0273-2289/96/5758—0293$8.25

Factors Contributing to the Loss of Ethanologenicity of *Escherichia coli* B Recombinants pLOI297 and KO11

HUGH G. LAWFORD* AND JOYCE D. ROUSSEAU

Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

ABSTRACT

To be economic and to be compatible with modern continuous bioconversion systems, it is imperative that the process organism exhibit an extremely high degree of stability. In the case of ethanol production from lignocellulosic biomass, functional stability of the potential process biocatalyst can be assessed in terms of the capacity to sustain high-performance fermentation during the continuous fermentation of biomass-derived sugars.

This investigation employed glucose- or xylose-limited chemostat culture to examine the functional stability of two patented, genetically engineered *E. coli*—namely *E. coli* B (ATCC 11303) carrying the *Zymomonas* genes for pyruvate decarboxylase and alcohol dehydrogenase II on a multicopy plasmid pLOI297 and a chromosomal *pet* integrant of strain 11303, designated as strain KO11. Both recombinants carry markers for antibiotic resistance and have been reported to exhibit genetic stability in the absence of antibiotic selection.

Chemostats were fed with Luria broth (LB) (with 25 g/L sugar) at a dilution rate of 0.14 and 0.07/h when the feed medium was glucose-LB and xylose-LB, respectively. The pH was controlled at 6.3. With glucose, both recombinants exhibited a rapid loss of ethanologenicity even when selection pressure was imposed by the inclusion of antibiotics in the feed medium. With strain KO11, increasing the concentration of chloramphenicol from 40 to 300 mg/L, resulted in a retardation in the rate of loss of ethanologenicity, but it did not prevent it. Under xylose limitation, the plasmid-bearing recombinant appeared to be stabilized by antibiotics, but this did not reflect genetic stability, since the slower-growing revertant was washed out at a dilution rate of 0.07/h. With both recombinants, interpretation of functional stability with xylose was complicated by the inherent ethanologenicity associated with the host culture.

Based on an average cost for large bulk quantities of antibiotics at $55/kg and an amendment level of 40 g/m^3, the estimated economic impact regarding the potential requirement for operational stabilization by antibiotics in a plant operating in batch mode varied from a maximum of 29¢/gal of E95 ethanol for antibiotic amendment of all fermentation media to a minimum of 0.45¢/gal where antibiot-

*Author to whom all correspondence and reprint requests should be addressed.

ics were used exclusively for the preparation of the inocula for every fourth batch fermentation cycle. The high degree of instability observed in these continuous fermentations does not auger well for the proposed potential industrial utility of these patented, genetically engineered constructs for the production of fuel ethanol from biomass and wastes.

Index Entries: Ethanol; xylose; stability; recombinant *E. coli*; strain KO11; antibiotics; *pet* operon.

INTRODUCTION

To be economic, it is imperative that the production of fuel ethanol from lignocellulosic biomass and wastes involve maximal efficiency of the conversion of all sugars to ethanol *(1,2)*. The five-carbon sugar xylose is a major component of hemicellulose *(3)*, which is hydrolyzed during pretreatment of lignocellulosic biomass *(4)*. Considerable research has been directed to the search for organisms capable of high-performance fermentation of biomass prehydrolysates. This search for xylose-fermenting ethanologenic microorganisms has produced several alternatives, including bacteria, yeasts, and fungi (for review, *see* ref. 5). In addition to natural isolates, several genetically engineered biocatalysts have been constructed for this purpose, and prominent among these have been the patented ethanologenic *Escherichia coli* cultures that carry genes for ethanol production, namely pyruvate decarboxylase *(pdc)* and alcohol dehydrogenase II *(adhB)* from *Zymomonas* mobilis *(6–8)*. In the early stages of developing ethanologenic recombinants, genetic engineering involved insertion of these genes (referred to as the *pet* operon) *(7)* on multicopy plasmids carrying genes responsible for resistance to tetracycline and ampicillin *(8)*. In one particular construct, designated as plasmid pLOI297, expression of the *pet* operon was under the control of the *E. coli* lac promoter *(9)*. The construction of this transformation vector is illustrated in Fig. 1A (for details, *see* ref. 9). Although the pioneering work was done with *E. coli* K12 *(10–12)*, a subsequent physiological assessment of growth characteristics of several different potential host cultures of *E. coli* identified the wild-type Luria strain B (ATCC 11303) as a "hardy strain and a suitable host for the *pet* plasmid pLOI297" *(13)*. For several years, we have been assessing the fermentation performance characteristics of this patented recombinant *E. coli* 11303:pLOI297 using both synthetic lab media *(14–18)* and biomass prehydrolysates prepared by different thermochemical processes from a variety of biomass/waste feedstocks, including both hardwood (aspen) *(19)* and softwood (pine) *(20)*, newsprint *(21)*, spent sulfite liquors *(22)*, and corn crop residues *(23)*.

Regarding plasmid stability, it has been claimed that "plasmid pLOI297 is stably maintained in ATCC 11303 in the absence of antibiotic selection" *(24)* based on the observation of 98% retention of antibiotic resistance for over 25 generations of growth with glucose in the absence of antibiotic selection *(9)*. Pyruvate decarboxylase is absent in *E. coli (25,26)*, and it is known that in order to function as an efficient ethanologen, there needs to be hyperexpression of the *pet* operon *(9)*. Although the replicon associated with pLOI297 ensures a high number of copies of the plasmid in the host (typically hundreds of copies per cell) *(27)* and consequently promotes high levels of activity of the *Zymomonas* ethanol production enzymes, the genetic instability associated with pUC-based plasmid constructs is also well known *(27,28)*. The potential for instability in recombinant 11303:pLOI297 is exacerbated

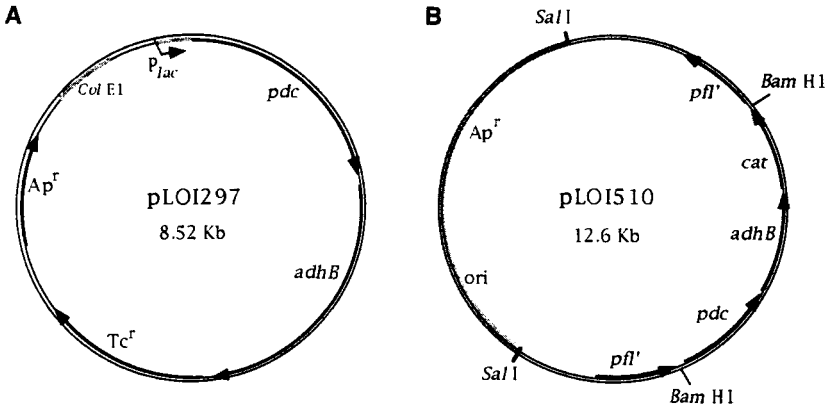

Fig. 1. The construction of vectors used to transform *E. coli* B (ATCC 11303). **(A)** Plasmid pLO12971—derived from the high-copy-number plasmid pUC18 with the *ColEI* replicon; **(B)** plasmid pLOI5102—Transformation via chromosomal integration was accomplished using a circularized 8.6-kbp derivative of pLOI510 containing promotorless *Zymomonas pdc* and *adhB* genes adjacent to *cat* (with a native promoter) inserted within a promotorless *E. coli* pyruvate formate lyase (the 4-kbp portion shown in gray was removed by *Sal* 1 digestion). Nomenclature: replicons *Col*E1 and ori—origins of replication; *pdc*, *Zymomonas* pyruvate decarboxylase; *adh*B, *Zymomonas* alcohol dehydrogenase II; Ap^r, β-lactamase (ampicillin resistance gene); Tc^r, tetracycline resistance gene; *pfl'*, split *E. coli* pyruvate formate lyase; *cat*, chloramphenicol acetyl transferase; P_{lac}, *E. coli* lac promotor; *Sal*1 and *Bam*H1, restriction endonucleases *(9,32)*.

by the fact that the host harbors low-copy-number cryptic plasmids *(29)*. Finally, high-copy-number plasmids are known to impose an energetic burden on the host *(30)*, and because energy is diverted from growth to plasmid maintenance, this effect is often reflected in reduced growth rate and cell yield *(31)*.

Recognizing that "plasmid-bearing recombinants are typically less stable than strains in which the foreign genes have been integrated into the host chromosome" *(32)*, Ingram and his associates at the University of Florida created a series of *pet* integrants *(32)*. One of the plasmids used for the purpose of inserting the *Zymomonas pet* genes into the chromosome of *E. coli* B ATCC 11303 is described in Fig. 1B (for details, *see* ref. *32*). Chromosomally integrated strains were constructed by inserting the *Zymomonas pdc* and *adhB* genes within the pyruvate-formate lyase gene *(pfl)* of the host *(32)*. However, it was discovered that single-copy inserts of the *pdc* and *adhB* genes did not result in the high level of activities of *Zymomonas* enzymes that had been achieved in multicopy plasmid-based recombinants *(32)*. The circularized fragment of DNA used to transform *E. coli* also contained the gene for chloramphenicol acetyl transferase *(cat)*, which is responsible for conferring resistance to chloramphenicol (Cm) (Fig. 1). Strain KO11 expresses high levels of both the *Zymomonas* genes and *cat*, and was a spontaneous mutant that was selected for resistance to high levels (600 μg/mL) of chloramphenicol. In addition, strain KO11 carries a mutation in its fumarate reductase gene, which impairs its ability to produce succinate as a fermentation end product *(32)*.

In a recent collaborative, international, interlaboratory study designed to compare, under standardized conditions, the fermentation performance of several different microorganisms in a xylose-rich medium consisting primarily of a dilute

acid hydrolysate of corn cobs, it was concluded that the *E. coli* recombinant strain KO11 "is currently the best known pentose-fermenting organism" *(33)*. This conclusion was based on a combination of criteria with a primary focus on ethanol yield and productivity. The issue of stability of this genetic construct was not addressed in this comparative study *(33)*. Likewise, in a recent review of hemicellulose conversion technology, McMillan *(2)* concluded that Ingram's recombinant *E. coli* is currently one of the "the best candidates" for converting hemicellulosic hydrolysates, but there was surprisingly no reference to the issue of stability of this candidate biocatalyst. A recently published technical and economic analysis of large-scale ethanol production from willow based on the use of recombinant *E. coli* KO11 admits to lack of information relating to the genetic stability of KO11 and its ability to sustain a high level of ethanologenicity during prolonged batch cycling and recirculation *(34)*. This study failed to consider the economic impact of the use of antibiotics to provide constant selective pressure as an aid to genetic stability *(34)*. Clearly, there is a deficiency in our knowledge regarding the issue of stability of these genetically engineered ethanologenic biocatalysts, and the purpose of this study was to extend our preliminary assessment of the stability of recombinant *E. coli* 11303:pLOI297 and the chromosomal integrant strain KO11 in which we used serial transfer cultivations with and without antibiotic selection *(35)*. Since the potential utility of these constructs relates principally to their ability to convert various biomass-based sugars to ethanol, we have chosen to assess the functional stability in terms of the ethanologenicity of the chemostat, whereby system stability is viewed as the capacity of the chemostat culture to sustain high fermentation performance with respect to the conversion of sugar to ethanol. Furthermore, continuous culture of recombinant ethanologenic *E. coli* is another area in which there is little available information and continuous fermentation offers significant potential for economic improvement through lowered capital and operating costs.

In this study, we used both glucose and xylose because these two sugars represent the major components of cellulose and hemicellulose, respectively *(3)*. The apparent linear growth pattern exhibited by recombinant *E. coli* in batch fermentations *(9,14,20)* made it difficult to predict an appropriate dilution rate for chemostat culture. Consequently, we have adopted a heuristic approach to the set point with respect to dilution rate. Based on our previous experience with batch fermentations, and knowing that growth with glucose was approximately two-fold faster than with xylose *(14)*, the dilution was set at 0.14 and 0.07/h for these sugars, respectively.

MATERIALS AND METHODS

Organisms

The wild-type, host culture, *E. coli* B (ATCC 11303) was obtained from The American Type Culture Collection (Rockland, MD). Recombinant *E. coli* B (ATCC 11303 carrying the *pet* plasmid pLOI297) *(9)* and the chromosomally integrated strain KO11 *(32)* were received from L. O. Ingram (University of Florida, Gainesville, FL). Cultures grown from single-colony isolates on selective antibiotic-containing agar medium were stored at –10°C in luria broth (LB) medium supplemented with glycerol (20 mL/dL) and sodium citrate (1.5 g/dL).

Continuous Culture Conditions

Continuous cultures were conducted in benchtop chemostats (Bioflo model C30, New Brunswick Scientific Co., Edison, NJ) with a working volume (V) of approx 350 mL. The chemostats were fed sterile LB medium *(36)* (containing approx 2.5 wt% sugar) at a constant flowrate (F) by means of peristaltic pump (dilution rate [D] where $D = F/V\ h^{-1}$). The temperature was 30°C, the agitation (multiple flat-blade turbines) was constant at approx 150 rpm and the pH was maintained at 6.3 by the addition of 2*N* KOH. In the case of recombinant 11303:pLOI297 selection medium, the LB medium was amended with 40 mg/L ampicillin (Ap) and 10 mg/L tetracycline (Tc). For recombinant KO11, Cm was used either at 40 or 300 mg/L as specified in the figure captions. Inocula were prepared as in phosphate-buffered LB in flask cultures described previously *(37)*.

Analytical Procedures

Growth was estimated turbidometrically at 550 nm. Compositional analyses of fermentation media and cell-free spent media were determined by high-performance liquid chromatography (HPLC) as described previously *(37)*.

Assaying Stability of Recombinant Cultures

Functional stability of recombinant *E. coli* cultures was assessed in practical terms as ethanologenicity of the culture and the degree of ethanol selectivity among metabolic end products. Genetic stability was assessed phenotypically by plating cultures on selective and nonselective agar. The phenotypic characteristics of the recombinant culture were related to antibiotic resistance, colony size, and morphology. Ethanologenic recombinants were recognized on antibiotic-selective media by the formation of distinctive large, yellowish, opaque colonies *(14,38)*.

Determination of Fermentation Parameters

Culture ethanologenicity was expressed in terms of the ethanol yield ($Y_{p/s}$), which was calculated as the mass of ethanol produced per mass of sugar consumed. The theoretical maximum ethanol yield for either glucose or xylose is 0.51 g EtOH/g sugar.

RESULTS AND DISCUSSION

Recombinant *E. coli* 11303:pLOI297

Figure 2 represents the continuous culture of recombinant *E. coli* B 11303:pLOI297 in LB with glucose (about 25 g/L) and is a plot of the trajectories for biomass (estimated by OD), effluent glucose, and the different catabolic end products (ethanol, lactic acid, and acetic acid). Flow of medium from the nutrient reservoir was initiated following overnight batch culture, and a period of time equal to 3 vol changes (21 h) was assumed to be sufficient for steady-state growth to be achieved in the selective medium, which contained both ampicillin and tetracycline. After 24 h of elapsed continuous fermentation, the feed was switched to a nonselective medium (25 g/L glucose in LB minus the antibiotics) (Fig. 2). The increase in lactic acid prior to the change over to the nonselective medium suggests instability even under the selective pressure of the presence of antibiotics in

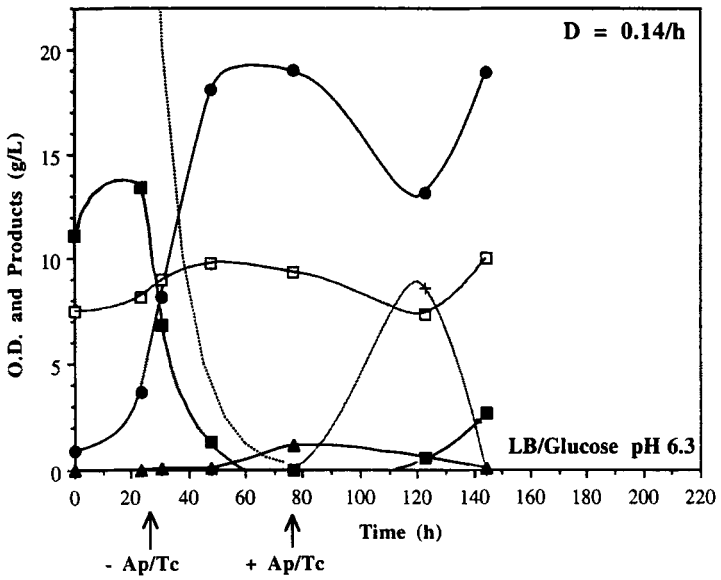

Fig. 2. Continuous culture of recombinant *E. coli* 11303:pLO1297. LB, containing glucose (25 g/L) and antibiotics (40 mg/L ampicillin and 10 mg/L tetracycline), was fed to the chemostat (350 mL constant working volume) at a fixed rate of 49 mL/h (D = 0.14/h). The pH was controlled at 6.3, and the temperature was constant at 30°C. The first arrow (-Ap/Tc) indicates the switch to a medium feed without antibiotics. The dashed line shows the theoretical dilution profile with respect to the concentration of ampicillin(*y*-axis as mg/L) in the chemostat. The second arrow (+Ap/Tc) indicates addition of antibiotics directly to the chemostat and the switch back to the medium containing antibiotics. Symbols: □, OD at 550 nm; +, glucose (g/L); ■, ethanol (g/L); ●, lactate (g/L); ▲, acetate (g/L).

the medium (Fig. 2). The trend to elevated lactic acid with concomitant fall in ethanol following the switch to the nonselective medium indicates a high level of functional instability under these conditions. The ethanol concentration falls to zero within 40 h of the switch to the nonselective medium, and this corresponds to only about eight generations. In fact, the apparent displacement of ethanol from the chemostat precedes that of the antibiotic (Fig. 2).

In this study, we have viewed the chemostat culture from a holistic perspective, and although functional instability almost certainly is a function of the genetic instability of this genetically engineered biocatalyst, we have not attempted to discern the mechanism(s) responsible for such instability. Since it is known that under similar conditions of pH-controlled batch culture, the wild-type host culture (ATCC 11303) exhibits a faster growth rate than recombinant 11303:pLOI297 *(31)*, it is reasonable to assume that the particular environmental conditions of the glucose-limited chemostat (D = 0.14/ h) offer a growth advantage to the revertant and that it rapidly displaces the recombinant culture *(39,40)*. The stoichiometric distribution of fermentation end products in the absence of antibiotics (Fig. 2) is consistent with what would be expected from glucose fermentation by the host culture *(31)*. In this investigation, plating of cultures on selective and nonselective agar was done only for the purpose of qualitative phenotypic screening. Plating of the 60-h chemostat culture revealed that a high proportion of the population

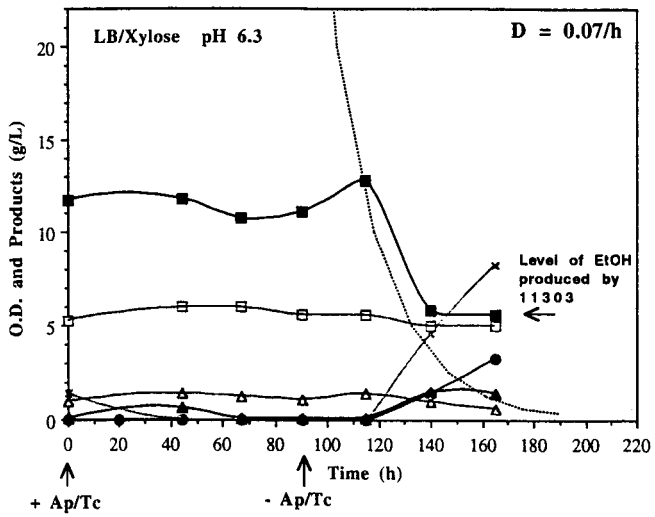

Fig. 3. Continuous culture of recombinant *E. coli* 11303:pLOI297. Conditions were as described in Fig. 2, except that the medium was LB with xylose (25 g/L) and antibiotics (40 mg/L ampicillin and 10 mg/L tetracycline). The dilution rate (D) was 0.07/h. The first arrow (-Ap/Tc) indicates the switch to a medium feed without antibiotics. The dashed line shows the theoretical dilution profile with respect to the concentration of ampicillin (*y*-axis as mg/L) in the chemostat. Symbols: □, OD at 550 nm; X, xylose (g/L); ■, ethanol (g/L); ●, lactate (g/L); ▲, acetate (g/L); △, succinate (g/L).

was not antibiotic-resistant (results not shown). That the culture was not completely devoid of antibiotic-resistant cells was indicated by the lack of "washout" and the partial recovery of ethanol that was observed following reintroduction of antibiotics into the system (Fig. 2).

The first *pet* recombinants of *E. coli* were based on high-copy-number plasmid constructs, and it was reported that the pLOI297 plasmid was "quite stable" in *E. coli* B (ATCC 11303) where plasmid stability was expressed as the percentage of cells retaining antibiotic markers after "25 generations" of growth in LB medium with glucose in the absence of antibiotic selection *(9)*. For recombinant 11303:pLOI297 under these specific assay conditions, it was reported that 98% of the cells retained antibiotic resistance, but the actual time period was not specified nor was the stability determined in terms of retention of culture ethanologenicity *(9)*.

The virtual disappearance of ethanol from the glucose-limited chemostat after only 3 d of operation does not suggest a stable biocatalyst and challenges the earlier claims *(9)* regarding the stability of this construct.

Figure 3 represents an experiment of similar design to that shown in Fig. 2, except that the LB medium contained xylose (about 25 g/L) and the dilution rate was 0.07/h. Over the initial 90-h period, the chemostat was operated under antibiotic selection, and the ethanol concentration was relatively constant at about 12 g/L corresponding to a xylose-to-ethanol conversion efficiency of 95% of the theoretical maximum (Fig. 3). However, when the antibiotics were absent from the feed medium, the decay in ethanol concentration paralleled that of the concentration of antibiotics in the chemostat (Fig. 3). The ethanol concentration decreased to a plateau value of about 6 g/L (Fig. 3), corresponding to an ethanol yield of 0.24 g/g

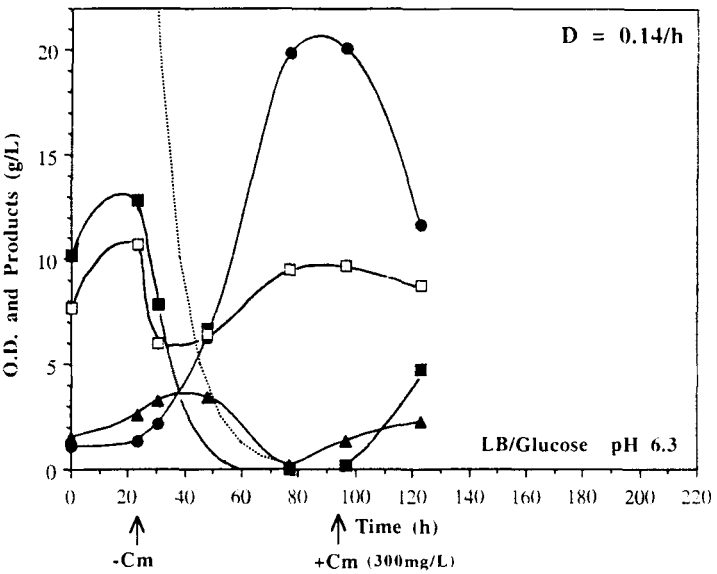

Fig. 4. Continuous culture of recombinant *E. coli* KO11. Conditions were as described in Fig. 2, except that the LB medium contained glucose (25 g/L) and Cm (40 mg/L). The dilution rate (D) was 0.14/h. The arrow at 24 h (-Cm) indicates the switch to a medium feed without Cm. The second arrow at 96 h (+Cm) indicates addition of Cm (final concentration of 300 mg/L) directly to the chemostat and the switch to LB medium containing 300 mg/L Cm. The dashed line shows the theoretical dilution profile with respect to the concentration of Cm (*y*-axis as mg/L) in the chemostat. Symbols: □, OD at 550 nm; ■, ethanol (g/L); ●, lactate (g/L); ▲, acetate (g/L).

xylose that is characteristic of xylose fermentation by the host culture *(8,31)*. Operationally, the xylose-limited chemostat under selective pressure appears to be stable over the 4-d period of assessment. However, this may not reflect genetic stability of the plasmid recombinant for the following reasons. It is known that *E. coli* does not grow well with xylose *(24,31)*, and furthermore, that the recombinant 11303:pLOI297 grows in LB-xylose at a rate that is considerably faster than the host culture *(31)*.Therefore, the *pet*-expressing culture would have an advantage in the chemostat whereby the slower-growing revertant host culture would be washed out *(39,40)*. In a separate experiment (results not shown), we determined that, at this dilution rate with xylose, steady-state growth was not possible with the host culture ATCC 11303. It follows that the apparent stability of the xylose-limited chemostat under selective pressure derives from its capacity for self-regulation with respect to maintenance of the ethanologenic strain.

Recombinant *E. coli* KO11

A similar experimental strategy was employed to assess the functional stability of the chromosomal integrant, strain KO11. Figure 4 represents the continuous culture of the recombinant in LB with glucose (about 25 g/L). Initially the medium contained chloramphenicol (Cm) at 40 mg/L; however, at 21 h, the feed was switched to a nonselective medium in which Cm was omitted. The trajectories for ethanol and lactic acid that are plotted in Fig. 4 are remarkably similar to those of Fig. 2. For purposes of comparison, all the plots have been made with both *x*-axis

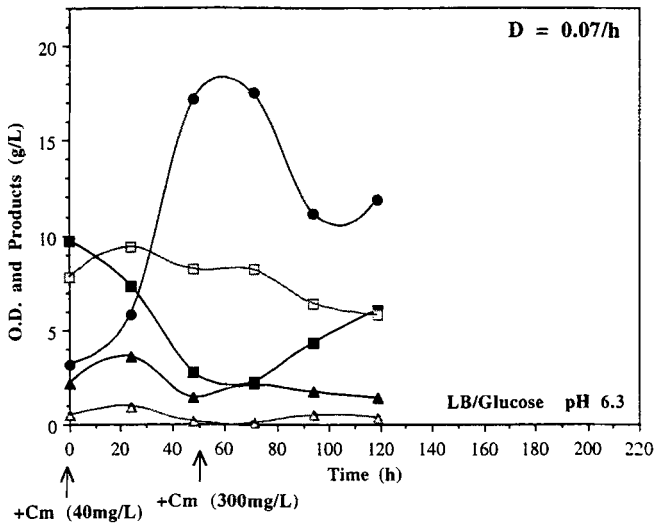

Fig. 5. Continuous culture of recombinant *E. coli* KO11. Conditions were as described in Fig. 4, except that the dilution rate was 0.07/h. Symbols: □, OD at 550 nm; ■, ethanol (g/L); ●, lactate (g/L); ▲, acetate (g/L); △, succinate (g/L).

and *y*-axis of the same scale and magnitude. The initial level of lactic acid is consistent with the known fermentation pattern for strain KO11 *(32)* and was not construed as indicative of instability during the initial period while the chemostat was under selective pressure with Cm in the medium (Fig. 4). The decrease in ethanol parallels the decrease in the concentration of Cm and seems to commence immediately on removal of the Cm from the medium being supplied to the chemostat. However, in comparing Figs. 2 and 4, it can be seen that the rate at which the ethanol decreases, and the lactic acid increases, is not as fast as with the plasmid-bearing recombinant. As was the case with the recombinant 11303:pLOI297, recovery of the system with respect to ethanol production is possible through the reintroduction of Cm (Fig. 4), which demonstrates that KO11 had not been completely eradicated at the time the antibiotic was added (96 h).

Figure 5 shows the effect of dilution rate on the stability of the glucose-limited chemostat with recombinant KO11. In the experiment shown in Fig. 5, the chemostat was operated at a dilution rate of only 0.07/h (compared to Fig. 4 where D = 0.14/h), and the decrease in ethanol along with the concomitant rise in lactic acid indicates functional instability even in the presence of 40 mg Cm/L. The ethanol yield fell to a value of 0.12 g/g by 48 h after flow was started, and this time period corresponds to <5 generations. Recovery was demonstrated by the introduction of 300 mg/L Cm (Fig. 5), suggesting that the system could be operated in a more stable fashion at this higher concentration of Cm. Figure 6 shows that this was not the case since the ethanol concentration began to decline after only 2 d of operation with 300 mg/L Cm in the medium. The higher level of antibiotic retarded the loss of ethanologenicity, but did not prevent it (Fig. 6).

Figure 7 shows the results of a continuous culture of recombinant KO11 in LB medium with xylose (about 25 g/L). The chemostat was operated at a dilution rate of 0.07/h, and for the first 2 d there was Cm (40 mg/L) in the feed medium during which time the ethanol decreased from 13 to 9 g/L (Fig. 7). There was very little

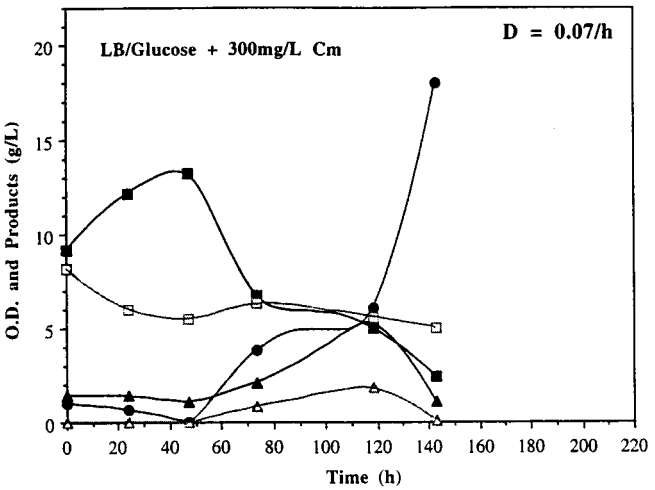

Fig. 6. Continuous culture of recombinant *E. coli* KO11. Conditions were as described in Fig. 5, except that the medium contained 300 mg/L chloramphenicol. Symbols are the same as in Fig. 5.

change in the ethanol concentration following the switch to medium without Cm, although there was a slow progressive increase in the concentration of both acetic acid and succinic acid (Fig. 7). Since strain KO11 is restricted from producing succinate because of a mutation in fumarate reductase (*frd*), the increase in succinate signals the presence of another population different from strain KO11. The pattern seen with xylose-limited KO11 in Fig. 7 is similar to that of the xylose-limited chemostat with plasmid recombinant (Fig. 3) in that the ethanol concentration decreases to a plateau that is defined by the ethanol yield characteristic of xylose fermentation by the host ATCC 11303.

Ethanologenic recombinant strain KO11 is characterized by its resistance to high levels of Cm and was derived from the *pet* integrant, strain KO3, which was selected for resistance to Cm at 40 mg/L (*29*). Whereas strain KO11 exhibits a characteristic very high degree of ethanol selectivity, strain KO3 produces a stoichiometric pattern of the fermentation end products that is very similar to the host culture (*32*). The distribution of metabolic end products resulting from xylose fermentation by strain KO3 has not been reported. Strain KO3 is distinguishable from the host culture by virtue of its resistance to Cm (*32*). The high frequency of mutation for hyperresistance to Cm (10^{-5}) that was observed in selecting strain KO11 from a culture of KO3 (*32*) suggests that the frequency of reversion could be of the same order. Hence, it is conceivable that the integrant KO3 strain could compete with strain KO11 in the chemostat that is being fed medium with 40 mg/L Cm.

These observations with recombinant KO11 challenge the claims of its designers regarding its "stability" (*32*) and call into question the generality of the presumption of stability of ethanologenic chromosomal integrants of other bacteria in which the genetic engineering was modeled after the work with *E. coli*. For example, strain P2 is a recombinant *Klebsiella oxytoca* M5A1 that expresses chromosomally integrated *Zymomonas* genes for ethanol production and is being used as a host strain for the expression of heterologous enzymes for hydrolyzing cellulose and xylan (*41*). However, a functional stability assessment for these constructs is lacking.

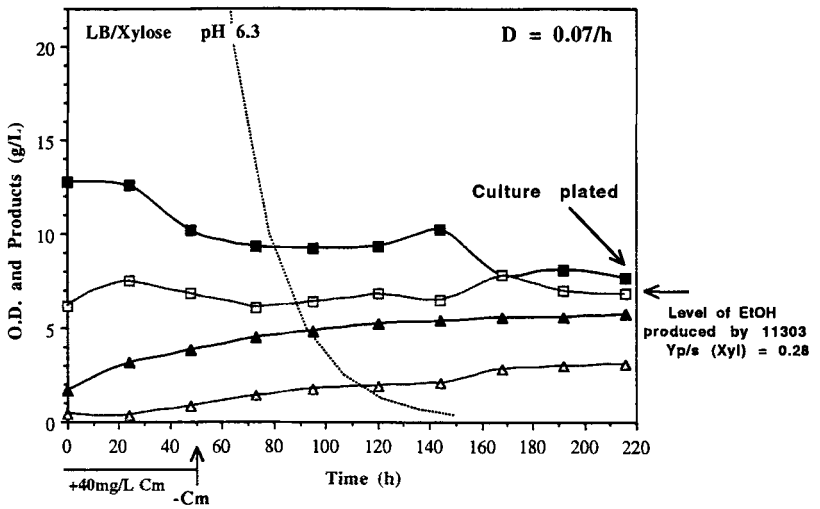

Fig. 7. Continuous culture of recombinant *E. coli* KO11. Conditions were as described in Fig. 4, except that the LB medium contained xylose (25 g/L) and the dilution rate was 0.07/h. The arrow at 50 h (-Cm) indicates the switch to a medium without chloramphenicol. The dashed line shows the theoretical dilution profile with respect to the concentration of Cm (*y*-axis as mg/L) in the chemostat. The arrow (right side) indicates the average level of ethanol produced under these conditions by the host culture *E. coli* B (ATCC 11303). Symbols are the same as in Fig. 5.

Although the stability of strain KO11 *per se* was not reported, the hyper-Cm-resistant integrant from which it was derived, namely strain KO4, was said to be stable after being serially transferred in glucose-LB without antibiotics for more than 68 generations, although the value of "generation time" was not specified *(32)*. Supposedly, strain KO11 differs from KO4 only by virtue of a mutation in the gene coding for *frd*, which is responsible for succinate production. Ohta et al. *(32)* described stability in terms of resistance to high levels of Cm (600 μg/mL) and phenotypic characteristics relating colony morphology on agar plates. According to these criteria of stability, 100% of the cells retained these traits after 68 generations *(32)*. The literature is remarkably silent with respect to the subject of the stability of ethanologenic recombinants, and claimed stability appears to have been tacitly assumed by several investigators who have independently endorsed strain KO11 in terms of its superiority as a xylose fermenter and suggested that it be considered as a candidate biocatalyst for hemicellulose utilization in large-scale fuel ethanol production facilities *(2,33,34)*.

The Economic Impact of a Requirement for Antibiotics on Cost of Fuel Ethanol

von Sivers et al. *(34)* recently published the results of their cost analysis of fuel ethanol production from lignocellulosic biomass based on using recombinant *E. coli* KO11. Their technical and economic analysis focused solely on the fermentation of prehydrolysate produced by steam-treated willow and estimated the total cost of producing fuel-grade E95 ethanol from xylose-rich prehydrolysate batch fermen-

tations to be 48¢/L (equivalent to $1.82/US gal). The cycle time for each batch fermentation was 55 h, and the plant produced 6.9 million liters of E95 ethanol/yr in 141 fermentation cycles. Pertinent to the results of our study with KO11 is the fact that it was assumed in their base case cost estimate that recombinant *E. coli* KO11 was sufficiently stable that only seven inoculations from pure culture would be required/yr. It was estimated that if the frequency with respect to the requirement for reinoculation changed from every 20 cycles to every 5 cycles, there would be an additional cost of 2¢/L of E95 raising the cost from 48 to 50¢/L. However, there was no mention regarding cost of antibiotics. Our results of the present study in combination with our preliminary findings with serial transfer fermentations in the absence of antibiotic suggest not only a more frequent requirement for inoculation, but also a possible involvement of antibiotics in all media. If Cm were to be used at the level of 40 mg/L in all media in the operation described by von Sivers et al. *(34)*, we estimate that the added cost of producing E95 ethanol would be 7.63¢/L (29¢/gal). This estimate is based on a bulk order cost of Cm at $55/kg. von Sivers et al. *(34)* used a pitch rate of 20% (to give an initial cell concentration of 1g dry wt/L), and even if there were a requirement to inoculate only every three cycles *(35)* and if Cm at 40 g/m^3 were used exclusively for the preparation of the inocula, there would still be an added cost of 0.45¢/gal E95. The stability of strain KO11 under the aerobic culture conditions proposed by these investigators remains to be assessed. Since strain KO11 appears to be maintained better at the higher level of 300 g/m^3 Cm, the economic impact would be increased proportionally by a factor of 7.5 times. The estimated economic impact regarding the potential requirement of stabilizing antibiotics is significant when viewed in terms of the cost estimates associated with nutritional supplements. von Sivers et al. *(34)* estimated the cost of using ammonia, phosphoric acid, and magnesium oxide as the sole nutritional supplements to be 10.6¢/gal E95. The results of our investigation being reported at this symposium indicate that corn steep liquor (CSL), at about 8 g/L, can supply all the nutritional requirements of recombinant *E. coli (31)*.The economic impact of CSL supplementation was estimated at 4.2¢/gal E95 ethanol. In the context of our findings with recombinant *E. coli* KO11, it is interesting to note that von Sivers et al. *(34)* made the following concluding remarks in their paper on technical and economic analysis:

> More experiments on both the laboratory and pilot scale are necessary to determnine how cell recirculation will affect the genetic stability of *E. coli* KO11. It is also necessary to establish whether *E. coli* KO11 is able to ferment equally well in a mineral salt medium as in the complex medium used in the laboratory experiments.

ACKNOWLEDGMENTS

This research was supported by The University of Toronto. We are grateful to Lonnie Ingram for the gift of the recombinant *E. coli* culture.

REFERENCES

1. Wyman, C. E. and Hinman, N. D. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 735–753.
2. McMillan, J. D. (1994), in *Bioconversion for Fuels*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, pp. 411–437.

3. Timell, T. E. (1967), *Wood Science Technol.* **1,** 45–70.
4. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
5. McMillan, J. D. (1993), Xylose fermentation to ethanol: a review; NREL TP-421-4944; National Renewable Energy Laboratory, Golden, CO.
6. Ingram, L. O., Conway, T., and Alterthum, F. (1991), United States Patent 5,000,000.
7. Ingram, L. O., Alterthum, F., Ohta, K., and Beall, D. S. (1990), in *Developments in Industrial Microbiology,* vol. 31, Elsevier Science, New York, pp. 21–30.
8. Ingram, L. O. (1991), in *Energy from Biomass and Wastes XIV,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 1105–1126.
9. Alterthum, F. and Ingram, L. O. (1989), *Appl. Environ. Microbiol.* **55,** 1543–1948.
10. Ingram, L. O. and Conway, T. (1988), *Appl. Environ. Microbiol.* **54,** 397–404.
11. Brau, B. and Sahm, H. (1986), *Arch. Microbiol.* **144,** 296–301.
12. Neale, A. D., Scopes, R. K., Wettenhall, E. H., and Hoogenraad, N. J. (1987), *J. Bacteriol.* **169,** 1024–1028.
13. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotechnol. Bioeng.* **38,** 296–303.
14. Lawford, H. G. and Rousseau, J. D. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 221–236.
15. Lawford, H. G. and Rousseau, J. D. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 185–204.
16. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 301–322.
17. Lawford, H. G. and Rousseau, J. D. (1993), *Biotechnol. Lett.* **15,** 615–620.
18. Lawford, H.G. and Rousseau, J. D. (1991), in *Energy from Biomass & Wastes XV,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 583–622.
19. Lawford, H. G. and Rousseau, J. D. (1991), *Biotechnot. Lett.* **13,** 191–196.
20. Lawford, H. G. and Rousseau, J. D. (1993), in *Energy from Biomass and Wastes XVI,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 559–597.
21. Lawford, H. G. and Rousseau, J. D. (1993), *Biotechnol. Lett.* **15,** 505–510.
22. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 667–685.
23. Lawford, H. G. and Rousseau, J. D. (1992), *Biotechnol. Lett.* **14,** 421–426.
24. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotechnol. Bioeng.* **38,** 296–303.
25. Neale, A. D., Scopes, R. K., and Kelly, J. M. (1988), *Appl. Microbiol. Biotechnol.* **29,** 162–167.
26. Sanbrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotech. Bioeng.* **38,** 296–303.
28. Tolan, J. S. and Finn, R. K. (1987), *Appl. Environ. Microbiol.* **53,** 2033–2038.
29. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugam, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 893–900.
30. DaSilva, N. A. and Bailey, J. E. (1986), *Biotechnol. Bioeng.* **28,** 741–746.
31. Lawford, H. G. and Rousseau, J. D. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 307–326.
32. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugam, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 893–900.
33. Hahn-Hägerdal, B., Jeppsson, H., Olsson, L., and Mohagheghi, A. (1994), *Appl. Microbiol. Biotechnol.* **41,** 62–72.
34. von Sivers, M., Zacchi, G., Olsson, L., and Hahn-Hägerdal, B. (1994), *Biotechnol. Prog.* **10,** 555–560.
35. Lawford, H. G. and Rousseau, J. D. (1995), *Biotechnol. Lett.* **17,** 751–756.
36. Luria, S. E. and Delbruck, M. (1943), *Genetics* **28,** 491–511.
37. Lawford, H. G. and Rousseau, J. D. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 179–195.
38. Ingram, L. O., Conway, T., Clark, D. P., Sewell, G. W., and Preston, J. F. (1987), *Appl. Environ. Microbiol.* **53,** 2420–2425.
39. Pirt, J. S. (1975), in *Principles of Microbe and Cell Cultivation,* Wiley, New York, pp. 200–202.
40. Bailey, J. E. and Ollis, D. F. (1977), in *Biochemical Engineering Fundamentals,* McGraw-Hill, New York, pp. 650–653.
41. Wood, B. E. and Ingram, L. O. (1992), *Appl. Environ. Microbiol.* **58,** 2103–2110.