions likely to be employed in a practical context: continuous culture (100-104) and pretreated substrates (9,207). Extensive strain development has been undertaken to increase selectivity in several species by mutagenesis combined with either screening (9,117), or selection (e.g. suicide selection on pyruvate or resistance to fluoro-substituted organic acids (173,190,203)). In the direct experience of the P.I. and Co-P.I. Demain, high selectivity proved to be an unstable characteristic of strains of *C. thermocellum* S7-19 (8) and *C. thermosaccharolyticum* strains HG-8 (89) and ATCC 7956 (173). An exhaustive continuous culture study of selected strains of *T. ethanolicus* (100-104) repeatedly found a significant decline in selectivity over time.

Metabolic approaches to selectivity enhancement have been pursued in Lynd's group with *C. thermosaccharolyticum* (67,125). Selectivity was found to be insensitive to mass action effects involving the concentrations of extracellular organic end-products. High (>10) selectivities were repeatedly obtained under specified transient conditions, but could not be maintained for any length of time in continuous cultures. Hill et al. (67) tested the hypothesis that ethanol selectivity is adjusted in relation to changes in the cellular ATP balance. Although such a relationship has been demonstrated for *Clostridium acetobutylicum* (142-144), we found with *C. thermosaccharolyticum* that only small differences in selectivity accompanied the transition from ATP-limited to ATP non-limited conditions brought on by changing from carbon limitation to either phosphate or ammonia limitation in batch or continuous cultures.

In light of the importance of ethanol selectivity and the unsatisfactory selectivities of current thermophilic strains known to the P.I.s, we believe that improving ethanol selectivity is the single most important research objective in order to make practical use of these organisms in the context of fuel ethanol production. The failure of conventional mutagenesis, strain selection, and metabolically-based environmental manipulation to yield stable high-yielding strains suggest that a molecular approach such as that proposed will be more fruitful.

**3. Ethanol Tolerance and Production of High Ethanol Concentrations.** Systems for producing cellulosic ethanol are likely to produce ethanol at concentrations  $\leq$  5 wt. % due to both biological and processing constraints. Although the maximum concentration of ethanol tolerated by industrial yeasts and *Zymomonas* is on the order of 10 wt.% at 30°C (76,110), the tolerance of microorganisms to ethanol decreases with increasing temperature (12,72,73,80,198). All SSF designs known to us operate at near the maximum temperature tolerated by the fermentative organism in order to maximize cellulase activity, consistent with the dominant cost impact of cellulase production relative to distillation. For example, the operating temperature in the NREL SSF design is 37°C, at which the maximum ethanol concentration tolerated by both yeast and *Z. mobilis* is reduced to 5 wt.%. On the processing side, cellulosic slurries become progressively more paste-like and difficult to handle at solids concentrations exceeding 15 wt.% (the NREL design uses 16 wt.%, (127)). At a representative carbohydrate content for a cellulosic feedstocks, ~ 2/3 on dry weight basis, a 15 wt.% feed corresponds to a potential ethanol concentration of 5 wt.%.

Fortunately, the penalty for operation at such modest ethanol concentrations is much smaller than often assumed. In the NREL base-case SSF design for example (127), ethanol recovery from a 4.5 wt.% broth using conventional distillation costs 7.8 cents per gallon (5.1 cents per gallon for energy and 2.7 cents per gallon for capital). In the advanced design, also featuring conventional distillation, ethanol recovery from a 5% broth costs 4.6 cents/gal. In comparison to selectivity, tolerance is clearly the less important parameter from an economic perspective (123).

Estimates of the tolerance of thermophilic bacteria to ethanol vary by over an order of magnitude (125). A key goal of the previous project was to narrow the uncertainty of such

estimates. Results from the previous grant period (Section A) indicate that the tolerance of *C. thermosaccharolyticum* to added ethanol is considerable (50% inhibition at 40 g/L ethanol and 55°C), a value somewhat higher than that of yeast and *Zymomonas* under SSF conditions. Moreover, thermophiles might be expected to be among the most ethanol tolerant organisms since the ability of membranes to tolerate high temperature and high ethanol concentrations is thought to have a common biophysical basis (80). At the same time, there have been no published reports of a thermophile producing over 30 g/L ethanol. Although the discrepancy between the tolerance to added ethanol and the maximum concentrations produced might be explained by a differential tolerance to added and produced ethanol, such a differential tolerance is not thought to be operative in other organisms (38,42,58,119) and has also been discounted based on theoretical grounds (76,119). Thus it would appear unlikely that the gap between the tolerance of *C. thermosaccharolyticum* to exogenously-added ethanol and the maximum reported concentrations of endogenously-produced ethanol is attributable to inherent biochemical limitations related to ethanol.

If ethanol is not the limiting factor in efforts to produce high ethanol concentrations with thermophiles, what is? As perhaps first observed by Monod (140), growth of microbial cultures generally ceases due to either exhaustion of a required nutrient, accumulation of an inhibitory metabolic product, or changes in the concentration of protons or other ions in the medium. In order to distinguish product inhibition from other growth-limiting factors, it is necessary to perform diagnostic experiments such as showing that growth continues when product is removed beyond that achieved in the presence of product, or that addition of product at low cell and substrate concentration is sufficient to reproduce limitation observed at high cell and substrate concentrations. For the most part, studies of ethanol production using thermophilic bacteria have not involved such diagnostic experiments. Moreover, in every case known to us where such diagnostic experiments have been performed, *it could not be demonstrated that ethanol was the factor limiting growth*. A case in point are the results from the previous grant (Section A), in which the inability of *C. thermosaccharolyticum* to utilize feed xylose concentrations > 75 g/L was clearly shown to be due to inhibition by KOH used for pH control and not due to inhibition by ethanol although the concentration of produced ethanol was 23 g/L.

Ethanol tolerance has long been thought to be a key limitation to using thermophiles for ethanol production in a practical context (27,123). However, our recent data provide significant reason to believe that this is not the case. To definitively establish the adequacy of thermophilic ethanol tolerance, it will be necessary to produce ethanol at the concentrations corresponding to the tolerance inferred from experiments involving exogenous added ethanol. Such realization of the ethanol production potential of thermophiles is an important goal of this proposal.

**4. End-Product Metabolism.** Ethanol-producing thermophilic bacteria are thought to ferment hexoses via the Emden-Meyerhoff-Parnas pathway, with the pentose phosphate pathway also utilized for C5 sugars (109,184). Although some interspecies variation exists, these organisms have in common an end-product metabolism consisting of a set of metabolic branches resulting in production of ethanol, acetic acid and lactic acid. Pathways and intermediates are presented in Fig. 2 from carbon-centered and electron-centered perspectives.

It is possible for thermophiles and other organisms with metabolism such as that shown in Fig. 2 to produce ethanol, acetic acid, or lactic acid as the sole organic fermentation product. For each of these products, balanced equations may be written that meet the basic requirements of fermentative metabolism: net production of ATP and regeneration of electron carriers (191). The possibility of a homoethanol fermentation results from the activity of ferredoxin-NAD oxidoreductase (Fig. 2), which allows electrons carried by reduced ferredoxin resulting from the action of pyruvate dehydrogenase to be transferred to NAD and subsequently to ethanol. Very high ethanol selectivities have been observed a sufficient number of times (16,29,116,117,202,203) to offer empirical confirmation that thermophiles can grow with ethanol as the only significant organic fermentation product.

As shown in Fig. 2, enzymes specific to the production of acetic acid include phosphotransacetylase (Pta) and acetate kinase (Ack), while lactic acid production requires the

activity of lactate dehydrogenase (Ldh). Key enzymes involved in production of acetylCoA (precursor for acetate and ethanol) include pyruvate dehydrogenase, hydrogenase, and ferredoxin-NAD oxidoreductase. Table 2 presents a summary of information for selected enzymes/genes. It may be noted that both yeast and *Zymomonas* do not produce ethanol via pyruvate dehydrogenase-mediated production of acetylCoA, but rather by pyruvate decarboxylase-mediated production of acetaldehyde. There is a general tendency of enzymes associated with formation of fermentation products to be present in single copies on the bacterial chromosome, as in the case of *pdc* and acetaldehyde dehydrogenase in *Z. mobilis* (6); *ldh*, *ack*, *pta*, and *pdc* from *E. coli* (99); and, based on the preliminary gene map of Wilkinson et al. (209), *ldh* and most other described enzymes associated with solvent formation in *C. acetobutylicum*. There are exceptions to the rule of single-copy genes, as in the case of *hyd* in many organisms (e.g. *E. coli* (99), *M. thermoautotrophicum* (5)), alcohol dehydrogenase in several organisms (6,24,32), and thiolase in *C. acetobutylicum* (211B, at least some strains).

Mutant strains of *E. coli* deficient in Pta, Ack, and Ldh have been obtained by classical techniques. For example, LeVine et al. (114) used monofluoroacetate to select for Pta-minus and Ack-minus mutants; mutants in these two enzymes were distinguished based on differential sensitivity to alizarin yellow. An Ldh-minus strain of *E. coli* was developed by Mat-Jan et al. (133) using chemically mutagenized pyruvate formate lyase-deficient *E. coli* and screening for lack of anaerobic growth on glucose plus acetate. In work during the previous grant period with both the strains developed by Levine et al. and that of Mat-Jan et al., we observed reversion to a non-mutant phenotype. The suitability of classical techniques in the context of the goals of this proposal is addressed in III.

**5. Heterologous Expression and Gene Transfer with *Clostridium*.** Several dozen genes have been cloned, generally in *E. coli*, from clostridia such as *C. acetobutylicum* and *C. pasteurianum* (48,172,212). These include most of the genes associated with formation of fermentation products (162). As a result, a significant body of information on gene expression and regulation is emerging for the mesophilic clostridia. Such information is available to a more limited but increasing extent for thermophilic clostridia, largely as a result of the interest in the *C. thermocellum* cellulosome (14,52). Because over a dozen genes encoding cellulase components have been cloned and sequenced, codon usage in this organism can readily be inferred. To our knowledge, no genes associated with formation of fermentation products in thermophilic ethanol-producing clostridia have been cloned.

Although heterologous expression of *Clostridium* genes in *E. coli* is becoming commonplace, the introduction and expression of genes in *Clostridium* is in a nascent stage of development. Other than our recent report of adenine methylase from *Enterococcus faecalis* expressed in *C. thermosaccharolyticum* (Section A), we know of no other reports of a foreign gene expressed in either of our target organisms: *C. thermocellum* or *C. thermosaccharolyticum*. Transformation of mesophilic clostridia has been approached via electroporation (3,88,157,218), chemical treatment (19,63,79,115,195,219), and conjugal mating (156,158). Electroporation is becoming increasingly standard in light of: 1) minimal difficulty with extracellular nucleases due to the brief incubation times involved; 2) no requirement for protoplast formation such as exists for transformation by techniques other than electroporation in most if not all clostridia (79,115); and 3) higher transfer efficiencies than conjugation (3,156,158). Neither *C. thermosaccharolyticum* or *C. thermocellum* had been transformed prior to our recent work (Section A). Restriction endonucleases have been shown to be significant barriers to transformation in some *Clostridium* strains (e.g. *C. acetobutylicum* ATCC 824 (136,137)). In our previous work (90), we showed that the restriction endonuclease system of *C. thermocellum* ATCC 27409 recognizes the sequence 5'-GATC-3', and that foreign DNA can be protected by using a host with the dam methylation system. We found no evidence of other restriction sequences recognized by this strain. *C. thermosaccharolyticum* ATCC 31960 does not exhibit restriction endonuclease activity.

**6. Pathway Engineering in Anaerobes.** Pathway (or metabolic) engineering is an emerging subdiscipline of biochemical engineering that has outstanding potential for future application (10,26,189). An increasing number of examples may be found in the literature, with a subset of these involving anaerobes (10). One focus of metabolic engineering involving

anaerobes includes redirection of end product metabolism as in enhanced ethanol production in *E. coli* (4,192) and *Klebsiella oxytoca* (44,151), over-production of solvent-producing genes in *C. acetobutylicum* (136,137), and enhanced production of 1,3 propanediol by *Klebsiella pneumoniae* (193). A second common focus is to broaden the range of substrates utilized, for example enabling *Zymomonas* (222) or *Saccharomyces* (68,69) to utilize xylose, and expressing a cellulase component in *C. acetobutylicum* (87). A major effort has been directed toward cloning catabolic genes and developing other requisite tools for pathway engineering in *C. acetobutylicum* (48,212). We have benefited considerably from information and investigators associated with this work - at times in close consultation with the groups of Papoutsakis (Northwestern), Bennett (Rice), and Young (Aberystwyth, Wales) - and expect this to continue.

**7. Homologous Recombination & Gene Knock-Out.** Homologous recombination is the basis for "gene knockout", a pathway engineering strategy in which a functional gene is replaced by a deleted and thus non-functional gene. This strategy has the advantages of being highly specific for the target gene, as well as being virtually irreversible as long as sufficiently large (e.g. several tens of base pairs) deletions are used. General considerations and mechanisms are reviewed elsewhere (45,47,220), with only selected aspects considered below. DNA exchange via homologous recombination has recently been reported in mesophilic clostridia (209; G. Bennett, personal communication).

Of particular interest here are genetic crossover events having homology-conferred specificity that result in insertion into the chromosome of an extrachromosomal DNA construct containing a deleted gene. Typically, the deleted gene is introduced on a nonreplicative plasmid, and selection for plasmid-borne markers is used to isolate cells that have integrated the construct into the chromosome (see Section IV.3b). As shown in Fig. 3, a single "Campbell-type" crossover involving a circular chromosome and a circular plasmid results in duplication of homologous DNA with intervening portions of plasmid DNA. Also illustrated in Fig. 3 is a double crossover event, in which the plasmid is excised and a portion of the target gene is now deleted from the chromosome. Finally, Fig. 3 demonstrates that the same end-point can be reached through a single crossover followed by intrachromosomal recombination. Whether they occur by two simultaneous events or by a single crossover followed by intrachromosomal recombination, all cells containing double-crossovers that express the antibiotic resistance marker R1 (Figure 3) are expected to have a non-functional copy of the target gene. Since the excision event associated with intrachromosomal recombination is effectively irreversible for a non-replicative plasmid, over time the entire population is expected to excise the plasmid DNA. Consistent with this expectation, progressive loss of repeated sequences resulting from "Campbell-type" single crossovers has been reported in the literature (45,59,111,164,187).

Frequencies of events associated with homologous recombination are presented in Table 3. As shown therein, plasmids with one or more homologous regions integrate into the chromosome with a reasonably high (typically  $> 10^{-3}$ ) frequency. Of particular note in the present context, Wilkinson et al. (209) report a homologous recombination frequency of  $\sim 10^{-2}$  for *C. beijerinckii*. A homologous sequence of  $> 0.5$  kb is sufficient to obtain significant integration (138,211,214). The data in Table 3 as well as the direct comparison of Pozzi and Guild (165) suggest that the integration frequency is not strongly affected by the presence of heterologous inserts of several kb. As presented in Table 3, single and double cross-over events generally have frequencies of the same order of magnitude, and intrachromosomal frequencies are sufficiently high to allow populations to shift toward a predominantly plasmid-excised genotype at rates compatible with practical exploitation. The fraction of double crossovers is generally quite high in the studies cited in Table 3, although we have observed somewhat lower (e.g.  $5 \times 10^{-2}$  to  $\leq 10^{-3}$ ) values in work with *Bradyrhizobium japonicum* (LeVier and Guerinot, unpublished).

### III. RESEARCH OBJECTIVES AND STRATEGIES

The premises of the proposed project, supported in the previous sections, are as follows:

- Biomass represents one of the most promising resources available to humankind as we seek to transform industrialized society to a reliance on sustainable sources of energy and materials.
- Consolidated bioprocessing (CBP) is a promising and perhaps under-studied strategy to develop the very low cost processes required for production of commodity products from biomass.
- Cellulosic ethanol is a transportation fuel with many desirable features and associated technology provides a model system for demonstrating the efficacy of the CBP strategy.
- Thermophilic bacteria are interesting and potentially significant biocatalysts generally, possess to an unusual extent the properties required for production of ethanol from cellulosic materials via CBP, and are also significant as a model system for CBP biocatalysts.
- The selectivity of thermophilic bacteria is too low for currently available strains to be useful in practical processes because of the production of organic acids (lactic and acetic)
- Recent data suggest that thermophiles are tolerant to high concentrations of added ethanol and may well have the capacity to produce high ethanol concentrations, although the latter has not yet been definitively established.

The following goals, based on the above premises, motivate the proposed project:

- 1) Develop and characterize strains of *C. thermocellum* and *C. thermosaccharolyticum* having ethanol selectivity similar to that of more conventional ethanol-producing organisms.
- 2) Realize and characterize the ethanol production potential of thermophiles.

Three-quarters of the proposed workplan (Section IV) is devoted to obtaining high selectivity strains, corresponding to Goal 1. Pursuant strategic elements include reliance primarily on gene knockout rather than overexpression, use of molecular tools to improve selectivity rather than classical techniques, identification of target genes and approaches for selectivity enhancement, and consideration of alternative routes for creating organisms upon which CBP processes can be based. These elements are considered respectively in the following paragraphs.

In general, altering flux in a branched pathway could be approached by either inactivating one or more enzymes in undesirable branches ("gene knockout") or by overexpressing enzymes in desired branches. Strategies to achieve overexpression include increasing gene copy number and increasing promotor strength. The proposed workplan relies on the gene knockout approach for two reasons. First, instability due to spontaneous mutations would appear to be a more significant problem for the overexpression strategy than for deletion strategy reliant on genetic deletions, as deleted strains should be stable indefinitely. For a strategy reliant on overexpression, spontaneous mutation of either promotor or structural genes can result in reduction of an overexpressed enzyme. In the common situation where the overexpression phenotype is of negative selective value, this phenotype will disappear from the population over time. Such instability has been observed for a genetically engineered *E. coli* strain which exhibits high ethanol selectivity as a result of overexpression of *Zymomonas* genes (107). The stability issue is particularly important for a commodity product such as ethanol, where the small manufacturing cost margin provides great incentive for continuous processing over long time periods and also makes impractical many approaches for maintaining a phenotype of negative selective value (122). The second reason we favor a deletion-based strategy is that it does not require an expression system in the target organism, and thus can be applied to organisms such as *Clostridium* for which limited information is available at the genetic level.

In addition to the molecular alternatives discussed above, classical techniques involving mutagenesis and selection are a potential route to increasing selectivity. Our choice to pursue molecular approaches is supported by the general lack of success and chronic instability observed for mutant thermophilic strains in which a product-minus phenotype was selected using classical techniques (Section II.2). Further, molecular approaches for selectivity enhancement are expected to have little if any effect on the chromosome at loci other than that of the target gene. This is a

second advantage relative to classical techniques, since classical techniques typically involve non-specific mutagenesis resulting in many mutations (generally deleterious) at sites other than the target genes. Finally, molecular approaches are seen as more widely applicable than classical techniques. Classical techniques are difficult to employ when a selection method is not available for the desired phenotype; the molecular approaches described herein are not subject to this limitation because direct selection for the desired phenotype is not involved.

As *Ldh* is the only enzyme specific to the formation of lactic acid (Figure 2), the *ldh* gene is the appropriate target to block lactic acid production using gene knockout. Likewise, the gene encoding acetate kinase, *ack*, and phosphotransacetylase, *pta*, are obvious candidates to block acetate production. Although blocking carbon-flux directly may be the most intuitive way to alter selectivity, blocking electron flux has the potential to be equally effective. In the case of hydrogenase, it is not possible to produce acetate with regeneration of oxidized ferredoxin unless hydrogen is produced. Knocking out hydrogenase is thus by itself a potential means to eliminate acetate formation, and is also a means to augment strains deleted in *ack* or *pta*. In addition to *ldh*, *ack*, *pta*, and *hyd*, we also intend to direct our cloning and gene-knockout efforts toward a fifth gene: pyruvate dehydrogenase, *pdh*. Our interest in *pdh* is two-fold. First, a cellulolytic derivative of *C. thermocellum* that produced lactic acid as the sole fermentation product would be interesting from an applied perspective and would demonstrate the potential breadth of the CBP approach. No cellulolytic homolactic acid fermenter has been described, and lactic acid is of considerable interest for production of biodegradable plastics (39). Second, a *Pdh*<sup>-</sup> strain could be a useful step in deriving a strain with high ethanol selectivity via a somewhat less direct strategy than those already described: 1) knockout *pdh*, resulting in a strain producing lactate as the sole end-product; 2) introduce *pdc*, e.g. from *Zymomonas*, on a replicative plasmid; and 3) knockout *ldh* and perhaps also acetaldehyde dehydrogenase\*. This strategy is intriguing because it would appear to avoid the possibility of acetate or hydrogen formation, mimic the biochemistry of the best known ethanol producers, and be unlikely to have stability problems since *Pdc* would be essential for survival. At the same time, a strategy involving substituting *Pdc* for *Pdh* is somewhat more speculative than the above-described approaches. Thus we envision pursuing this strategy only through step 1 during the proposed project, unless other alternatives are tried without success. The resultant *Pdh*<sup>-</sup> strain could be an important point of departure for subsequent studies targeting production of either ethanol or lactic acid.

There are two general strategies for creation of organisms upon which CBP processes can be based: 1) begin with an organism having excellent product production properties and confer (or improve) desired substrate utilization properties; 2) begin with an organism having excellent substrate utilization properties and improve (or confer) desired product production properties. An example of strategy 1 would be to introduce the capacity to express and excrete a functional cellulase system into yeast or *Zymomonas*. Improving the ethanol selectivity of thermophilic bacteria, the focus of this proposal, is an example of strategy 2. While each strategy has points in its favor, both are worthy of pursuit in our view, especially given the overall potential of the CBP concept. It may well be that the most advantageous of these two strategies will ultimately prove dependent upon the particular product considered.

Goal 2, realization and characterization of the ethanol production potential of thermophiles, corresponds to documentation of the maximum concentration of ethanol that can be produced by thermophiles with confirmation of ethanol limitation by diagnostic experiments (Section A). As presented in Section II.3, all studies known to us that systematically investigated the cause of thermophilic growth limitation at high concentrations of produced ethanol have found factors other than ethanol to be responsible. If such inhibiting or limiting factors can be identified and alleviated, it is reasonable to expect that ethanol can be produced at higher concentrations than reported to date for thermophiles. In particular, it is quite possible that thermophiles will be able to produce ethanol at concentrations corresponding to the tolerance they exhibit with respect to

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\* A strain deleted in *pdh* and acetaldehyde dehydrogenase might well require acetate to synthesize acetylCoA for biosynthetic purposes. However, acetate could easily be supplied in laboratory medium, and is likely to be present in hydrolysates resulting from biomass pretreatment.

added ethanol. Confirmation by diagnostic experiments is necessary because various forms of growth limitation can easily be mistaken for ethanol limitation.

#### IV. WORKPLAN.

The workplan consists of four tasks: 1) optimization of electrotransformation; 2) cloning catabolic genes, 3) generation of modified thermophilic strains, and 4) fermentation studies dedicated to realization of the ethanol production potential of thermophiles and characterization of modified strains. We plan to proceed in an integrated fashion, with personnel from the three P.I.'s labs collaborating on all tasks. Task 1 is to be led by Lynd's lab, in which the electroporation protocols for thermophiles were devised (90b), with support from Guerinot's lab, which developed the first electroporation protocol for *Bradyrhizobium japonicum* (57B) and collaborated on our development of an electroporation protocol for *C. thermosaccharolyticum*. Task 2 is to be jointly undertaken by all three labs. Demain's group has considerable experience cloning genes from *C. thermocellum* in *E. coli*, including 7 distinct cellulase components (81,92,93,169,170), Guerinot's group has experience with the cloning techniques we propose to use (28,49,56,57,179,217), and Lynd's group has used PCR to synthesize fragments of genes coding for catabolic enzymes in thermophiles (Section A). Preparation of plasmid constructs, the first part of Task 3, will be led by Guerinot's lab, which has successfully generated gene knockout constructs in *B. japonicum* using techniques similar to those proposed here (57, unpublished). Introduction of constructs into thermophiles will be done in Lynd's lab. Finally, fermentation studies will be carried out in Lynd's lab with support from Demain's lab, both of which have extensive experience involving continuous culture and industrial microbiology of thermophilic ethanol producing bacteria. A work schedule is presented in Table 4.

Both *C. thermocellum* and *C. thermosaccharolyticum* will be investigated throughout, as a combination of these or similar organisms is necessary in order to have a thermophilic system capable of processing both hexose and pentose sugars (Section II.1). Furthermore, the utilization of two organisms will provide two different systems in which various techniques and approaches might succeed, as well as opportunities to transfer success from one system to the other. We propose to use *C. thermocellum* ATCC 27405 and *C. thermosaccharolyticum* ATCC 31960, as we have experience with these strains and they appear to have properties competitive with other available strains. Over the years, our labs have been active in developing and refining growth medium for thermophiles (13,77,199, Section A), as well as continuous cultivation of thermophiles (12,67,125,128). The Lynd lab has developed four generations of solids-delivery devices for continuous culture on solid substrates (128,186 & unpublished), with the most recently developed system capable of delivering paper sludge.

**Task 1. Optimization of Electrotransformation.** As described Section A, we have clearly demonstrated transformation of *C. thermosaccharolyticum* and have also obtained presumptive evidence that we have successfully transformed *C. thermocellum*. However, for both organisms, transformation frequencies appear to be quite low. Using plasmid pCTC1 and the conditions described (Section A) we reproducibly obtained 0.5 to 50 transformants/ $\mu$ g DNA (depending on the DNA source), and frequencies on the order of  $3 \times 10^{-9}$  or less transformants/viable cell. These values correspond to about one transformant per electroporation event, and may be compared to  $1.7 \times 10^5$  transformants/ $\mu$ g for *C. acetobutylicum* transformed with pFNK3 (137). It may not be strictly necessary to increase the frequency of transformation in order to implement the gene knockout-based strategies we propose, because even low frequency transformation with a replicative plasmid containing a deleted copy of a target gene will provide ample opportunity for homologous recombination to occur. However, it would be convenient to increase transformation frequencies, and it is reasonable to expect such an effort to be successful. Finally, developing more efficient transformation techniques would represent a general contribution to the molecular "toolbox" available for manipulation of thermophiles.

General approaches to increasing the transformation frequency include manipulation of electroporation conditions and use of alternative plasmids. We propose to examine both. In our previous work we have found that the state of the cells (fresh or frozen) and incubation temperature (45 or 60°C) had little impact on the transformation frequency of *C.*

*thermosaccharolyticum*. Buffer composition had an absolute impact, however, as we were unable to obtain any thermophilic transformants (although controls were positive) with the sucrose-phosphate buffer often used for transformation of mesophilic clostridia (3,17,137,163). In addition, both Southern blots and retransformation experiments indicated that the plasmid was replicated independently of the chromosome in this organism (Section A). Similar experiments will be undertaken in the course of Task 1 with *C. thermocellum*. In addition, we will measure both transformation frequency and viability in relation to field strength (up to the limit at which arcing occurs), for transformation of both *C. thermosaccharolyticum* and *C. thermocellum* using pCTC1. Finally, we will consider the impact of variables such as cell age, the DNA/cell ratio, preincubation with DNA, the length of the post-electroporation recovery period, and buffer composition. Conditions will be identified that give maximum transformation frequency.

Once we have optimized electrotransformation with pCTC1, we will then turn our attention to alternative plasmids. We envision testing on the order of a dozen different plasmids developed as shuttle vectors between Gram positive and Gram negative organisms, chosen from an ever-growing list (e.g. (15,17,20,60,84,86,88,94,129,137,139)). The group of plasmids selected for testing will be chosen to represent a diversity of features, with particular emphasis on including plasmids that replicate with both single-stranded and theta replicons, and plasmids that originate from gram positive organisms other than *Clostridium* (pCTC1, with which we have had success to date, is in this category) as well as plasmids originating from *Clostridium* - ideally including thermophiles. We have assembled a collection of 8 plasmids of potential interest in this context, have identified several more plasmids we would like to obtain, and are in touch with several labs that are actively developing additional plasmids. By testing such a diversity of plasmids, we will both maximize the likelihood that we will obtain one or more plasmids compatible with high frequency transformation, and we will also put ourselves in a position to deduce which features are associated with such high frequencies. We will produce plasmids in restriction-compatible host cells, and will use optimal electroporation conditions determined using pCTC1; conditions will not in general be reoptimized for each plasmid as this would be too time consuming.

**Task 2. Cloning of Catabolic Genes.** As discussed in Section III, we have an interest in cloning the catabolic genes *ldh*, *pta* *ack*, *hyd*, and *pdh*. Our preferred approach to cloning involves PCR amplification of a target gene fragment which is then used to screen a genomic library. We will definitely pursue this approach for the *ack* and *hyd* genes, for which we already have PCR fragments. Once genomic library inserts containing the *ack* gene (identified by library screening) are recovered, we will examine sequences adjacent to the *ack* gene to see if they also contain *pta*. This possibility is consistent with *ack* and *pta* being adjacent in several organisms (82,181,197). We are actively working to amplify PCR fragments from *ldh* and *pdc*. If such fragments are obtained, we will pursue a PCR-based cloning approach for these genes also. In the perhaps unlikely event that we are not successful in obtaining *ldh* and *pdc* PCR fragments, we will investigate alternatives such as: 1) complementation (transformation of a gene library into a host deficient in the target gene under consideration), 2) heterologous probing (use of available genes or gene sequences as probes to screen genomic libraries), and 3) protein isolation followed by microsequencing, synthesis of corresponding DNA fragments based on codon usage, and use of DNA fragments either as PCR primers or heterologous probes. In the present context, we rank these alternatives in the order indicated.

Obtaining PCR fragments for *ldh* and *pdc* will involve the steps we have applied successfully for *ack* and *hyd*. Primer sequences of 20 to 25 bp will be selected that exhibit a highly conserved amino acid sequence among different species, while also avoiding particularly degenerate codons (with due consideration to codon usage). Typically, 3 different primers will be synthesized for each target gene to maximize our chance of success. PCR conditions will be optimized for each primer pair, resultant products will be sequenced, and the deduced amino acid sequences will be compared to previously-reported sequences. Because of the high degree of amino acid sequence conservation among various members of the gene families in which we are interested, we will rely on sequence similarity to tell us whether we have indeed amplified the correct genes. After verifying by Southern blot that the PCR products are amplified from *Clostridium*, we will clone the PCR fragments to ensure permanent stocks of each fragment. We

will then use our cloned PCR products to prepare probes for screening of *C. thermocellum* and *C. thermosaccharolyticum* genomic libraries. We have made 4 such libraries in the course of previous work, and can prepare additional libraries or obtain them from colleagues as needed. The size range of the ligated fragments (4 to 10 kb) is sufficient to code for one or more proteins with a combined molecular weight in the range of 148,000 to 370,000. Given the reported molecular weights of the proteins encoded by our target genes (Table 2), this insert size should ensure that we have a good chance of recovering intact genes or even operons.

Positive clones from genomic library screening will be further characterized as follows: Enzyme activity assays will be conducted to determine if the enzyme is functionally expressed and whether positive clones have elevated activity. Such activity would be convenient, in that it would allow us to readily confirm that deletions render the gene inactive (Task 3), but is not strictly necessary in order to pursue a gene knockout strategy. A restriction map of the insert region will be generated, and will be compared to that generated from probing Southern blots of genomic DNA to make sure that the insert region is colinear with that of the genome (i.e. that no rearrangements have occurred). Based on the DNA hybridization data, appropriate fragments will be sub-cloned and sequenced. The amino acid sequences encoded by the structural gene(s) will be aligned with those encoded by other known genes. From restriction mapping and DNA sequencing data, we should also be able to determine whether any of our genes are arranged in operons. Northern blot analysis will be used to determine the size of the transcript. The 5' end of the gene(s) will be mapped using S1 protection and/or primer extension analysis (7). Once the start of transcription has been determined, we will be in a position to construct both transcriptional and translational fusions for use in gene expression studies. Such fusions will be valuable tools generally, and will allow us to monitor expression of one gene in response to the deletion of another gene. For example, if we knock out *ack*, does *ldh* expression change? The Guerinot lab has considerable experience with gene fusion technology (159,160).

**Task 3. Generation of modified thermophilic strains.** This task involves two distinct phases: preparation of deleted constructs and introduction of constructs into thermophiles. Both phases are much simplified by our elucidation of restriction endonuclease systems operative in *C. thermocellum* and *C. thermosaccharolyticum* and the knowledge that use of a *Dam*<sup>+</sup> *E. coli* host for propagation of plasmid constructs avoids restriction endonuclease attack in *C. thermocellum* (Section A).

*A. Preparation of deleted constructs.* The overall goal of this subtask is to alter target gene inserts so that: 1) target genes are inactivated by a deletion of  $\geq 100$  bp; 2) deletions are flanked by homologous regions on the order of 1 to 2 kb.

After restriction mapping of inserts, deletions  $\geq 100$  bases will be generated at various locations using either pairs of conveniently-located restriction sites or by exonuclease III digestion from a single compatible restriction site. An *Em*<sup>r</sup> marker originating from pAM $\beta$ 1, shown to be effective in our transformation work (Section A), will be inserted at or near the site of deletion within the insert. A second antibiotic resistance marker will be inserted exterior to the insert. Based on the sensitivity of *C. thermosaccharolyticum* to thiamphenicol (Section A), we would expect at this point to use the chloramphenicol acetyl transferase (*cat*) gene of either *Staphylococcus aureus* (168) or *Clostridium difficile* (213). New information could of course suggest alternative antibiotics and/or resistance markers. Internally deleted DNA inserts will be reintroduced into *E. coli* on a plasmid, with the choice of plasmid (e.g. replicative or non-replicative in thermophiles) influenced by considerations described below (Task 3.B). Antibiotic-resistant transformants will be tested for the presence of the *C. thermocellum* insert, and when appropriate eliminated target enzyme activity.

At this point, we anticipate having a circular construct suitable for use in Task 3B. As presented at the top of Fig. 3, the construct includes: 1) a segment of *C. thermocellum* DNA containing an internally-deleted and non-functional target gene; 2) an *Em*<sup>r</sup> marker located at or near the deletion site; and 3) a second marker effective in conferring antibiotic resistance to thermophiles, presumptively *cat*, associated with vector sequences.

*B. Introduction of deleted constructs into thermophiles.* Using optimized procedures (Task 1) and DNA prepared from a restriction-compatible host strain, the deleted constructs generated in Task 3A will be introduced by electroporation into *C. thermocellum* and *C. thermosaccharolyticum*. Several strategies for detecting various types of recombinants are possible, with the preferred strategy depending on both the efficiency of electroporation, the frequency of plasmid integration, and the fraction of integrants having double crossovers.

We would prefer to use non-replicative plasmids for introduction of deleted constructs into thermophiles, because they allow an unambiguous selection of organisms with plasmid DNA integrated into the chromosome. Transformants with the construct incorporated into the genome will be selected based on resistance to antibiotic 1 ("R1", Figure 3). The next step is then to distinguish among R1 integrants those that have a single crossover event, expected to have a functional gene, and those that have the desired double crossover event, expected to have only a deleted non-functional gene (see Figure 3). Cells with single crossovers should be resistant to both antibiotic 1 and antibiotic 2 (R1/R2) whereas cells with double crossovers should have a R1/S2 phenotype. Thus screening based on antibiotic resistance is a possibility, and reported homologous recombination frequencies (Table 3) support the feasibility of this strategy. Presumptive double crossovers will be confirmed via Southern blotting. If the fraction of double crossovers proves much lower than indicated in Table 3, as our experience indicates can occur in some cases (K. LeVier and M. Guerinot, unpublished), we will investigate positive selection for double crossovers based on use of a vector which will allow us to select against the retention of vector sequences in the host genome. A number of such positive selection, allelic exchange vectors have now been constructed which permit genetic modification of a variety of gram negative and gram positive organisms (18,43,182,183), and their use is under active study in the Guerinot lab.

Because cells with plasmid DNA integrated into the chromosome (whether via double or single crossovers) will only be a fraction of cells into which an intact plasmid is successfully delivered, the use of non-replicative plasmids requires that the electroporation efficiency be reasonably high. In the event that only very low electroporation efficiencies are obtained, we may choose to use a replicative plasmid for introducing deleted genes (e.g. pCTC1 in *C. thermosaccharolyticum* or a plasmid identified in Task 1B). Use of a replicative plasmid has the advantage that even if the transformation frequency is very low, once transformed the deleted plasmid is available to participate in homologous cross-over in many billions of cells. The disadvantage of replicative plasmids in this context is that an R1 phenotype does not necessarily indicate that plasmid integration has occurred. If replicative plasmids are used, it is likely that we will investigate plasmid curing, either by maintenance in the absence of antibiotic selection for a time or by other methods. Most if not all known replicative plasmids in *Clostridium* are somewhat unstable, and are lost in the absence of selective pressure (139,219). Thus it may not be difficult to obtain a culture enriched in cells that have integrated plasmid DNA and do not have autonomously-replicating plasmids. When such a culture is returned to antibiotic selection, only integrants should survive. Distinction of single and double crossovers would then proceed as described in the preceding paragraph.

Colonies of *C. thermocellum* in which a double-crossover event has been identified will be screened for inability to produce products (either acetate or lactate) in batch culture. The likelihood that the above-described approach will result in the desired strains with little or no production of organic end-products is enhanced by the tendency of genes associated with formation of fermentation products to be present in single copies, at least in other bacteria (Section II.4). If we do encounter residual production of targeted fermentation products in strains in which a double crossover has been confirmed, we will look for evidence of multiple gene copies using Southern blots. A variety of approaches could be pursued to knockout a redundant gene copy, such as using high antibiotic concentrations to select for multiple integrants (59), use of single-stranded (expected to be particularly recombinogenic (75)) plasmids, and repeating the procedure with different markers.

**Task 4. Fermentation Studies.** This phase of the project will be oriented around two goals. Task 4A is dedicated to realization of the ethanol producing potential of thermophiles. Task 4B involves characterization of modified strains.

*A. Realization of ethanol production potential.* As developed in previous sections, there is substantial uncertainty with respect to the maximum concentrations of ethanol that thermophiles are capable of producing, ( $E_{max}$ ). Furthermore, there is reason to expect that thermophiles may be able to produce higher concentrations than have been hitherto documented. To resolve the uncertainty over  $E_{max}$ , it is necessary to demonstrate limitation of a thermophilic culture by the ethanol it produces. All attempts by the P.I., and perhaps others, to demonstrate such limitation have been stymied by either nutritional deficiency or inhibition due to neutralizing agents. We will proceed by using continuous cultures, which our results suggest are more conducive to utilization of high substrate concentrations than batch cultures. We will work first with xylose-grown monocultures of *C. thermosaccharolyticum*, with which we have already made much progress, and will proceed to co-cultures of *C. thermocellum* and *C. thermosaccharolyticum* grown on paper sludge. The former system is easier to work with, and is thus suitable for tackling generic issues (for example involving medium formulation or avoidance of inhibition by neutralizing agents). The latter is more challenging to work with because it involves solid substrates, but it represents a more complete embodiment of the CBP strategy.

For both systems, we intend to increase the feed substrate concentration,  $S_0$ , to progressively higher feed substrate concentrations. Eventually, evidence of limitation will be encountered, as indicated by failure to realize a steady state with complete substrate utilization when the culture is subjected to a step-wise increase in  $S_0$ . When this occurs, we will perform diagnostic experiments to determine the most likely cause of limitation. Typically this will involve a repeat of the step-wise  $S_0$  increase in the presence of factors that might either exacerbate or alleviate the limitation, for example increased concentrations of one or more nutrients, products, or salts produced by neutralizing agents. Recently we have used this procedure to determine that continuous culture of *C. thermosaccharolyticum* at a feed xylose concentration of 75 g/L (and an ethanol concentration of 25 g/L) is limited by potassium ion resulting from neutralization with KOH and that ethanol at this concentration plays little or no role in inhibiting the culture (Section A).

In light of our finding that cations introduced in the course of neutralization are inhibitory, an immediate challenge is to circumvent this inhibition. Since our results show that even large amounts of added buffer (e.g. 100 g/L MOPS) have a minor impact on base demand, use of alternative neutralizing agents would appear to be preferred rather than use of alternative buffers. Our results suggest that NaOH is no better, and perhaps worse, relative to KOH. Furthermore, the organism is rather sensitive to high ammonia concentrations, so this would not appear to be an attractive base either. However, we have performed very promising experiments using  $Ca(OH)_2$  slurries. To date,  $Ca(OH)_2$  has been found to be an effective neutralizing agent in continuous *C. thermosaccharolyticum* cultures with feed xylose concentrations up to 50 g/L; higher concentrations have yet to be attempted. We see  $Ca$  inhibition as very unlikely because its concentration is sharply limited by the solubility of  $CaCO_3$ , which forms as a precipitate in the fermentor but does not appear to pose operational problems. We intend to investigate use of  $Ca(OH)_2$  as a neutralizing agent, both alone and in conjunction with lesser amounts of other bases (KOH, NaOH,  $Mg(OH)_2$ ). Use of a mixture of bases is attractive because of reports that mixtures of cations are less inhibitory than single cations (2,95,185), and because some mixture components are soluble thus reducing the quantity of precipitates involved for a given degree of neutralization. Ultimately, the solution to the neutralization problem is to eliminate formation of organic acids, the goal of Tasks 1 through 3. The incentive to solve the neutralization problem is that this provides a direct path to characterizing the ethanol production potential of thermophiles.

Successful completion of this subtask will involve characterization of the maximum ethanol concentrations that can be produced by both *C. thermosaccharolyticum* monocultures as well as *C. thermocellum/C. thermosaccharolyticum* cocultures. Based on our recent results and our understanding of the mechanisms of ethanol inhibition, we think it distinctly possible that ethanol concentrations  $\geq 40$  g/L can be produced by thermophiles. Confirmation of this hypothesis would be a significant applied development. From a fundamental point of view, the matter of distinguishing product inhibition from other forms of growth limitation is of interest because it

can be subtle, because it has received relatively little attention, and because its relevance is widespread.

*B. Characterization of modified strains.* As they become available, modified strains obtained in the course of Task 3 will be characterized. We intend to focus on qualities such as long-term stability of product-minus phenotypes in continuous culture, and the ability of modified strains to utilize high substrate concentrations and to produce high concentrations of ethanol. If time permits, performance of modified strains on substrates of applied interest other than paper sludge (e.g. pretreated hydrolysates) will also be investigated. Modified strains may have unexpected features, which could have both applied and fundamental significance. The metabolic branches involved in end-product formation in thermophilic anaerobes appear to readily accommodate flux changes; that is, they are "flexible" in the terminology of Stephanopoulos and Vallino (189). However, the proposed strategy will effectively remove some (but not all) options for the cell to process catabolic intermediates and regenerate oxidized electron carriers. Under such conditions, accumulation of metabolic intermediates (e.g. pyruvate (40)) is a possibility. Such accumulation may be deleterious, and thus may be eliminated due to natural selection. For example, a strain deleted in hydrogenase could be a powerful means by which to select strains unable to synthesize acetate, since only by avoiding acetate production could such a strain prevent the depletion of oxidized NAD (see Figure 2). We routinely observe extensive and reproducible evolution in continuous culture with respect to traits such as ethanol tolerance and substrate affinity (12), and will pay particular attention to the behavior of modified strains over the prolonged time periods (e.g. hundreds of hours) possible in a chemostat. The phenomenon of evolution in part of a pathway induced by a modification in another part of the pathway would be interesting to document. Work on non-modified strains carried out in Task 4A will provide a frame of reference for evaluating modified strains.

## V. JUSTIFICATION AND SIGNIFICANCE.

Consistent with the co-submittal of this proposal to both the NSF and the DOE, the described work is motivated by a mixture of applied and fundamental goals. Most directly, the proposal targets production of ethanol from cellulosic biomass. We readily acknowledge that the potential of cellulosic ethanol has to date been anticipated to a greater extent than it has been realized. Yet ethanol is generally accepted as an excellent fuel and a versatile chemical feedstock, and we know of no challenge to the assertions that cellulosic ethanol has a decidedly favorable energy balance and a huge potential resource base. Finally, it is becoming increasingly clear that cellulosic ethanol is a leading option available for reduction of transportation sector greenhouse gas emissions. A mounting body of evidence supports the expectation of significant warming due to greenhouse gases (66), the United States has made international commitments to reduce emissions of greenhouse gases (35), and the transportation sector is the fastest-growing source of greenhouse gas emissions (35). Recently, a comprehensive analysis by a Policy Advisory Committee to President Clinton on Reducing Greenhouse Gas Emissions from Personal Vehicles found ethanol from cellulosic biomass to be one of the two largest potential contributors to reducing greenhouse gas emissions in the first quarter of the twenty first century (113,131).

The real issue is not the desirability of cellulosic ethanol, but rather the cost. The challenge of achieving the very low costs necessary to be cost-competitive with petroleum-derived products is not specific to ethanol, but is common to a whole family of renewable, biomass-derived materials. Not only is this dilemma common to high volume/low unit-value "biocommodity" products, but the solution may in many cases be common as well: consolidated bioprocessing (CBP). A sensitivity analysis conducted by the P.I. in conjunction with NREL (127) found CBP to offer a larger cost advantage than any of the several other process improvements considered. Although this analysis involved ethanol, we see no reason that the relative benefits of the CBP approach would be less for other biocommodity products. The alternative to CBP - producing cellulase in a dedicated process step as in the "SSF" strategy - may well prove to be too expensive except in niche markets. Thus we see consolidated bioprocessing as a particularly promising strategy which is generically applicable to biomass-derived products.

Biomass-derived products are of strategic importance to the country. The United States has historically been a world leader in agriculture. Although this has engendered many benefits, our ability to produce food and fiber has also resulted in a chronic problem of overcapacity that has persisted for most of this century and is predicted to worsen. As a result many see development of new, non-food uses for agricultural products as a major priority (167). Our nation also has a strong technical position in biotechnology related to production of pharmaceuticals and other high value products. Recently, there has been increasing acceptance of the notion that there also may be large opportunities in "industrial biotechnology" involving production of commodity products (132,221). We have a significant opportunity to enhance international competitiveness by building upon existing strengths in pharmaceutical biotechnology and agriculture to establish a similar leading position in industrial biotechnology.

From a more fundamental perspective, the gene knockout strategy we propose to use is applicable in principle to manipulation of any branched pathway. Examples of such pathways include amino acids, as well as antibiotics and other secondary metabolites. Compared to an over-expression strategy, the deletion strategy may be advantageous because: 1) it may be more practical to delete one activity essential to an undesired branch rather than overexpress several activities associated with a desired branch; 2) the deletion strategy does not require an expression system in the target organism, and thus can be applied to organisms such as *Clostridium* for which limited information is available at the genetic level; 3) instability due to spontaneous mutations would appear to be a more significant problem for the over-expression strategy than for elimination of undesirable enzyme activities by deletion. We note that the issue of stability is far more important for biocommodity products than for pharmaceuticals (122).

Recently, pathway (or metabolic) engineering has emerged as an important area of biotechnology (10,189,193). Although the field is reasonably advanced at the theoretical level (53,83,114b,205,206), the number of experimentally-verified case-studies is limited. Moreover, most of the available examples of metabolic engineering: 1) use host organisms for which the genetics are relatively well understood; 2) use overexpression rather than gene deletion; and 3) involve and require plasmids that replicate in the host and expression of plasmid-borne genes. As the proposed work has none of these features (with the possible exception of 3), we anticipate that it will add to the diversity as well as the number of successful metabolic engineering case studies.

The thermophilic (62,65,208) and hyperthermophilic (1,21,85,121) bacteria have been recognized for over a decade as being of interest as sources of heat-stable biocatalysts in a variety of contexts. Of particular note, a thermophilic enzyme is an integral component of polymerase chain reaction (PCR) technology (50). In light of the potential of thermophiles and the preliminary state of knowledge of the genetics of these organisms, the proposed work involving DNA transfer and recombination, gene cloning and characterization, and pathway engineering are of general interest.

Finally, we believe that the proposed work serves NSF goals in terms of development of human resources. Our three research groups will continue to work very closely together, with bi-monthly meetings, personnel exchange where appropriate, joint participation on student committees, and joint publication. The collaboration fostered by the proposed project provides a rich educational environment that combines elements of biotechnology including molecular biology, biochemical engineering, and microbial physiology. Perhaps the best indication of this is the performance over the previous grant period, in which two students (one a woman) were placed in Senior Scientist positions (Taryn Klapatch at Merck and David Hogsett at Bioenergy Inc.), and five undergraduates, including four women and an African American, were availed of significant research opportunities. The project's human resource contributions will be further expanded by the addition of Co-P.I. Mary Lou Guerinot, who is an active supervisor of both graduate and undergraduate students; many of them women.

TABLE 1. CONTINUOUS FERMENTATION OF PAPER SLUDGE BY A  
*C. THERMOCELLUM/C. THERMOSACCHAROLYTICUM* CO-CULTURE<sup>1</sup>

<u>COMPOUND</u>	<u>CONCENTRATION (G/L)</u>		<u>CONVERSION</u> <u>(% THEORETICAL)</u>
	<u>FEED</u>	<u>PRODUCT</u>	
CELLULOSE (FROM SLUDGE)	10.3	0.72	93
XYLAN (FROM SLUDGE)	2.4	0.21	91
XYLOSE (ADDED)	4.3	0.26	94
ETHANOL	-	5.32	61
ACETATE	-	1.93	

<sup>1</sup> P. VAN WALSUM, UNPUBLISHED. DATA ARE AVERAGES OF STEADY-STATE DATA POINTS, FOR GROWTH IN GBG MEDIUM (128) AT A RESIDENCE TIME OF 24 HOURS

Table 2. Properties and availability of information for target enzymes/genes.

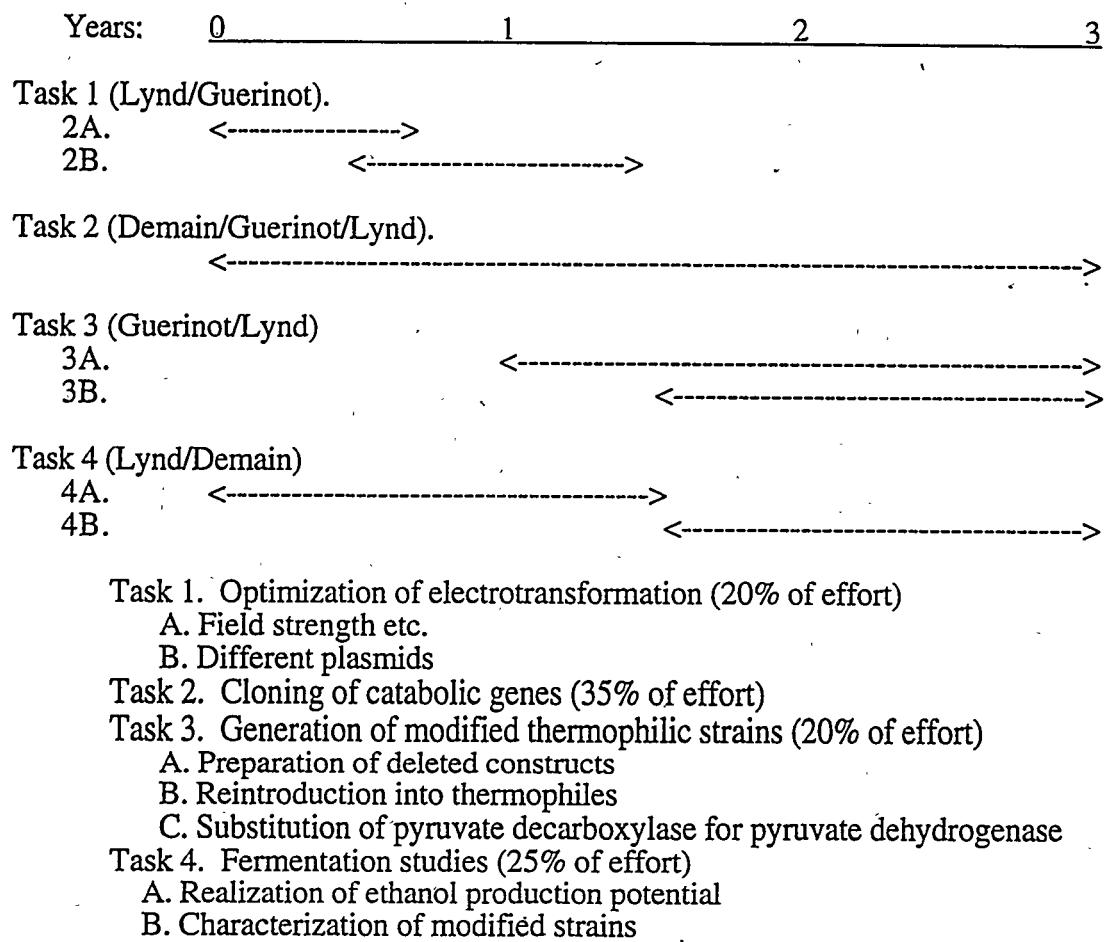
<u>Enzyme/Gene</u>	<u>Bacterial sequences (GenBank) Number</u>	<u>Size</u>	<u>E. coli</u> Strains suitable for complementation	Comments (with particular attention to <i>Clostridium</i> and thermophiles)
Acetate Kinase	3	1.2 to 1.9 kb	TA3515 (114) TA3516 (114) KH103 (82)	Monomeric protein 30 to 44 kDa, sometimes as dimer & tetramer (22,106,134,146,176). Purified, <i>Clostridium thermoaceticum</i> (176). Cloned & sequenced, <i>Methanosaerina thermophila</i> (106). Butyrate kinase cloned & sequenced, <i>C. acetobutylicum</i> (155,201).
Phosphotrans- acetylase	2	1.0 to 2.1 kb	TA3514 (114) TA3516 (114) KH103 (82)	Protein 35 to 88 kDa, apparently as monomer (46,106,216). Purified, <i>Clostridium thermoaceticum</i> (46). Cloned & sequenced, <i>Methanosaerina thermophila</i> (106). Phosphotransbutyrylase cloned & sequenced, <i>C. acetobutylicum</i> (155,201)
l-Lactate Dehydrogenase	17	0.93 to 0.97 kb	FMJ139 (133) FMJ144 (133)	Monomeric protein 33 to 40 kDa, sometimes as dimer & tetramer (11,97,118,154,178,188). Purified (55) & cloned (36), <i>C. acetobutylicum</i> . Cloned & sequenced in <i>Bacillus stearothermophilus</i> (11,223), <i>Streptococcus thermophilus</i> (74), <i>Thermus aquaticus</i> (154).
Hydrogenase	21	0.9 to 8 kb (1.7 to 2.3 kb in <i>C. acetobutylicum</i> , <i>C. pasteurianum</i> )	AK23, SE-3-1 (30) AK11,AK22 (31) AP3 (135) (among many)	Several functionally-distinct enzymes present in most organisms. Large literature includes aerobic lithotrophs and nitrogen fixers. <i>C. pasteurianum</i> and <i>C. acetobutylicum</i> enzymes about 60 kDa, sequenced (145, 175). 3 subunits cloned & sequenced in <i>Methanobacterium thermoautotrophicum</i> (5).
Pyruvate Dehydrogenase	9	1.1 to 2.7 kb (for subunits)	<i>E. coli</i> YYC202 (64)	A complex of three distinct subunits with individual molecular weights 65 to 150 kDa (23,61,112,200). All three subunits purified in <i>E. coli</i> (200), sequenced in <i>Acetiligenes eutrophus</i> (64). Single protein purified in <i>B. stearothermophilus</i> (61).

Table 3. Selected Parameters for Integration of Plasmid DNA into the Bacterial Chromosome by Homologous Recombination

Parameter	Value	Organism/vector	Comments <sup>1</sup>	Reference
<b>Integration frequency<sup>2</sup></b>				
10 <sup>-2</sup>	<i>Bacillus subtilis</i>	1 to 15 kb homology		153
10 <sup>-2</sup>	<i>Clostridium beijerinckii</i>	Homologous segments as small as 336 bp		209
10 <sup>-3</sup> to 10 <sup>-2</sup>	<i>Escherichia coli</i>	5.8 kb homology; 0.2 to 6 kb insert		166
10 <sup>-4</sup> to 3 x 10 <sup>-3</sup>	<i>Escherichia coli</i>	5 to 7 kb homology; mtl CAD genes		108
10 <sup>-3</sup> to 10 <sup>-2</sup>	<i>Lactobacillus plantarum</i>	2 to 5 kb homology; no insert		177
1 to 7 x 10 <sup>-3</sup>	<i>Lactococcus lactis</i>	~1 kb homology		171
10 <sup>-2</sup>	<i>Salmonella typhimurium</i>	17 kb homology; intact che gene		59
10 <sup>-2</sup>	<i>Salmonella typhimurium</i>	15 kb homology; 2.2 kb deletion		59
<b>% double crossovers<sup>3</sup></b>				
80	<i>Bacillus subtilis</i>	2.4 kb homology; complements pheA <sup>-</sup> host		54
73	<i>Bacillus subtilis</i>	2 & 3.3 kb flanking homology; 1 kb insert		150
25	<i>Bacillus subtilis</i>	0.5 kb flanking homology; ~5kb insert		187
14	<i>Lactococcus lactis</i>	~2.8 kb flanking homology; 3.7 kb insert		33
2	<i>Lactococcus lactis</i>	3.7 and 1.6 kb flanking homology; 1.2 kb insert		111
>99	<i>Streptococcus pneumoniae</i>	2 kb flanking homology; 1.8 kb insert		165
<b>Intrachromosomal recombination frequency<sup>4</sup></b>				
10 <sup>-4</sup>	<i>Bacillus subtilis</i>	3.7 to 3.8 kb homology; no intervening sequence		149
10 <sup>-4</sup>	<i>Bacillus subtilis</i>	0.25 kb homology; 4.8 kb intervening sequence		152
10 <sup>-3</sup>	<i>Bacillus subtilis</i>	0.67 or 1.2 kb homology; 5 kb intervening seq.		164
10 <sup>-4</sup>	<i>Bacillus subtilis</i>	0.5 kb homology; ~5 kb insert		187
10 <sup>-5</sup> to 4 x 10 <sup>-4</sup>	<i>Bacillus subtilis</i>	3.4 kb homology; ~5 kb insert		196
10 <sup>-2</sup>	<i>Escherichia coli</i>	5.8 kb homology; 0.2 to 6 kb intervening seq.		166
2 x 10 <sup>-2</sup>	<i>Lactococcus lactis</i>	~2.8 kb homology; 3.7 kb insert		33
3 to 9 x 10 <sup>-6</sup>	<i>Lactococcus lactis</i>	1.6 kb homology; 4.1 kb insert		111

<sup>1</sup> Values for "homology" refer to the length of the entire homologous region (which are repeated in the case of intrachromosomal recombination); values for "flanking homology" refer to the length of the flanking regions on either side of a marker or insert. <sup>2</sup> Fraction of cells containing transformed DNA that incorporate DNA into the chromosome, generally calculated from the frequency of integration for a nonreplicative plasmid relative to the transformation frequency for a replicative plasmid. <sup>3</sup> Fraction of cells that incorporate DNA into the chromosome that do so by an apparent double crossover event. <sup>4</sup> Given repeated sequences generated by a single Campbell-type crossover event, the fraction of cells per generation that excise the intervening sequence.

**Table 4. Work Schedule.**



# FIGURE 1: CONSOLIDATION OF BIOLOGICALLY - MEDIATED EVENTS IN BIOMASS ETHANOL PRODUCTION

<u>BIOLOGICALLY - MEDIATED EVENT</u>			
PROCESSING STRATEGY <sup>1</sup> (EACH BOX REPRESENTS A BIOREACTOR NOT TO SCALE)			
<u>SHF</u>	<u>SSF</u>	<u>SSCF</u>	<u>CBP</u>
CELLULOSE PRODUCTION			
CELLULOSE HYDROLYSIS			
FERMENTATION OF C <sub>6</sub> SUGARS			
FERMENTATION OF C <sub>5</sub> SUGARS			

<sup>1</sup>SHF: SEPARATE HYDROLYSIS AND FERMENTATION.

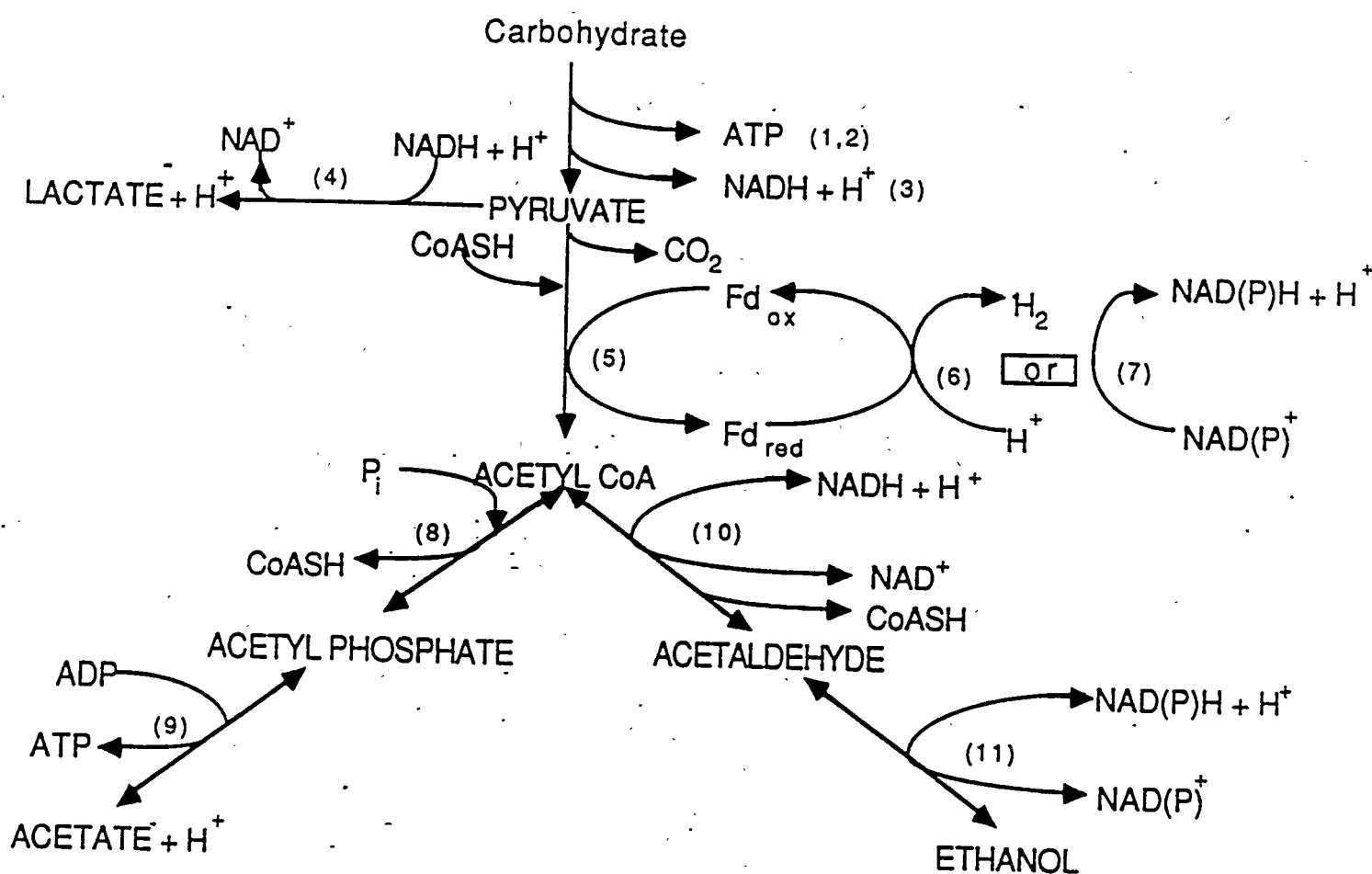
SSF: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION, SEPARATE PENTOSE FERMENTATION.

SSCF: SIMULTANEOUS SACCHARIFICATION AND CO- FERMENTATION.

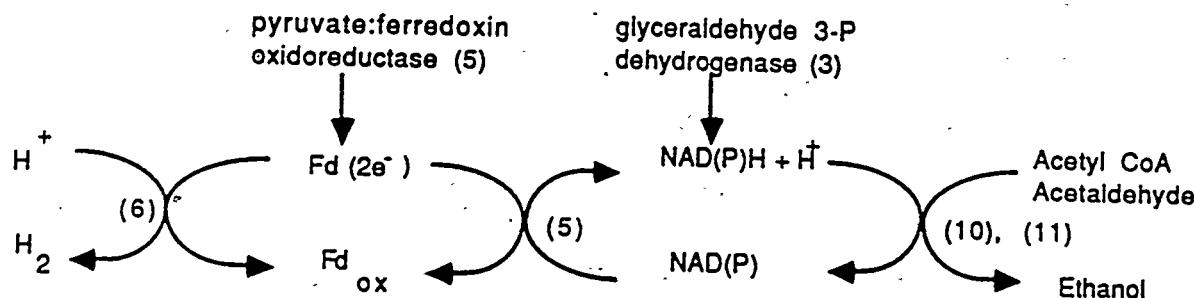
CBP: CONSOLIDATED BIOPROCESSING.

**Figure 2. Metabolic pathways for production of ethanol, acetate, and lactate by ethanol-producing, thermophilic bacteria**

**A: Carbon-Centered Perspective**

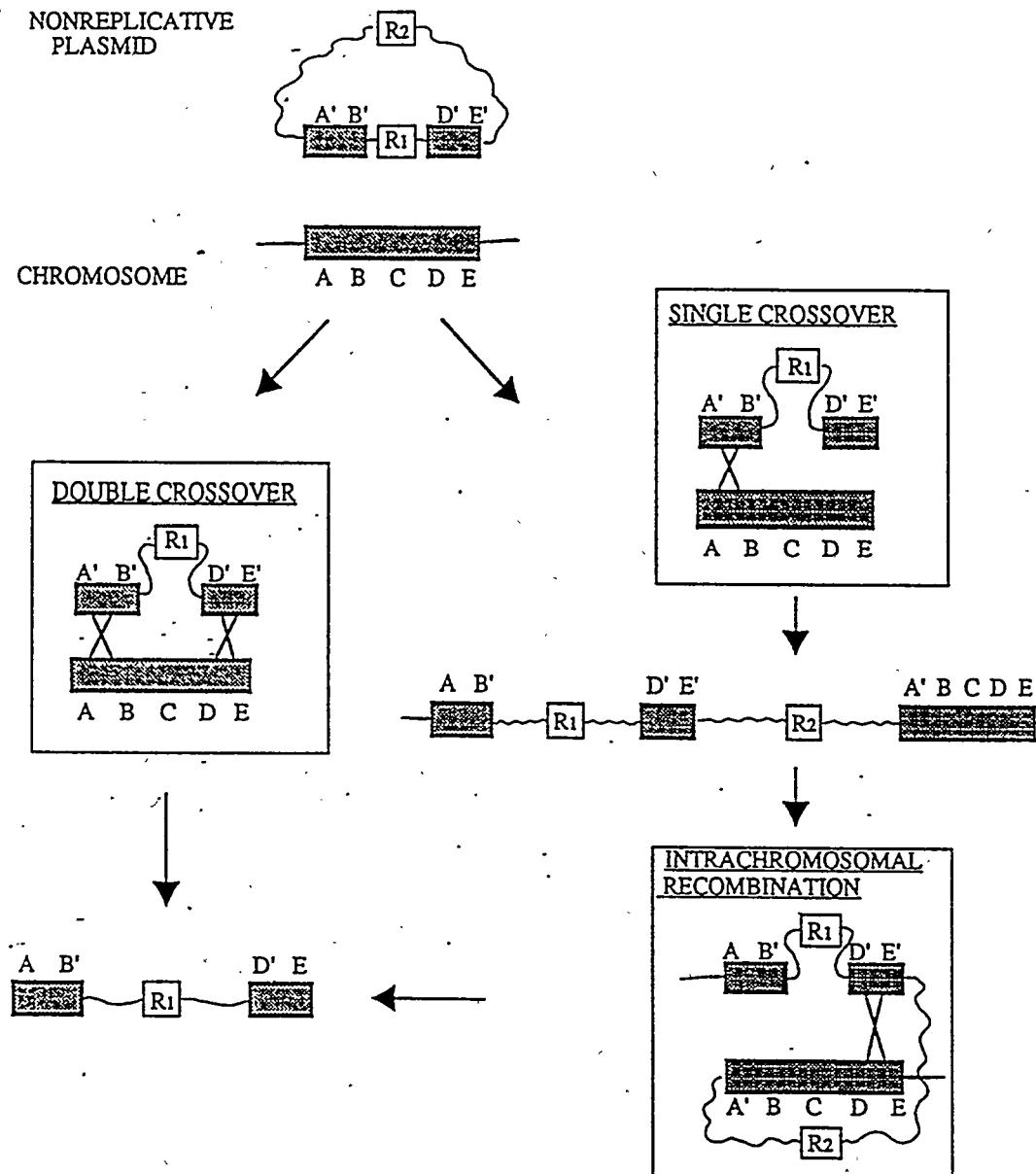


**B: Electron-Centered Perspective**



- (1) phosphoglycerate kinase (2) pyruvate kinase (3) glyceraldehyde 3-P dehydrogenase
- (4) lactate dehydrogenase (5) pyruvate:ferredoxin oxidoreductase (6) hydrogenase
- (7) ferredoxin:NAD oxidoreductase (8) phosphotransacetylase (9) acetate kinase
- (10) acetaldehyde dehydrogenase (11) alcohol dehydrogenase

Figure 3. Gene substitution by homologous recombination.



$R_1$  and  $R_2$  are antibiotic resistance determinants. Dark segments denote homologous regions of the DNA.  $A, B, C, D$ , and  $E$  denote locations on the genome, with prime superscript indicating DNA originating from the plasmid. In the example shown; the plasmid has a deletion at location  $C$ . Arrangements other than those shown are possible, e.g. a single crossover between  $D$  and  $E$ s and intrachromosomal recombination between  $A$  and  $B$  resulting in a functional gene with some plasmid DNA incorporated.

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