Journal of Industrial Microbiology & Biotechnology (2001) 27, 322–328

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Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated *solR* gene

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The effect of *solR* inactivation on the metabolism of *Clostridium acetobutylicum* was examined using fermentation characterization and metabolic flux analysis. The *solR*-inactivated strain (SolRH) of this study had a higher rate of glucose utilization and produced higher solvent concentrations (by 25%, 14%, and 81%, respectively, for butanol, acetone, and ethanol) compared to the wild type. Strain SolRH(pTAAD), carrying a plasmid-encoded copy of the bifunctional alcohol/aldehyde dehydrogenase gene (*aad*) used in butanol production, produced even higher concentrations of solvents (by 21%, 45%, and 62%, respectively, for butanol, acetone, and ethanol) than strain SolRH. Clarithromycin used for strain SolRH maintenance during SolRH(pTAAD) fermentations did not alter product formation; however, tetracycline used for pTAAD maintenance resulted in 90% lower solvent production. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 322–328.

Keywords: *Clostridium acetobutylicum*; metabolic engineering; flux analysis; solvents; *solR*; tetracycline; clarithromycin

Introduction

Clostridium acetobutylicum, a Gram-positive, spore-forming, obligate anaerobe, has long been of industrial interest for the production of the solvents acetone and butanol from renewable sources [8]. Due to its ability to catabolize a wide range of substrates into solvents, *C. acetobutylicum* was in widespread use internationally for fermentative production of acetone and butanol between 1910 and 1960. However, unprecedented growth of the petroleum industry during the middle of the 20th century led to a drastic decline in industrial *C. acetobutylicum* fermentations since it was more economical to produce solvents from petroleum-based sources [8]. Metabolic engineering of this organism to construct superior solvent-producing strains may be the key to a resurgence of industrial-scale clostridial fermentations.

While the majority of genes directly responsible for formation of acids and solvents in this strain have been cloned and studied, less is known about mechanisms for controlling gene expression in solventogenic clostridia. Recently, a putative repressor of solvent formation genes, SolR, was identified and a molecular characterization of *solR*-modified strains was reported [16]. Inactivation of the *solR* gene (located on the pSOL1 megaplasmid) [6], using genomic integration of a non-replicative plasmid, resulted in the generation of strains which produce higher levels of solvents. Preliminary characterization of one of these strains (renamed SolRB, formerly Mutant B) included a single batch fermentation [16]. The present study focuses on detailed fermentation characterization and metabolic flux analysis [2] of a second strain (SolRH; formerly Mutant H) and a derivative of that strain [SolRH(pTAAD)]. The latter strain carries an additional plasmid pTAAD-encoded copy of the *aad* gene, which encodes a bifunctional butanol formation enzyme [15]. SolRH was selected as the host strain in this study since it has a defined genetic modification; one non-replicative plasmid is integrated into the chromosome of SolRH in contrast to strain SolRB, which has multiple non-replicative plasmids integrated into its chromosome [16]. Additionally, the tetracycline minimum inhibitory concentrations (MICs) in SolRH was less than 5 μg/ml compared to 10 μg/ml in SolRB (data not shown). Thus, use of the tetracycline resistance plasmid pTAAD (as reported here) was more practical in strain SolRH. Strain SolRH(pTAAD) was constructed in order to determine if an increased *aad* gene dosage would further enhance the solvent-producing capabilities of strain SolRH. The use of the antibiotics clarithromycin and tetracycline for selection was also examined to examine their effects on product formation.

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Received 12 September 2000; accepted 21 July 2001

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 1.

Growth conditions

Escherichia coli was grown aerobically at 37°C in Luria–Bertani (LB) medium, and *C. acetobutylicum* was grown anaerobically at 37°C in Clostridium Growth Medium (CGM [18,22]). Colonies of *E. coli* and *C. acetobutylicum* were obtained on agar-solidified LB or Reinforced Clostridial Medium (RCM; Difco, Sparks, MD), respectively. For recombinant strains, liquid media were appropriately supplemented with erythromycin (Em; 100 μg/ml), tetracycline (Tc; 10 μg/ml), and chloramphenicol (Cm; 35 μg/ml); 40 μg/ml of erythromycin and 10 μg/ml of tetracycline were used in solid media as needed. For bioreactor fermentations,

Table 1 Bacterial strains and plasmids

Strain/Plasmid	Relevant characteristics[a]	Source or reference
Strains		
C. acetobutylicum		
ATCC 824		ATCC[b]
SolRH (formerly Mutant H)	ATCC 824 *solR*::pO1X MLS^r (single copy of plasmid integrated into genome), MIC 5 μg/ml	[16]
SolRB (formerly Mutant B)	ATCC 824 *solR*::pO1X MLS^r (multiple copies of plasmid integrated into genome), MIC 10 μg/ml	[16]
E. coli		
ER2275	*rec*A *lac*Z *mcr*BC	NEB[c]
Plasmids		
pAN1	Cm^r, Φ3TI	[13]
pAM620	Tc^r	[19]
pIM13	MLS^r	[16]
pIMP1	Ap^r MLS^r	[10]
pJC4	MLS^r Tc^r	[10]
pO1X	MLS^r	[16]
pTAAD	Ap^r Tc^r *aad*	This study
pTLH1	Ap^r Tc^r	This study

[a]Abbreviations: *solR* putative repressor of *sol* operon genes; MLS^r, macrolide lincosamide streptogramin B-resistant; *rec*A, homologous recombination abolished; *lacZ*, β-galactosidase; *mcrBC*, methlycytosine-specific restriction system; Cm^r, chloramphenicol-resistant; Φ3TI, Φ3T methylase; Tc^r, tetracycline-resistant; Ap^r, ampicillin-resistant; AAD, *aad*, alcohol/aldehyde dehydrogenase.
[b]American Type Culture Collection, Manassas, VA.
[c]New England Biolabs, Beverly, MA.

erythromycin was substituted by its pH-stable derivative clarithromycin (100 μg/ml). *E. coli* strains were stored long-term at -85°C in LB medium with 10% glycerol. For long-term storage of *C. acetobutylicum*, strains were maintained as spores on RCM agar at a pH of 6.8, or frozen at -85°C in CGM with 15% glycerol.

Plasmid DNA isolation and manipulation

The isolation of plasmids from *E. coli via* the alkaline lysis method and further manipulations of *E. coli* plasmid DNA were performed using standard protocols [9]. A modified alkaline lysis method was used for isolation of plasmid DNA from *C. acetobutylicum* [7]. A previously published method [14] was used for electrotransformation of *C. acetobutylicum* with methylated plasmid DNA [13] using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA).

Construction of plasmids

Plasmid pTAAD was generated by cloning a 3.06-kb DNA fragment containing *aad* gene with its two promoters into the tetracycline-resistant *E. coli–C. acetobutylicum* shuttle vector pTLH1 [7]. These tetracycline-resistant vectors were suitable for use in the MLS^r (macrolide lincosamide streptogramin B-resistant) clostridial strains.

Fermentations and analytical methods

Fermentations of *C. acetobutylicum* strains and product concentration analysis were performed as previously reported [3,7]. Batch fermentations were performed in either a BiostatM (Braun Biotech, Allentown, PA) or a BioFloII bioreactor (New Brunswick Scientific, Edison, NJ) with working volumes of 1.5 and 4.0 l, respectively. After inoculating the growth medium (CGM pH 6.2), the culture pH was allowed to fall to 5.0, at which point it was controlled through addition of 6 M NH_4OH. Approximate measurement errors (SEM) were: glucose, acetone, ethanol, butanol, and acetoin, 3%; acetate and butyrate, 5%.

Metabolic flux analysis

Metabolic flux analysis involves the calculation of specific (per unit cell mass) *in vivo* intracellular reaction rates for various enzymatic reactions (which will be referred to as "fluxes"). These fluxes [in units of mmol $(g\ cells)^{-1}\ h^{-1}$] were calculated from substrate utilization and product formation data using a system of linear equations developed from the metabolic reaction stoichiometry, a widely used and validated technique [1–3,7,17]. A mass extinction coefficient of 51 $(g\ cells)^{-1}\ cm^{-1}$ was used to convert optical density measurements (A_{600}) into cell dry weight concentrations ($g\ cells\ l^{-1}$). The flux analysis was performed using software developed specifically for analysis of *C. acetobutylicum* fermentation data [2,17], and the pathways fluxes considered are depicted in Figure 1. For these analyses, the fermentation times were re-scaled [1,3,7]: the normalized scale, T_N, is set such that $T_N=0$ h at $A_{600}=1$. On this time scale, the transition from exponential phase to stationary phase typically occurs at $T_N=10$ h. Therefore, the stages of the fermentations are classified as "Early" for $T_N<10$ h, and "Late" for $T_N>10$ h. The calculation of both kinetic and integral fluxes and the errors of such calculations have been discussed previously [1,3,7].

Results and discussion

Strain SolRH [16] and newly developed strain SolRH(pTAAD) were characterized using fermentation experiments and metabolic flux analysis to determine the impact of two genetic alterations, the

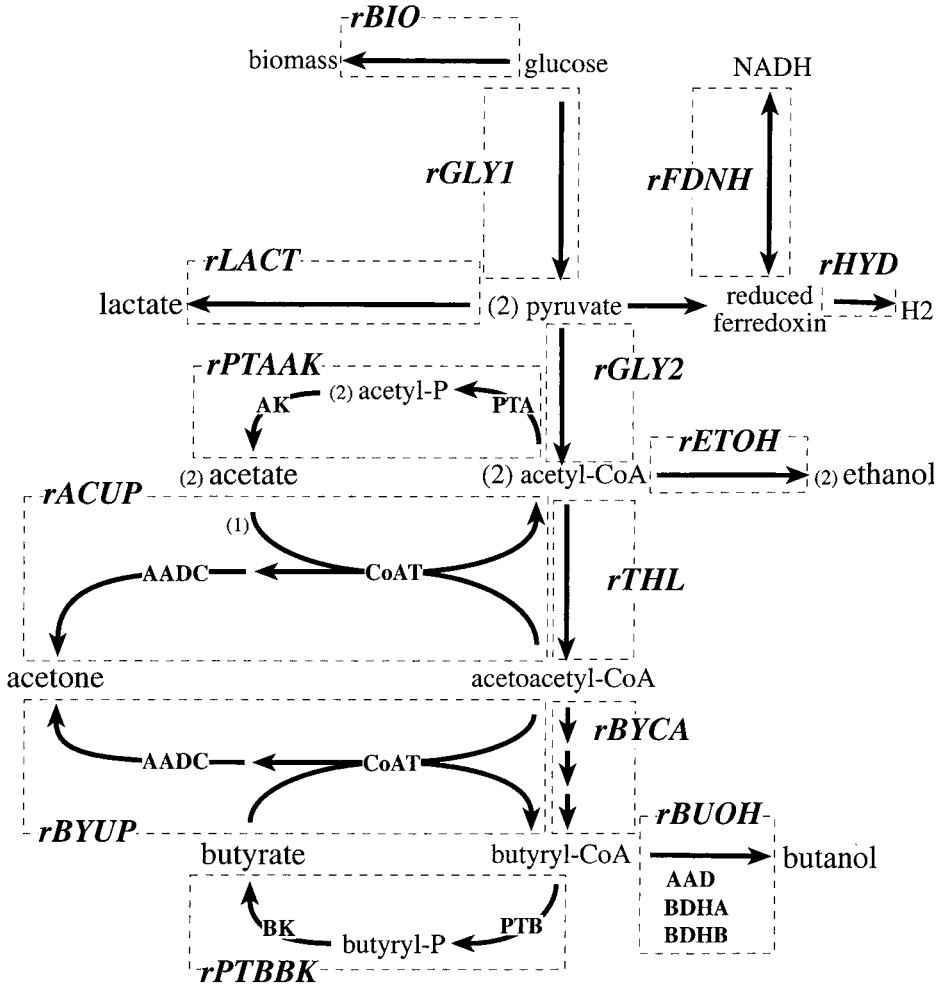

Figure 1 *C. acetobutylicum* primary metabolic pathways and corresponding *in vivo* fluxes. The conversion between major carbon containing species is depicted without cofactors. Selected enzymes are shown in bold and abbreviated: PTA, phosphotransacetylase; AK, acetate kinase; CoAT, CoA transferase; AADC, acetoacetate decarboxylase; PTB, phosphotransbutyrylase; BK, butyrate kinase, AAD, alcohol/aldehyde dehydrogenase (DH); BDHA and BDHB, butanol DHG isozymes A and B. Reactions involved with the intracellular fluxes (e.g., rBIO and rHYD) examined here are indicated by dashed boxes. Further details on these metabolic fluxes can be found in Refs. [2,3,7,17].

solR inactivation alone and in combination with increased *aad* gene dosage.

Fermentation and flux analysis of solRH versus wild type (WT): effect of solR inactivation

Fermentation characterizations showed that inactivation of *solR* in strain solRH resulted in altered product concentration profiles compared to the WT organism, *C. acetobutylicum* ATCC 824 (Table 2). Although peak butyrate levels did not differ significantly between strains SolRH and WT, final SolRH butyrate concentrations were 68% lower than WT values (13 *vs.* 41 mM). Both maximum and final acetate levels were slightly higher in SolRH compared to WT. *solR* inactivation had a profound effect on solvent formation, resulting in final butanol, acetone, and ethanol concentrations that were 25%, 14%, and 81% higher, respectively, than WT strain concentrations.

Metabolic flux analysis of the fermentation data was used to further characterize strain SolRH. The key pathway fluxes (i.e., specific intracellular reaction rates) discussed (Figure 1) are rGLY1 (glucose utilization), rPTAAK and rPTBBK (acid formation pathways), rACUP, rBYUP (acid re-utilization), and rBUOH (butanol formation). Strain SolRH exhibited a consistent elevation in rGLY1 from T_N=0 to 5 h (Figure 2A). This increased flux of glucose utilization was also reflected by elevated acid formation fluxes (rPTAAK and rPTBBK) from T_N=0 to 5 h compared to the WT strain (Figure 2B and C). In addition, strain SolRH exhibited an earlier reversal (indicated by negative flux values) of flux through the butyrate formation pathway (rPTBBK). The most dramatic effect of the *solR* inactivation was on solvent formation fluxes. Acetone formation flux is the sum of the acetate re-utilization flux (rACUP) and the butyrate re-utilization flux (rBYUP) and showed significantly higher peak levels compared to the WT strain. The acetate re-utilization flux, rACUP, in strain SolRH reached a peak value twice that of the WT strain (Figure 2D). The butyrate re-utilization flux, rBYUP, in strain SolRH reached a peak value up to 60% higher than in the WT (Figure 2E). In addition, the peak in rBYUP was 5 h earlier in strain SolRH than in the WT strain. However, the elevated acetone formation fluxes in strain SolRH were sustained for a shorter period of time than in the WT strain. The butanol formation flux reached a peak up to 30% higher (but

Table 2 Product formation in fermentation experiments at pH 5.0

Fermentation characteristics	Strains			
	WT (control)	SolRH	SolRH(pTLH1) (control)	SolRH(pTAAD)
Doubling time (h)	1.2±0.02	1.4±0.01	2.5±0.02	2.1±0.02
Max A_{600}	8.8±1	9.4±1	12±1	11±1
Butanol (mM)	158±4	197±12	195±8	238±13
Acetone (mM)	85±4	97±4	99±1	141±20
Ethanol (mM)	16±1	29±4	18±3	47±1
Acetate (mM)				
Peak	71±5	84±2	67±2	87±2
Final	60±4	68±9	65±4	85±4
Butyrate (mM)				
Peak	76±4	74±6	79±2	97±2
Final	41±2	13±3	20±6	12±2

Data are shown as mean±SEM values from two experiments. Glucose was fed in strain SolRH(pTAAD) fermentations to prevent glucose depletion and premature termination of the fermentation.

was sustained for a shorter period of time) in strain SolRH than in the WT strain (Figure 2F).

In addition to the time profiles, an integral flux analysis (see Materials and Methods; data not shown) further reinforced the observations discussed above, but also showed significant differences that are not apparent from the kinetic analysis: the central pathways fluxes (rGLY1, rGLY2, rTHL, and rBYCA) were twice as high, and acid formation fluxes, rPTAAK and rPTBBK, were 120% and 840% higher, respectively, in strain SolRH than in the WT strain. Solvent formation fluxes, rACUP, rBUOH, and rETOH, were *ca.* 130%, 150%, and 400%, respectively, higher in strain SolRH. While acetone formation *via* butyrate re-utilization

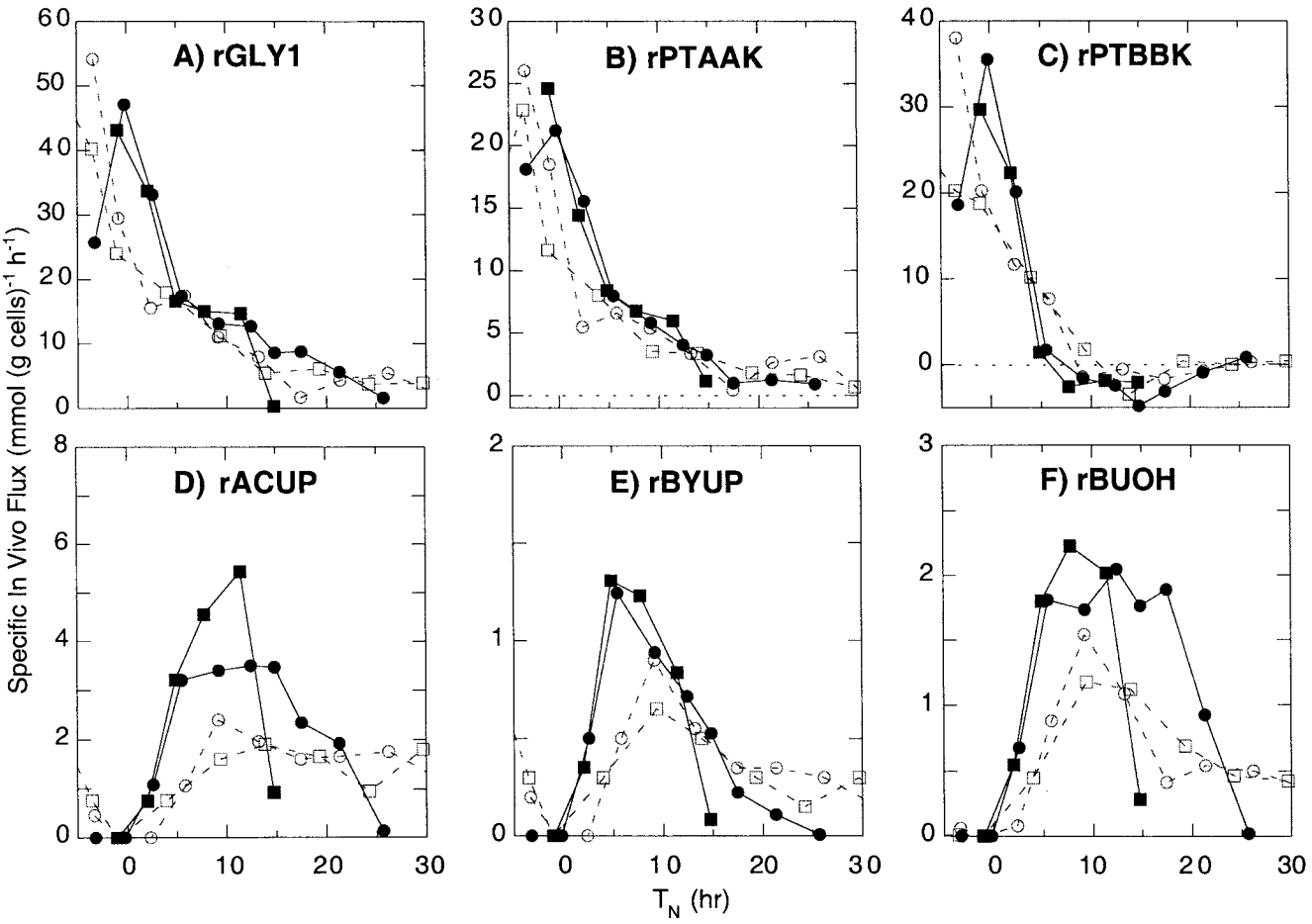

Figure 2 Time course profiles of metabolic fluxes in cultures of strain SolRH (solid symbols) and of the WT (open symbols). Different symbols represent data from the two replicate experiments of Table 2.

(rBYUP) was elevated by *ca.* 60%, this pathway is responsible for only a small amount of the carbon reutilization in strain SolRH. The calculated metabolic pathway fluxes were used to estimate changes in the split ratio at the butyryl−CoA branchpoint. During the stationary phase of the WT strain, the central pathway (rBYCA) provided 82% of the carbon used in butanol formation, while rBYUP and rPTBBK provided 10% and 8%, respectively. In contrast, in strain SolRH, rBYCA provided 65% of the carbon, while rPTBBK provided 28% of the carbon used for butanol formation. In fact, this reversal of the butyrate formation pathway is largely responsible for the 150% increase in rBUOH, while glucose utilization increased by only 100%.

Effects of tetracycline and clarithromycin on product formation

Initially, two antibiotics were used for maintenance of *C. acetobutylicum* strains SolRH(pTLH1): the control strain carrying a plasmid without the cloned *aad* gene and SolRH(pTAAD). Clarithromycin (100 μg/ml), a stable derivative of erythromycin, was used to select for the host strain SolRH, and tetracycline use (10 μg/ml) assured presence of the replicative plasmids pTLH1 and PTAAD. Clarithromycin resistance was conferred by the presence of the non-replicative plasmid integrated into the genomic *solR* [16], while tetracycline resistance was conferred by the *tetM* gene on the multicopy vectors pTLH1 and pTAAD. During fermentations, the use of two antibiotics coincided with a drastic decrease in growth and solvent production. Thus, experiments were designed to determine the effects of tetracycline and clarithromycin use separately and together (Table 3).

When tetracycline was used during SolRH(pTAAD) fermentations, cell densities were decreased, peak butyrate concentrations were increased by over 35%, while final butyrate values exceeded the control values by over 1000% (Table 3). Peak and final acetate concentrations were not significantly affected. Tetracycline inhibition of solvent production was severe: butanol, acetone, and ethanol concentrations were decreased by 92%, 94%, and 90%, respectively, compared to the no-antibiotic control (Table 3).

Table 3 Tetracycline (Tc) and clarithromycin (Clt) effects on strain SolRH(pTAAD) product formation

Parameter or compound	Number of experiments			
	2	1	1	1
Antibiotic	Tc+Clt	Tc	Clt	None
Doubling time (h)	2.2±0.05	2.3	1.8	1.8
Max OD	5.5±0.4	5.2	11	11
Butanol (mM)	4.4±16	17	209	216
Acetone (mM)	20±9	7	109	119
Ethanol (mM)	5±0.5	4	40	46
Acetate (mM)				
Peak	132±10	84	83	88
Final	115±7	83	83	82
Butyrate (mM)				
Peak	146±2	118	88	74
Final	118±9	118	2	10

Fermentation experiments were carried out at pH 5.0. There was no glucose fed into these fermentations. For the duplicate experiment, values are reported as mean±SEM.

When tetracycline and clarithromycin were used in combination, acetate and butyrate levels were both significantly elevated. Final butanol, acetone, and ethanol concentrations were all reduced by *ca.* 80%, and cell densities significantly decreased (Table 3).

Patterns of product formation when clarithromycin alone was used during the SolR(pTAAD) fermentation were similar to the control fermentation with no selective pressure (Table 3).

These results indicate that the use of clarithromycin to select for SolRH(pTAAD) does not alter product formation patterns, growth rates, or cell densities in this organism. In contrast, the data show that the use of tetracycline in SolRH(pTAAD) fermentations, alone and in combination with clarithromycin, inhibits solvent production and enhances acidogenesis. Although tetracycline is useful for selection of plasmid-carrying strains, use of this antibiotic drastically reduces solvent production and is therefore not appropriate for use in fermentations for solvent production. Loss of this family of plasmids during batch fermentations of recombinant *C. acetobutylicum* strains in the absence of antibiotics as a selection pressure is known to be minimal [11].

Another example of inhibition of product formation in the presence of tetracycline involves the use of strain 34, a derivative of *C. acetobutylicum* ATCC 824 with a single chromosomal insertion of the tetracycline resistance conjugative transposon Tn916. When tetracycline was used to select for strain 34, no butyraldehyde dehydrogenase activity was detected and no butanol was produced (C. Cass, unpublished results). Tn916 has been used extensively for generating clostridial strain mutants, including solvent formation mutants (e.g., Ref. [12]). Our data suggest that the use of tetracycline-resistant Tn916 in generating solvent formation mutants may be inappropriate. Use of tetracycline and its derivatives in various microbial systems inhibits several types of enzymes, including some dehydrogenase systems [4]. While the mechanism by which tetracycline affects product formation is not proven, it is possible that tetracycline interferes with the dehydrogenase activity required for solvent production in recombinant strains of *C. acetobutylicum* such as SolRH(pTAAD).

Fermentation and flux analysis of strains solRH (pTAAD) versus solRH(pTLH1): effect of plasmid-encoded aldehyde alcohol dehydrogenase

Strain SolRH(pTAAD) was characterized in order to determine the effect of pTAAD-encoded aldehyde alcohol dehydrogenase [5] on product formation in strain SolRH (Table 2). No antibiotics were used during SolRH(pTLH1) fermentations. However, selective pressure was applied for maintenance of strains SolRH(pTAAD) and SolRH(pTLH1) until the time that reactors were inoculated at the start of each fermentation. Comparative plating studies (data not shown) on selective and non-selective media indicated that the plasmid-carrying strains were stable throughout the course of fermentations without selective pressure.

Fermentations of strain SolRH(pTAAD) were performed at pH 5.0 based on other studies showing that pH=5.0 resulted in higher solvent production than either pH 4.7 or 5.5 (data not shown). These SolRH(pTAAD) cultures were supplemented with glucose during mid to late exponential growth to avoid premature termination of solvent production due to glucose exhaustion. Concentrated glucose (3.8 M) was added when the residual glucose concentration dropped below 150 mM in order to keep glucose

concentration above 100 mM until solvent production began to plateau. Equivalent glucose supplementation of either the WT or SolRH(pTLH1) strains did not affect solvent production (data not shown). The results of the SolRH(pTAAD) fermentations were compared to the control strain SolRH(pTLH1), which was developed to account for host–plasmid effects [21] using a plasmid without a cloned clostridial gene. Inactivation of *solR*, combined with increased *aad* gene dosage, resulted in a strain [SolRH(pTAAD)] with improved solvent-producing ability. At final concentrations of 248 mM (17.6 g/l) butanol, 141 mM (8.2 g/l) acetone, and 47 mM (2.2 g/l) ethanol, total solvent production (28 g/l) by SolRH(pTAAD) far exceeded the 19 g/l solvents produced by the WT. It should be noted that *aad* overexpression in the WT strain (ATCC 824) did not result in increased solvent formation [14]. This suggests that the inactivation of *solR* is necessary for the positive effect of *aad* overexpression on solvent production.

Maximum and final acetate concentrations produced by SolRH(pTAAD) were 30% higher than control values. Peak butyrate levels were 23% higher in SolRH(pTAAD) than in the control; however, final butyrate concentrations were 40% lower. This implies better re-utilization of butyrate by strain SolRH(pTAAD). Solvent production was significantly increased in strain SolRH(pTAAD) fermentations compared to control [SolRH(pTLH1)] fermentations. Final butanol, acetone, and ethanol concentrations were elevated by 22%, 42%, and 161%, respectively. While the SolRH(pTAAD) fermentation doubling time was 16% lower than the doubling time of the control, maximal optical densities did not vary significantly.

Kinetic metabolic flux analysis (Figure 3) indicated noticeable differences between strains SolRH(pTAAD) and SolRH(pTLH1). Glucose utilization in SolRH(pTAAD) was transiently elevated at the shift to the stationary phase (Figure 3A). A similar trend was observed in the other central pathway fluxes (data not shown). A stationary phase elevation was also observed in rPTAAK fluxes, but not in rPTBBK fluxes (Figure 3B and C). Instead, the butyrate formation pathway supported increased butyrate re-utilization. As with strain SolRH, solvent formation fluxes showed the most dramatic differences. The peak acetone formation fluxes were evaluated in strain SolRH(pTAAD) by up to 200% and 35% for rACUP and rBCUP, respectively, compared to strain SolRH(pTLH1) (Figure 3D and E). The peak butanol formation flux, rBUOH, was elevated by up to 200% during the stationary phase (Figure 3F), and the ethanol formation flux was significantly elevated (data not shown). It should also be noted that while strain SolRH(pTAAD) exhibited higher peak fluxes, strain SolRH(pTLH1) sustained the fluxes for a longer period of time.

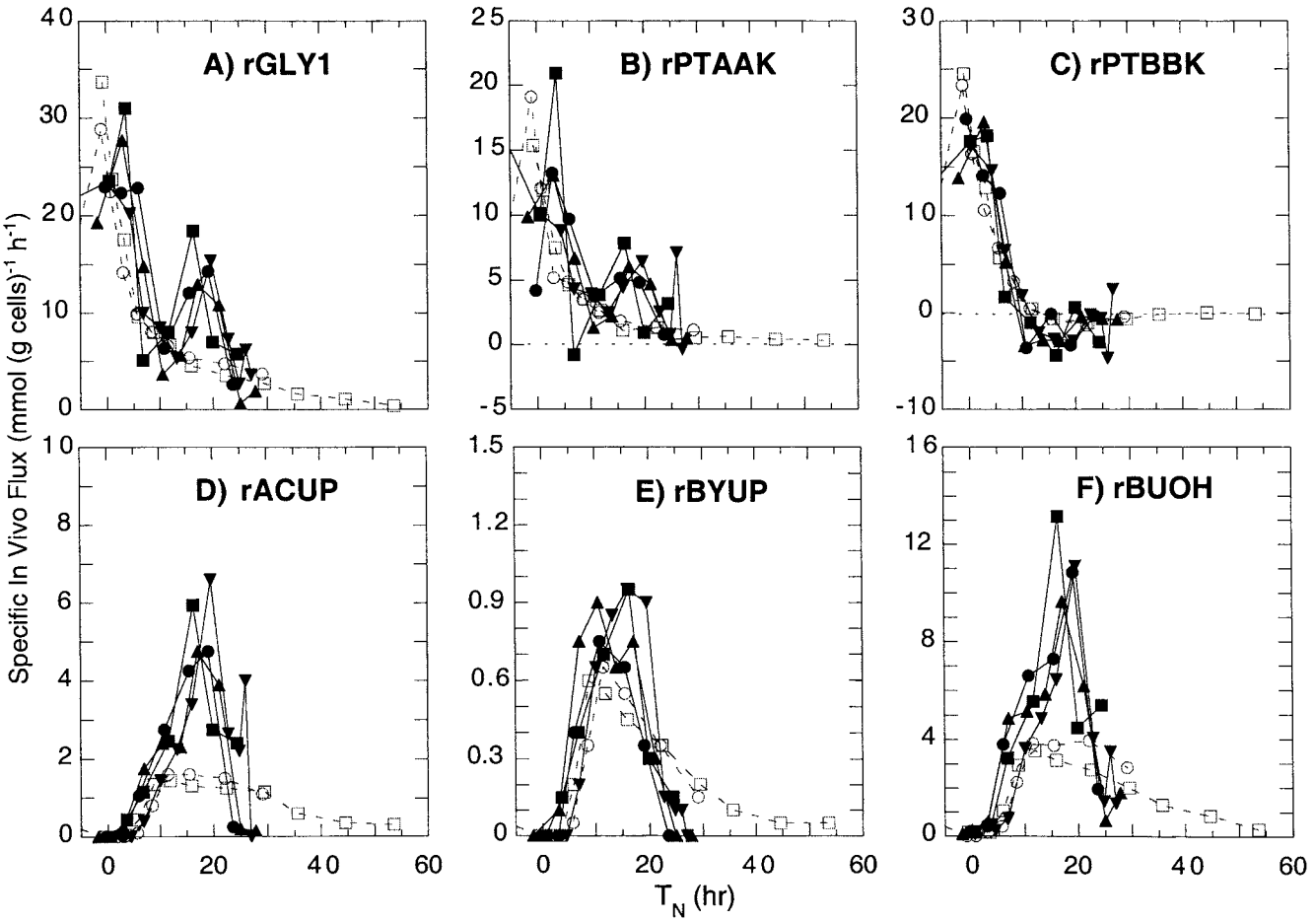

Figure 3 Time course profiles of metabolic fluxes in strains SolRH(pTAAD) (solid symbols) and SolRH(pTLH1) (open symbols). Different symbols represent data from the two replicate experiments of Table 2. For strain SolRH(pTAAD), data from two additional replicates (not shown on Table 2) are also plotted.

As before, integral flux analysis (data not shown) was used to detect differences between SolRH(pTAAD) and SolRH(pTLH1) that are less noticeable from the kinetic flux analysis. After the shift to stationary phase, The differences between strains SolRH(pTAAD) and SolRH(pTLH1) were most pronounced: glucose utilization (rGLY1) and the cental pathways (rGLY2, rTHL, and rBYCA) were *ca.* 100–130% higher, and the acid formation fluxes, rPTAAK and rPTBBK, were elevated by 180% and 225%, respectively, in strain SolRH(pTAAD). The acetone formation fluxes, rACUP and rBYUP, were elevated by 160% and 70%, respectively, although rBYUP played a lesser role than rPTBBK in butyrate re-utilization. Finally, the alcohol formation fluxes, rBUOH and rETOH, were elevated by 125% and 370%, respectively.

The results of the fermentation characterizations and the metabolic flux analyses clearly illustrate the potential for rational design of superior solvent-producing strains through manipulation of key regulatory and product formation genes. Although the putative role of *solR* as a transcriptional repressor is now being challenged [20], our results show that *solR* plays a significant role in regulating solvent formation.

Acknowledgements

Supported by National Science Foundation grant BES-9905669, and National Institutes of Health Predoctoral Biotechnology Training grant GM-08449. We thank Abbott Laboratories (Abbott Park, IL) for donation of clarithromycin.

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