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Comparison of Growth, Acetate Production, and Acetate Inhibition of *Escherichia coli* Strains in Batch and Fed-Batch Fermentations

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The growth characteristics and acetate production of several *Escherichia coli* strains were compared by using shake flasks, batch fermentations, and glucose-feedback-controlled fed-batch fermentations to assess the potential of each strain to grow at high cell densities. Of the *E. coli* strains tested, including JM105, B, W3110, W3100, HB101, DH1, CSH50, MC1060, JRG1046, and JRG1061, strains JM105 and B were found to have the greatest relative biomass accumulation, strain MC1060 accumulated the highest concentrations of acetic acid, and strain B had the highest growth rates under the conditions tested. In glucose-feedback-controlled fed-batch fermentations, strains B and JM105 produced only 2 g of acetate · liter^{-1} while accumulating up to 30 g of biomass · liter^{-1}. Under identical conditions, strains HB101 and MC1060 accumulated less than 10 g of biomass · liter^{-1} and strain MC1060 produced 8 g of acetate · liter^{-1}. The addition of various concentrations of sodium acetate to the growth medium resulted in a logarithmic decrease, with respect to acetate concentration, in the growth rates of *E. coli* JM105, JM105(pOS4201), and JRG1061. These data indicated that the growth of the *E. coli* strains was likely to be inhibited by the acetate they produced when grown on media containing glucose. A model for the inhibition of growth of *E. coli* by acetate was derived from these experiments to explain the inhibition of acetate on *E. coli* strains at neutral pH.

Media used for the high-density growth of *Escherichia coli* (20, 22), as well as for the production of recombinant DNA products by *E. coli* strains (4, 25), typically include substantial concentrations of glucose, since this is an inexpensive and readily utilizable carbon and energy source. Growth of *E. coli* on excess glucose under aerobic conditions, however, causes the formation of acidic by-products, of which acetate is the most predominant (1, 4, 9, 17, 20, 23, 25). This glucose-mediated aerobic acidogenesis, known as aerobic fermentation (4, 11, 16) or the bacterial Crabtree effect (10, 25), is most readily observed when *E. coli* is grown at high growth rates in the presence of high glucose concentrations (10, 11, 16). These conditions are prevalent in fed-batch cultures in which glucose is fed in a nonlimiting manner to aerobic fermentation cultures to obtain high cell densities (17, 20, 22). The production of acidic by-products, especially acetate, is a major factor in the limitation of high-cell-density growth (1, 4, 17, 20, 26). Moreover, the accumulation of acetate during recombinant *E. coli* fermentations has been correlated with a reduced production of recombinant protein (8, 19), demonstrating the importance of choosing host strains and growth conditions which minimize acetate accumulation. Although several studies have been carried out to determine the effect of fermentation conditions on the accumulation of acetate and other by-products (1, 4, 8, 10, 12, 17, 19, 20, 23, 25–27), no previous studies have compared the sensitivity of various *E. coli* strains to the bacterial Crabtree effect. In this study we compare several commonly used strains of *E. coli* with respect to growth rate, biomass yield, and acetate formation, with the goal of determining the strains most (or least) likely to yield good productivity in initial scale-up conditions. Such data should be helpful in choosing strains for rDNA fermentations.

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MATERIALS AND METHODS

Strains and medium composition. *E. coli* strains and plasmids used in this study are listed in Table 1. For maintenance, cultures were grown for 24 to 48 h on plates containing solidified (1.5% agar) SD-7 medium (see below) at 37°C and then stored at 4°C for about 2 weeks before subculture to fresh media. A glucose-yeast extract medium (SD-7 medium [Table 2]), based on mass balance with respect to cell composition (31) and biomass yields from major elements (23), was developed for the growth of the *E. coli* strains. SD-7 medium was titrated to pH 7.0 with 5 M NH_4OH before autoclaving, and the glucose and $MgSO_4$ were autoclaved separately from the remaining components and added after cooling. The trace-element solution contained the following (in grams per liter of 5 M HCl): $FeSO_4 \cdot 7H_2O$, 40.0; $MnSO_4 \cdot H_2O$, 10.0; $Al_2(SO_4)_3$, 28.3; $CoCl \cdot 6H_2O$, 4.0; $ZnSO_4 \cdot 7H_2O$, 2.0; $Na_2MoO_4 \cdot 2H_2O$, 2.0; $CuCl_2 \cdot 2H_2O$, 1.0; and H_3BO_4, 0.5.

For fermentation experiments, SD-7 medium was modified by reducing the ammonium and magnesium content to prevent precipitation (SD-8 base medium [Table 2]). SD-8 medium was used for batch fermentations and as the initial medium for fed-batch fermentations. The remaining nutrients added in the fed-batch fermentations were supplied in the various feed solutions by a computer-controlled scheme (described below).

Shake flask experiments. Growth characteristics and the effects of varying the acetate concentrations on these characteristics were evaluated by using a final volume of 27 ml in 300-ml flasks. The flasks were autoclaved empty and dried overnight at 85°C. Before the experiment, 25 ml of sterile SD-7 medium (containing 2 to 5 g of glucose · liter^{-1} as designated) were pipetted into each of the shake flasks, which were equilibrated to 37°C by shaking in an orbital shaker (250 rpm) for 2 h. A seed flask containing 25 ml of SD-7 medium (with 2 g of glucose · liter^{-1}) was inoculated with 1.0 ml of an overnight culture which had been grown in 5 ml of tryptic soy broth (Difco Laboratories, Detroit,

TABLE 1. *E. coli* strains and plasmid used in this study

Strain or plasmid	Genotype	Source[a]
Strains		
B	Wild type	OSU 333
CSH50	F⁻ Δ(*lac proAB*) *strA thi ara*	C. J. Daniels
DH1	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44* λ⁻	J. S. Lampel
HB101	F⁻ *hsd-20 recA13 ara-14 proA2 lac-41 galK2* Str *xyl-5 mtl-1 supE44* λ⁻	J. S. Lampel
JM105	F⁻ Δ(*lac proAB*) *lacI*^{q} *thi repsL endA1 slcB15 hadR4 traD36 proAB*Δ(ZM15)	Pharmacia
JRG1046	F⁻ *pta-39 trpR80 iclR17* λ⁻	CGSC 5992
JRG1061	F⁻ *ack-11 trpA9761 trpR72 iclR7 gal-25* λ⁻	CGSC 5993
MC1060	F⁻ Δ(*lacI-lacY*)*74 galE15 galK16 relA1 rpsL150 spoT1 hsdR2* λ⁻	CGSC 6648
W3100	F⁻ *gal* λhft	OSU 395
W3110	F⁻ [r⁻ m⁻]	OSU 389
Plasmid		
pOS4201	pKK223-3 with *denC* at *Sma*I site[b]	D. H. Dean

[a] Abbreviations: OSU, The Ohio State University culture collection; CGSC, *E. coli* Genetic Stock Center (B. J. Bachmann).
[b] The *denC* gene encodes delta endotoxin from *Bacillus thuringiensis*.

Mich.). The culture in the seed flask was grown to mid-exponential phase (Klett values of 50 to 100), and then 1.0 ml of this seed culture was used to inoculate each experimental flask.

Acetate stock solutions were prepared in concentrated form so that 0.1 to 1.0 ml of the stock solution could be added to the experimental shake flask to give the desired final concentration. The acetate stock solution contained 380.0 g of sodium acetate · liter^{-1} (270.2 g of acetate ion · liter^{-1}) so that 0.1, 0.2, 0.5, and 1.0 ml yielded final concentrations of 1, 2, 5, or 10 g of acetate anion · liter^{-1}, respectively, in 27-ml (final volume) cultures. The volumes were equalized to 27 ml by addition of sterile water. In experiments in which the acetate was added at mid-logarithmic phase, both the acetate additions and the water blanks were added after the experimental cultures had been grown to mid-exponential phase.

TABLE 2. Components of media used to grow *E. coli* in batch and fed-batch fermentations

Component	Amt component (g/liter) added to medium listed for purpose mentioned		
	SD-7 medium	SD-8 medium	
		Base and initial medium	Components added in feed solutions
NH_4Cl	7.0	7.0	28.0
KH_2PO_4	1.5	7.5	0.0
Na_2HPO_4	1.5	7.5	0.0
K_2SO_4	0.35	0.85	0.0
$MgSO_4 \cdot 7H_2O$	0.17	0.17	0.86
Trace elements[a]	0.8	0.8	3.2
Yeast extract	5.0	10.0	15.0
Glucose			
Batch	1.0–5.0	20.0	
Fed-batch		1.0	99.0

[a] Milliliters of trace elements solution, prepared as described in Materials and Methods, added per liter of medium or feed solution.

Once the flasks were equilibrated and the initial amounts of acetate were added, the turbidity was measured in a Klett-Summerson photometer to determine the background values (with a distilled water blank). Each flask was then inoculated, the contents were mixed, and 1.0 ml was removed for later analysis of initial conditions. A turbidity measurement was taken to begin the experiment. Growth of the strains in these shake flask experiments was monitored every 30 min until early stationary phase. Another 1.0-ml sample was taken to determine final conditions, and the remainder of the culture was harvested for final dry-weight determinations. Samples were either stored on ice and assayed the same day or frozen at −20°C and assayed the next day.

Fermentations. Seed cultures for fermentations were started by picking an isolated colony from a plate and transferring by loop to a 1-liter flask containing 500 ml of SD-7 medium with 2 g of glucose · liter^{-1}. Flasks were incubated overnight at 37°C at 200 rpm on an orbital shaker. The entire contents of the flask were used to inoculate the fermentor, which for batch culture conditions contained SD-8 base medium.

The fermentation hardware has been extensively described elsewhere (18, 28). Fermentors (MF-214; New Brunswick Scientific Co., Inc., Edison, N.J.) equipped with 14-liter vessels (working volume, 10 liters) were used throughout this study. Control units (MRR-1; B. Braun Biotech, Bethlehem, Pa.) were used to monitor dissolved oxygen and pH in the fermentations with polarographic dissolved-oxygen probes (model 40180-02; Ingold Electrodes, Inc., Andover, Mass.) and autoclavable pH probes (model 465; Ingold), respectively. All fermentations were carried out under the following growth conditions: temperature, 37°C; pH, direct-digital controlled at 7.0; dissolved oxygen, controlled by using the increase in agitation from 300 to 600 rpm, followed by addition of mixtures of air and pure oxygen to 1 vol/vol/min (total flow) to maintain relative dissolved oxygen above 20% of saturation (18, 28).

Glucose-feedback-controlled fed-batch fermentations. A computer-assisted on-line glucose analyzer was developed for the combined model-based and glucose feedback control of fed-batch fermentations, as described previously (18). The system consisted of the prototype model 2000 glucose analyzer (Yellow Springs Instruments, Inc., Yellow Springs, Ohio), a filtration system (Megaflow TM-10; New Brunswick) containing a 10-in^2, 0.2-μm-pore-size filter, and high-speed circulation pump (model 7520-25; Cole-Parmer Instrument Co., Chicago, Ill.). Modifications to the previously described system for this study included a higher sampling rate (2 min) and a 0.2-μm-pore-size filter (Gelman Sciences, Inc., Ann Arbor, Mich.) between the glass tee union and the two-way acrylic valve. The setpoint for glucose control in the fed-batch fermentations described herein was 1.0 ± 0.2 g · liter^{-1}. When the glucose concentration remained within this window, the pump rate remained the same.

For fed-batch fermentations, three feed solutions were used. (i) Feed no. 1 contained the following (in grams per liter): glucose, 200; $MgSO_4 \cdot 7H_2O$, 0.85; (ii) feed no. 2 contained the following (in grams per liter): glucose, 780; $MgSO_4 \cdot 7H_2O$, 8.58; and (iii) feed no. 3 contained the following (in grams per liter): NH_4Cl, 280; yeast extract, 150; it also contained 32 ml of the trace-element solution. All components of the feed solutions dissolved during autoclaving and remained in solution upon cooling. A feeding strategy was devised which began with SD-8 base medium in the vessel, with the exception that the initial glucose concentra-

tion was 1.0 g · liter^{-1} (Table 2), and feed no. 1 was added based on glucose feedback control set at 1.0 g · liter^{-1} until the culture reached about 9 g of dry cell weight (DCW) · liter^{-1}. At that point, feeds no. 2 and 3 were added in place of feed no. 1 to keep up with the requirements of the high-density fermentations. In all cases, the glucose solutions were fed at a rate based on glucose requirements calculated from previously run fermentations. Glucose feedback control was based on the on-line glucose concentration measurements as previously described (18).

Analytical procedures. Cell growth was monitored by measuring culture turbidity with a Klett-Summerson colorimeter using a red filter and by dry weight of biomass, determined as described previously (28). Off-line glucose analysis was carried out by using an analyzer (model 27; Yellow Springs Instruments) calibrated with either 2.0 or 5.0 g of glucose standards · liter^{-1}. Samples were clarified by centrifugation in a microcentrifuge and diluted in distilled water if necessary.

Fermentation broth samples were prepared for acetic acid analysis by precipitation of macromolecules at pH 2.0 by the addition of 50 μl of 70 mM H_2SO_4 per 1.0 ml of sample at room temperature. The precipitate was pelleted for 2 min in a microcentrifuge, and the supernatants were filtered through a 0.2-μm-pore-size, 13-mm-diameter filter (Gelman). The acetic acid produced in fermentation and shake flask experiments was quantitated by high-pressure liquid chromatography. The system consisted of Beckman/Altex model 100A pumps, model 420 pump controller, and model 400 solvent mixer; an injector with a 20-μl loop (model 7125; Rheodyne Inc., Cotati, Calif.); and Teflon tubing (inner diameter, 0.18 mm; Alltech Associates Inc., Deerfield, Ill.) was used throughout the system. An organic acid analysis Aminex ion-exchange column (7.8 by 300 mm; model HPX-87H; Bio-Rad Laboratories, Richmond, Calif.) was used, and organic acids were separated by using 3.5 mM H_2SO_4 at pH 2.0 at a flow rate of 0.7 ml · min^{-1} at room temperature. The elution was monitored with a Hitachi model 160-40 variable-wavelength spectrophotometer set at 210 nm, and A_{210} peaks were recorded and integrated with either a model 3390A or model 3396 integrator (Hewlett-Packard Co., Palo Alto, Calif.). Organic acid standards were prepared from reagent-grade chemicals dissolved in high-pressure liquid chromatography-grade water. Standards were injected under the same conditions as the fermentation samples, and the retention times were compared. The identification of acetate in samples of fermentation broth was confirmed by cochromatography.

RESULTS

Shake flask experiments. The growth rate, yield, final biomass, and acetate production by eight *E. coli* strains were compared after growth of the strains under identical conditions in shake flasks containing SD-7 medium plus 5 g of glucose · liter^{-1} (Table 3). Under the conditions tested, the growth rates of the strains ranged from 0.85 h^{-1} (generation time, 49 min) to 1.42 h^{-1} (generation time, 29 min), the final dry cell weights ranged from 1.16 to 1.87 g · liter^{-1}, the yields (grams of biomass per gram of glucose utilized) ranged from 0.54 to 0.77, and 0.30 to 0.92 g of acetate · liter^{-1} was produced (Table 3). The strains could be divided into two categories based on their growth rates in SD-7 medium: fast-growing strains (strains B, W3110, W3100, and CSH50) and slow-growing strains (strains JM105, DH1, MC1060, and HB101). The faster-growing strains generally produced more acetate and had lower biomass yields from glucose than did the slower-growing strains. The major exceptions to these generalized relationships were found with strain MC1060, which, although slow-growing, produced the largest amount of acetate of any strain tested, and strain B, which maintained both high growth yields and high growth rates (Table 3).

TABLE 3. Comparison of the growth parameters of several *E. coli* strains grown in shake flasks containing SD-7 medium[a]

Strain	Initial specific growth rate (h^{-1})	DCW (g/liter)	Yield (g of cells/g of glucose consumed)	Amt of acetate produced (g/liter)
B	1.42	1.76	0.63	0.79
W3110	1.29	1.34	0.57	0.50
W3100	1.13	1.61	0.54	0.79
CSH50	1.00	1.45	0.60	0.62
JM105	0.93	1.69	0.71	0.35
DH1	0.91	1.16	0.64	0.51
MC1060	0.90	1.42	0.60	0.92
HB101	0.85	1.87	0.77	0.30

[a] SD-7 medium in these experiments contained 5 g of glucose · liter^{-1} (autoclaved separately and added after cooling). The data represent the average of three separate experiments, and in no case was the standard error above 5%.

Effect of acetate additions to shake flask cultures. The addition of sodium acetate to shake flask cultures of the acetate kinase mutant, strain JRG1061, the low-acetate-producing strain, JM105, and a recombinant strain, JM105 (pOS4201), in SD-7 medium (containing 2 g of glucose · liter^{-1}) reduced the growth rates of the organisms logarithmically (Fig. 1). The *ack* (acetate kinase minus) mutant strain, JRG1061, which should have a reduced ability to assimilate acetate (9), was inhibited by acetate to the same extent as was strain JM105. Therefore, the ability with which a strain

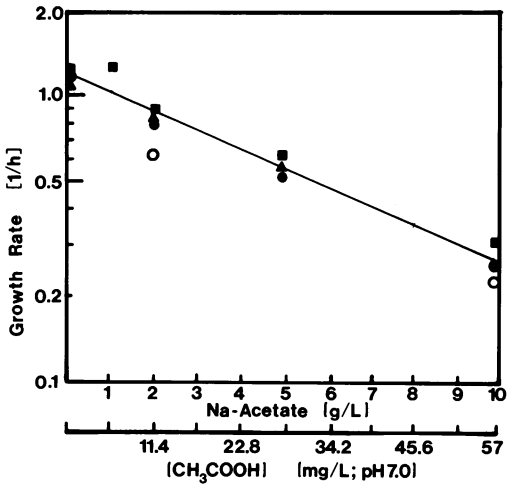

FIG. 1. Effect of exogenously added acetate concentration in SD-7 medium (containing 2 g of glucose · liter^{-1} [pH 7.0]) on the growth rates of *E. coli* strains. The acetate was added just before inoculation. Symbols: ■, JM105; ▲, strain JM105(pOS4201); ●, strain JRG1061; ○, data showing the same effect on strain JM105 of acetate added at mid-logarithmic growth phase. Each datum point represents triplicate values with less than 2% error. The line was drawn by calculated linear regression for data from strain JM105 (r = 0.9997). The concentration of the protonated form of acetate at pH 7.0 was calculated by using the Henderson-Hasselbalch equation.

TABLE 4. Comparison of the growth characteristics of several *E. coli* strains grown in batch cultures of SD-8 medium in 10-liter stirred-tank fermentors

Strain	Specific growth rate (h^{-1})	Glucose consumption rate (h^{-1})[a]	Yield (g of cells/g of glucose consumed)	Final DCW (g/liter)	Acetate production	
					Initial rate (h^{-1})[a]	Final concn (g/liter)
B	1.14	1.09	1.02	9.3	0.50	1.75
JRG1046	1.02	1.38	0.61	9.6	0.71	1.60
JRG1061	0.89	0.56	0.33	8.3	0.39	3.03
JM105	0.79	0.44	0.64	10.8	0.62	1.20
MC1060	0.77	0.89	0.56	8.0	0.78	5.12
JB101	0.64	0.85	0.73	5.4	0.57	0.88

[a] Calculated as slope = log [(grams of acetate per liter at time point T_2 − grams of acetate per liter at time point T_1)/(time point T_2 − time point T_1)], which yields h^{-1}.

metabolized acetate did not apparently influence the inhibitory effect of acetate on that strain. Similarly, the growth rate of the recombinant strain, JM105(pOS4201), was inhibited to the same extent by the added acetate, indicating that the presence of the plasmid did not alter the level of toxicity of acetate to strain JM105 (Fig. 1). Moreover, the inhibition of growth of *E. coli* by acetate was independent of the age of the culture (Fig. 1). When sodium acetate was added at mid-exponential phase to make 10 g · $liter^{-1}$ (final concentration), both the growth rate and yield (grams of DCW per gram of glucose utilized) of strain JM105 were reduced by more than 50%, compared with control cultures in which only buffer was added at the same time (data not shown).

Batch fermentations. Four strains were further compared in 10-liter batch fermentations with SD-8 medium: (i) a slow-growing, high-acetate producer (strain MC1060); (ii) a slow-growing, low-acetate producer (strain HB101); (iii) a fast-growing, high-acetate producer (strain B); and (iv) a fast-growing, relatively low-acetate producer (strain JM105). In addition to these four strains, two *E. coli* mutant strains defective in acetate metabolism, JRG1046 and JRG1061 (15), were included in these batch fermentation experiments.

With the six strains of *E. coli* that were grown in SD-8 base medium in batch fermentations, wide ranges of growth rates (0.79 to 1.14 h^{-1}), glucose consumption rates (0.44 to 1.09 h^{-1}), acetate production rates (0.39 to 0.78 h^{-1}), biomass yield on glucose (0.33 to 1.02 g · g^{-1}), and amount of acetate produced (0.88 to 5.12 g · $liter^{-1}$) were observed. The growth rates of the six strains grown in SD-8 base medium in batch fermentation (Table 4) were 15 to 25% lower than the growth rates for the same strains grown in SD-7 medium in shake flasks (Table 3). There was generally an inverse relationship between the growth rates and the yields from glucose, which was similar to the shake flask data (Table 3) and data obtained in other investigations (10). One exception to these trends was that strain B maintained a high biomass yield from glucose during rapid growth. Unexpectedly, strains MC1060 and JRG1061 produced large amounts of acetate while growing relatively slowly.

Fed-batch fermentations. The growth parameters and acetate production of four strains were evaluated under glucose feedback-controlled conditions in which the glucose setpoint was 1.0 ± 0.2 g · $liter^{-1}$. The actual glucose control was not quite as tight as that set by the computer and was usually within 1.0 ± 0.5 g · $liter^{-1}$; this was due to the changes in growth rates by the strains used during the fermentations. Under these glucose-controlled conditions, strain JM105 grew to 31 g · liter of DCW $liter^{-1}$ (Fig. 2A) with a biomass yield of 0.42 g of DCW · g of glucose $consumed^{-1}$ and a productivity of 3.47 g of DCW · $liter^{-1}$ · h^{-1}. The initial growth rate was 0.69 h^{-1} for the first 5 h; however, the growth rate continually declined for the last 4 h of the experiment. Acetate was accumulated by JM105 to a final concentration of about 2 g · $liter^{-1}$.

In fed-batch cultures, strain B grew at a rate of 1.03 h^{-1} during the initial growth phase, and growth continued to about 30 g of DCW · $liter^{-1}$ (Fig. 2B). Because of the rapid growth, the proportional control of the feed pumps could not maintain the feed rate to meet the glucose demand of the culture, and therefore the culture became glucose limited by 3.5 h. Biomass productivity was 3.75 g of DCW · $liter^{-1}$ · h^{-1}, and about 2 g of acetate · $liter^{-1}$ was accumulated. However, during the period of glucose limitation, the acetate produced at the beginning of the experiment was consumed. Once relieved from glucose limitation, after about 6 h of growth, acetate again began to accumulate.

Only 9.1 g (DCW) of strain HB101 · $liter^{-1}$ was produced under fed-batch culture conditions similar to those described for strains JM015 and B (Fig. 2C). Glucose concentration was well controlled near the setpoint, and, similarly to strains JM105 and B, only about 2.0 g of acetate · $liter^{-1}$ was produced. The accumulation of acetate continued throughout the experiment at a nearly constant rate of 0.2 h^{-1}.

Strain MC1060 grew to about 10 g of DCW · $liter^{-1}$ in 9 h (Fig. 2D). Even though good control of glucose concentration was achieved, 8 g of acetate · $liter^{-1}$ was accumulated. The rate of acetate accumulation by strain MC1060 in fed-batch cultures was similar to that of batch fermentations (Table 4), but because of the longer period of growth in fed-batch cultures, more acetate was accumulated.

DISCUSSION

Growth of *E. coli* strains in batch and fed-batch fermentations. Of the six strains of *E. coli* grown in batch fermentations, strain JM105 had the lowest glucose consumption rate and a relatively low growth rate, and it produced the second smallest amount of acetate of all strains tested, which may explain why cell densities of JM105 were higher than those of the other K-12 derivatives tested. Strain JM105 was unique in that a biphasic growth curve was measured during batch fermentations, while the glucose consumption rate remained constant (18). A similar biphasic growth curve was observed previously in the high-cell-density growth of other *E. coli* strains (1).

Strain B grew at the highest rate and had the highest biomass productivity of all strains tested. Strain B has been used to produce the highest recorded cell densities of *E. coli* in fed-batch fermentations (125 g · $liter^{-1}$ [20]; 90 g · $liter^{-1}$ [22]), whereas there is only one report of an *E. coli* K-12 derivative grown as dense as 78 g · $liter^{-1}$ in fed-batch fermentations (1). On the other hand, in a comparison of the high-density growth of a few *E. coli* strains, Bauer and Ziv (7) found that *E. coli* W, a strain unrelated to *E. coli* K-12 (5), achieved both higher biomasses and higher growth rates than *E. coli* B grown at 30°C under identical high-cell-density fed-batch growth conditions.

Strains HB101 and MC1060 did not grow to densities greater than 10 g of DCW · $liter^{-1}$ under fed-batch conditions similar to those which supported 30 g of strains JM105 and B · $liter^{-1}$ (Fig. 2). Since the fed-batch cultures of HB101, JM105, and B produced approximately the same amount of acetate, other genetic factors probably were

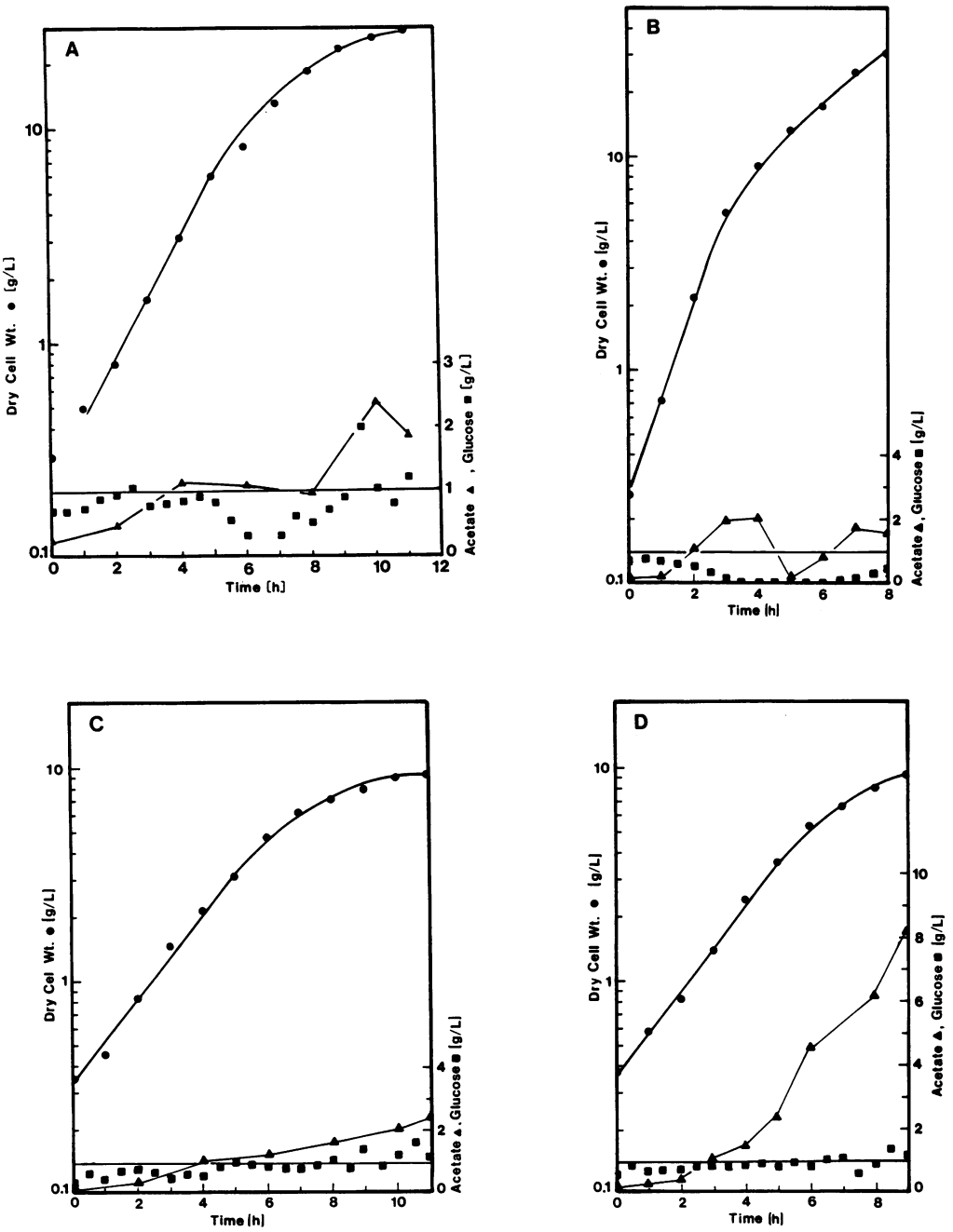

FIG. 2. Growth of *E. coli* strains in SD-8 medium in glucose feedback-controlled fed-batch fermentations. In all cases, the soluble glucose setpoint was controlled at 1.0 g · liter^{-1} (horizontal line). Symbols: ●, DCW (grams per liter); ■, soluble glucose concentration (grams per liter); ▲, soluble acetate concentration (grams per liter). (A) Strain JM105; (B) strain B; (C) strain HB101; (D) strain MC1060.

involved in the limitation of the growth of HB101. On the other hand, the fed-batch culture of strain MC1060 accumulated large amounts of acetate, even though glucose concentrations were maintained at low levels. In previous studies with other *E. coli* strains, the production of acetate was limited by controlling glucose at concentrations slightly higher than the levels maintained in this work (1). The acetate production rate exhibited by strain MC1060, however, was only slightly higher than that of other strains which did not accumulate as much acetate (Table 4). In batch culture fermentations of strain MC1060, the level of acetate did not decrease once the glucose was completely consumed (data not shown), indicating that the accumulation of acetate to higher concentrations in MC1060 cultures may be due to reduced enzyme activity in the acetate reassimilation pathway. Once glucose has been consumed, most *E. coli* cultures reutilize acetate (1, 4, 9, 12) by an activated tricarboxylic acid cycle (2, 10).

Acetate production by acetate metabolism mutants. The *ack* (acetate kinase-minus) strain, JRG1061 (15), accumulated substantial amounts of acetate, which was expected since *ack* mutants were previously shown to accumulate at least as

much acetate as wild-type strains did (9). The production of acetate by an *ack* strain was probably due to the spontaneous hydrolysis of acetyl phosphate, which occurs at relatively high rates at 37°C under physiological conditions (9). Similarly, the absence of acetate kinase would possibly slow the reassimilation of acetate by strain JRG1061. Even though acetyl coenzyme A (acetyl-CoA) synthetase is considered to be the primary route for acetate assimilation (21), the maximal velocity of acetyl-CoA formation by this enzyme is too low to support the growth of *E. coli* (9). Moreover, acetyl-CoA synthetase is inhibited by glucose (9), suggesting that the acetate kinase-phosphotransacetylase route may be important in the reassimilation of acetate, albeit with low affinity (9).

The production of 1.6 g of acetate · liter^{-1} by the *pta* (phosphotransacetylase-minus) mutant strain, JRG1049, was unexpected since Brown et al. (9) differentiated *pta* and *ack* mutants based on the inability of the former to produce acetate. It is possible that reversal of the acetyl-CoA synthetase reaction is responsible for this acetate production; however, since acetyl-CoA synthetase is repressed by glucose (9), it should not have been active under the growth conditions used. Acetyl-CoA synthetase mutants have not yet been isolated (21), so the acetate production by a combined *pta* and acetyl-CoA synthetase mutant cannot be measured as yet.

Inhibition of growth by acetate. It is well established that *E. coli* cultures growing aerobically in the presence of excess glucose produce acidic by-products, particularly acetate (1, 3, 4, 8–11, 17, 19, 20, 22, 23, 26, 27). This glucose-mediated aerobic acidogenesis is known as the bacterial Crabtree effect (10, 25). The formation of acetate has been suggested to be caused by an imbalance between glucose metabolism and respiration (3, 13), a condition in which influx of carbon into the cell exceeds demands for biosynthesis (12), the presence of excess NADH (10), the repression of tricarboxylic acid cycle enzymes (2, 14, 25), or uncoupled metabolism (26). It is likely that all of these interrelated causes are involved.

The acetate produced by *E. coli* cultures growing on excess glucose has been suggested to cause an increased uncoupling in metabolism (26, 27). Because of its weak lipophilic nature, the protonated form of acetate is able to cross the cell membrane (6, 24) and act as an uncoupler of the proton motive force (specifically ΔpH) (24, 26, 27). This toxic effect has been clearly demonstrated at pH values at or below the pK_a of the acid (24, 29); however, evidence now suggests that acetate is toxic to cell growth at neutral pH when sufficient amounts accumulate in the medium (1, 4, 17, 20, 22, 26). Acetate exists at neutral pH in both the ionized (CH_3COO^-) and protonated (CH_3COOH) states. The more lipophilic protonated form can pass through the lipid membrane to the interior of the cell, where it dissociates at the higher internal pH (ca. 7.5) to CH_3COO^- and H^+, thereby decreasing the intracellular pH (24, 27). When the protonated acid moves across the membrane into the cells, additional protonated acid is formed in the medium by the equilibrium, causing a net electroneutral hydrogen ion influx (24). The overall external pH would not change drastically, because of the large volume of buffered medium, so the decrease in intracellular pH would cause an uncoupling effect. The homeostatic mechanisms of *E. coli* require energy to adjust to this decrease in intracellular pH, even in the absence of an overall change in proton motive force (27). Thus for rapid growth, *E. coli* apparently requires not only a large $\Delta\tilde{\mu}_{H^+}$, but also maintenance of an optimum intracellular pH (27).

Our results suggest two phenotypes of *E. coli* with respect to acetate metabolism: (i) strains that produce acetate and then reassimilate it once glucose is depleted (e.g., strains JM105 and B), and (ii) strains that produce acetate, but cannot reassimilate it (e.g., strain MC1060 and JRG1061). The growth rates of strains from both groups were inhibited equally by acetate. Thus, we have developed a growth model based on the data obtained for acetate inhibition of growth rate at pH 7.0. Growth in the presence of acetate is mathematically defined as follows:

$$X = X_0 e^{\mu' t} \quad (1)$$

where X is the biomass concentration (in grams per liter), t is time, X_0 is the initial biomass, and μ' is the instantaneous growth rate evaluated as a function of acetate inhibition. The amount of acetate produced was calculated as a function of the biomass concentration by using the following equation:

$$A = [k_1 \cdot (X - X_0)] - A_0 \quad (2)$$

where A is the acetate concentration (in grams per liter), A_0 is the initial acetate concentration, and k_1 is the stoichiometric production constant (grams produced per gram of biomass). Acetate inhibition (I) of the growth rate at pH 7.0 was evaluated from the relationship shown in Fig. 1 as follows:

$$I = k_2 A/\mu \quad (3)$$

where k_2 is the logarithmic function of growth inhibition versus acetate concentration and μ is the rate of growth in the absence of acetate. The new growth rate is defined as follows:

$$\mu' = I \cdot \mu \quad (4)$$

Iterative calculations of equations 1 through 4 were carried out with the initial conditions of $X_0 = 0.1$ g · liter^{-1} and $A_0 = 0.0$ g · liter^{-1} and the following parameters: $\mu = 1.28$ h^{-1}; $k_1 = 0.5$ g of acetate produced · g of DCW^{-1} (1); and $k_2 = 10^{(-0.06A + 0.107)}$. Each iteration was taken to be 1 h of growth. The biomass and acetate production predicted by this model are given in Fig. 3 and are typical of the results of our batch fermentations. In the first 4 h, the inhibition of growth by acetate is negligible. After 4 to 5 h, when the acetate concentration would be 5.3 to 9.8 g · liter^{-1}, the inhibition of growth by acetate becomes apparent. By using the Henderson-Hasselbalch equation and a pH of 7.0, the calculated concentration of the protonated form of acetate would be 30 to 56 mg · liter^{-1} at 4 and 5 h, respectively. This model fits the data for growth of MC1060 (Fig. 2D), for which the rate of growth appears constant until acetate has accumulated to approximately 7 g · liter^{-1} (ca. 40 mg of the protonated form · liter^{-1}), at which point the growth of MC1060 was visibly affected. This acetate inhibition model and our data together suggest that acetate is inhibitory very early in the growth of the culture, but that the inhibitory effect is not visualized until acetate has accumulated to ca. 5 g · liter^{-1} (ca. 29 mg of CH_3COOH · liter^{-1} [pH 7.0]). This level of total acetate is similar to the inhibitory levels reported by Landwall and Holme (17) and Doelle and co-workers (10, 11, 16). Similarly, Baskett and Hentges (6) found that ca. 45 mg of undissociated acetate · liter^{-1} caused a logarithmic decrease in the number of viable cells of *Shigella flexneri* and that 1.8 mM (108 mg · liter^{-1}) undissociated acetate completely inhibited the growth of *S. flexneri*. Weiner and Draskoĉzy (29) also found that ca. 125 mg of protonated acetate · liter^{-1} reduced the oxidative metabo-

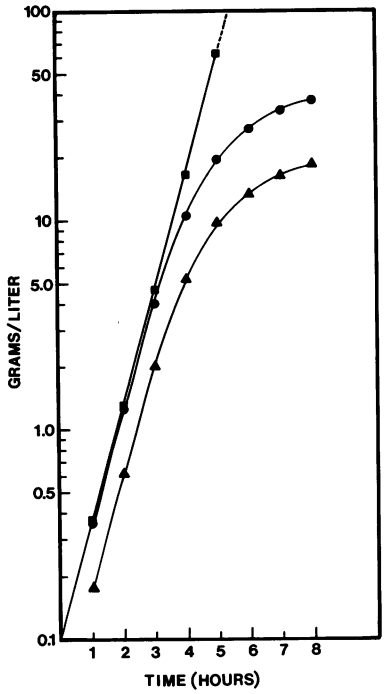

FIG. 3. Acetate inhibition model based on data presented in Fig. 2 and derived from equations 1 through 4 in the text. Symbols: ■, DCW (grams per liter) of cultures not inhibited by acetate (control); ●, DCW of cultures inhibited by acetate; ▲, concentration of acetate (grams per liter).

lism of an *E. coli* strain by 20%. Considering our data and information from the literature (6, 24, 27, 29), it appears that concentrations of undissociated acetic acid above ca. 30 mg · liter^{-1} would visibly inhibit the growth rate of *E. coli*.

Use of process control to eliminate the bacterial Crabtree effect. The ability of microorganisms to uncouple catabolism from anabolism is very important to those interested in process control (1, 4, 13, 18, 20, 30). It is becoming apparent that the physiological state of the host cell greatly affects the expression of cloned products and thereby the productivity of the fermentation process (8, 25, 30). Specifically, there appears to be a link between the accumulation of acetate caused by the bacterial Crabtree effect and several important factors of rDNA fermentations, such as final cell concentration, cell yield, genetic stability, and product stability (30).

Even though the level of glucose in our fed-batch fermentations was controlled at about 1.0 g · liter^{-1}, high levels of acetate were still produced by most of the cultures tested. Doelle and co-workers (11, 16) have suggested that both rapid growth and high glucose levels contributed to the bacterial Crabtree effect. Using turbidostat cultures at constant biomass, Doelle et al. (11) found that switchover from normal coupled respiration to the bacterial Crabtree effect occurred at 1.5 to 2 g of glucose · liter^{-1}. Our data suggest that in rapidly growing fed-batch cultures of *E. coli*, the switchover threshold concentration of glucose may be lower than 1.0 g · liter^{-1}. Growth inhibition due to the buildup of these toxic by-products can be reduced either by controlling the amount of carbon substrate available by increasingly sophisticated fed-batch techniques (12, 18, 20) or by removing the by-product from the culture broth once formed by using dialysis and filtration techniques (17). We are currently developing an on-line adaptive control fed-batch process that maintains glucose at 0.40 ± 0.05 g · liter^{-1} (G. L. Kleman, J. Chalmers, and W. R. Strohl, manuscript in preparation). It is hoped that this system, in conjunction with changes in other parameters such as the controlled pH value, will be able to control fed-batch cultures in such a way as to reduce or eliminate the bacterial Crabtree effect.

It is apparent from the data shown herein that each strain of *E. coli* responds differently to its environment and that the growth characteristics of the strains differ greatly under similar fermentation conditions. These differences should be taken into account when choosing the host strain for rDNA product fermentation. Concurrently, more efficient fermentation procedures that maintain glucose at near-limiting levels must be developed to eliminate the bacterial Crabtree effect by strains that are highly sensitive to glucose concentration.

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