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Effects of mutations in acetate metabolism on high-cell-density growth of *Escherichia coli*

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To study the role played by acetate metabolism during high-cell-density growth of *Escherichia coli* cells, we constructed isogenic null mutants of strain W3100 deficient for several genes involved either in acetate metabolism or the transition to stationary phase. We grew these strains under identical fed-batch conditions to the highest cell densities achievable in 8 h using a predictive-plus-feedback-controlled computer algorithm that maintained glucose at a set-point of 0.5 g/l, as previously described. Wild-type strains, as well as mutants lacking the σ^s subunit of RNA polymerase (*rpoS*), grew reproducibly to high cell densities (44–50 g/l dry cell weights, DCWs). In contrast, a strain lacking acetate kinase (*ackA*) failed to reach densities greater than 8 g/l. Strains lacking other acetate metabolism genes (*pta, acs, poxB, iclR,* and *fadR*) achieved only medium cell densities (15–21 g/l DCWs). Complementation of either the *acs* or the *ackA* mutant restored wild-type high-cell-density growth. On a dry weight basis, *poxB* and *fadR* strains produced approximately threefold more acetate than did the wild-type strain. In contrast, the *pta, acs,* or *rpoS* strains produced significantly less acetate per cell dry weight than did the wild-type strain. Our results show that acetate metabolism plays a critical role during growth of *E. coli* cultures to high cell densities. They also demonstrate that cells do not require the σ^s regulon to grow to high cell densities, at least not under the conditions tested. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 421–430.

Keywords: acetate metabolism; acetate mutants; glucose-controlled high cell density fermentation; fed-batch fermentation; rpoS

Introduction

Escherichia coli remains the best established and most valuable host system for expression of recombinant proteins [23,33]. Nevertheless, several physiological aspects of its growth (e.g., the propensity to accumulate extracellular acetate) limit the ability to achieve optimal expression and accumulation of several recombinant proteins under high-cell-density growth conditions [2,15,23,33]. Aerobic cultures growing at or near μmax excrete acetate as a result of "glucose overflow" metabolism [14,19,23,25,28,33]. This acetate excretion occurs when the carbon flux into central metabolic pathways exceeds the cells' biosynthetic demands and the capacity for energy generation [9,14,32]. It is generally thought that saturation of the tricarboxylic acid (TCA) cycle and/or the electron transport chain results in such overflow metabolism [10,23,34]. Cultures grown to normal densities, such as those grown in Luria–Burtani (LB) medium, generally result in maximum concentrations of 1–2 g of acetate per liter. On the other hand, cultures grown to high-cell-density (i.e., in excess of ca. 40 g dry cell weight (DCW)/l) [23,28,33] accumulate acetate to concentrations greater than 8 g/l [19,23].

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Acetate concentrations above ca. 1 g/l are toxic to both the growth of recombinant *E. coli* strains and their production of heterologous proteins [1,2,6,13–15,26,34]. Acetate specifically inhibits the consumption of both glucose and oxygen [19,34] and inhibits specific growth rate (μ) noncompetitively, with a K_i of 9 g/l [34]. To avoid these inhibitory effects, researchers have tried a wide variety of strategies to reduce acetate accumulation in high-cell-density fed-batch fermentations. These include various glucose feeding strategies [17–20,23], limitation of growth rate by substrate-limited fed-batch schemes [21,23], utilization of alternative feeds such as glycerol [12,23], and use of *E. coli* strains lacking phosphotransacetylase, one of the key enzymes involved in acetate production [2,6,12]. Recombinant methods also have been used. In one case, carbon flow from pyruvate was diverted by recombinant *Bacillus subtilis* acetolactate synthase to acetolactate, which was converted to the noninhibitory waste product, acetoin [1]. In a second recombinant approach, acetate production was reduced fourfold by redirecting the carbon flux through overexpression of the gene encoding phosphoenolpyruvate carboxylase and constitutive expression of the glyoxylate bypass through deletion of one of its regulators, *fadR* [10].

Acetate production from the metabolic intermediate, acetyl-SCoA, occurs only through the activities of phosphotransacetylase (Pta) (phosphate acetyltransferase; EC 2.3.1.8) and acetate kinase (AckA) (EC 2.7.2.1). Metabolic utilization of acetate, on the other hand, occurs via activation of acetate to acetyl-SCoA by two distinct pathways. One pathway also utilizes the enzymes AckA and Pta, both of which possess K_m values for their substrates in the 7–10 mM range [4,22]. The second pathway, generally considered glucose-repressible and acetate-inducible, uses the high affinity (K_m for acetate of 200 μM) enzyme acetyl-SCoA

synthetase (Acs) (acetate:CoA ligase [AMP forming]; EC 6.2.1.1) to scavenge low concentrations of acetate [22]. We observed previously that glucose-fed high-cell-density cultures of *E. coli* strain W3100 go through a "metabolic switch", in which the cultures change from a glucose-consuming, acetate-producing state, through a short pause, to an acetate-plus-glucose consuming state [19]. Concomitant with this metabolic switch, we observed a fourfold increase in the activity of isocitrate lyase (ICL), the first enzyme of the glyoxylate shunt (GS) [19]. While the underlying mechanism for this metabolic switch has not been elucidated, our results indicated that acetate metabolism played a critical role during fermentation to high cell densities. To investigate this acetate-associated metabolic switch further, we generated a collection of isogenic mutants of *E. coli* strain W3100, each lacking a different acetate-associated enzymatic activity (*pta*, *ackA*, *acs*, *poxB*, *iclR*, *fadR*, or *rpoS*). We compared the growth of each mutant strain to that of its isogenic wild-type parent during glucose-controlled high-cell-density fermentation (GC-HCDF). We also monitored their excretion and resorption of acetate. Our data indicate that all aspects of acetate metabolism play integral roles in growth of *E. coli* to high cell densities. The σ^s regulon, in contrast, seems to be unnecessary.

Materials and methods

Organisms and inoculum preparation

All strains used are derivatives of the *E. coli* K-12 strain W3100 (ATCC 14948) and are listed in Table 1. Isogenic derivatives were constructed by generalized transduction with phage P1kc [31]. All strains were maintained at -70°C for long-term storage and at 4°C on agar plates containing solidified Luria–Burtani (LB) medium between experiments. When appropriate, plates contained 10 μg of kanamycin/ml.

Plasmids pSR30 and pKK7 carry the wild-type alleles of *acs* and *ackA*, respectively, each under the control of its native promoter [22]. Complementation tests were performed by introducing each plasmid via transformation into cells deleted for either *acs* or *ackA*. To maintain selective conditions, transformants were maintained on LB agar plates containing 50 μg of ampicillin/ml.

Fed-batch fermentations

The glucose feedback computer-controlled system and the optimized algorithm parameters (proportional constant [K_c], 0.5; number of points used in determining the rolling linear regression, 5) used for high-cell-density fed-batch fermentations was described in detail by Kleman *et al.* [17] and updated by Kleman and Strohl [19] and Kleman *et al.* [20].

Table 1 *E. coli* strains used in this study

Strain	Relevant genotype	Source or reference
W3100	F$^-$ gal hft	ATCC 14948
AJW1387	W3100 Δ*acs*::Km	This study
AJW1388	W3100 *pta*::Tn*phoA*′-9	This study
AJW1392	W3100 *ackA*::Tn*phoA*′-2	This study
AJW1483	W3100 Δ*rpoS*::Km	This study
AJW1507	W3100 *fadR*::Tn*5*	This study
AJW1508	W3100 *poxB*::Tn*5*	This study
AJW1509	W3100 *iclR*::Tn*5*	This study

A 5 ml volume of LB liquid medium was inoculated from LB agar plates and incubated for 8 h at 35°C with shaking at 200 rpm on a rotary shaker. The entire volume of this culture was then used to inoculate seed cultures, consisting of 500 ml of trypticase soy broth. These seed cultures were then incubated for 16 h at 35°C with shaking at 200 rpm on a rotary shaker before being used to inoculate 6 l of fermentation broth in the fed-batch fermentation system. The composition of this fermentation broth has been described previously [17,19,20]. The composition of the four feed solutions added during fed-batch fermentation were as follows: (i) a 2-l solution of glucose (70 g/l); (ii) a 2-l solution of glucose (375 g/l) and $MgSO_4 \cdot 7H_2O$ (0.85 g/l); (iii) a 2-l solution of glucose (780 g/l) and $MgSO_4 \cdot 7H_2O$ (0.85 g/l); and (iv) a 1-l solution of casein hydrolysate (CE90M, Deltown, Fraser, NY) at 400 g/l, glucose (375 g/l) and trace elements [17,18] at 32 ml/l. The feed pumps were calibrated and the feed solutions added to the vessel as described by Kleman *et al.* [17]. To maintain selective pressure on plasmids during fermentation, ampicillin was added with the inoculum to a final concentration of 100 mg/l. An additional 500 mg of ampicillin was added to the fourth feed solution [20].

The conditions for fed-batch fermentations were: temperature, 35°C; pH, 7.0, adjusted with NaOH as required; soluble glucose level, controlled on-line at 0.5 g/l as previously described [19,20]; gas flow to the fermentor, 10 l/min; agitation rates, initially 400 rpm, increased manually during early stages of the fermentation to 600 rpm to maintain dissolved oxygen (dO_2) at 70% relative to saturation. When a dO_2 of 70% relative to saturation was not achievable using an agitation rate of 600 rpm and 10 l of air/min, pure O_2 was added to the inlet gas as needed to maintain the set-point dO_2. dO_2 never decreased below 50% in any of the fermentations described.

We stopped all fermentations at 8 h (i.e., shortly after the metabolic switch from net acetate production to net acetate utilization [19]). At 9 h, under these growth conditions, dissolved oxygen began to influence the results. At this point, even the addition of 20 l/min of pure O_2 to the fermentor could not keep dO_2 levels above 50% relative to saturation [17–20].

The figures and data shown in this paper represent duplicate samples derived from representative fermentations for each condition. Each fermentation experiment was reproduced in triplicate.

Culture and broth analysis

DCW was measured for each time point as described previously [17–20]. Fermentation broth samples were prepared for acetic acid analysis by precipitation of macromolecules at pH 2.0 with an equal volume of 0.02 N H_2SO_4 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 (dia) filter (Gelman, Ann Arbor, MI).

Acetic acid produced during fermentation was quantified by high-pressure liquid chromatography (HPLC) as described previously [19]. The system consisted of a Waters (Milford, MA) 600E multisolvent delivery system and a Waters U6K injector; stainless-steel or polyetheretherketone (PEEK) (Alltech Associates, Deerfield, IL) tubing was used throughout. An HPLC water jacket (Alltech Associates) and a circulating water bath (Lauda type K2; Brinkmann, Westbury, NY) maintained the

column temperature at 55°C. An organic acid analysis Phenomenex ion-exchange column (7.8×300 mm; Phenomenex, Torrance, CA) was used. Organic acids were separated using a mobile phase of 0.01 H_2SO_4 (pH 2.0) at a flow rate of 0.7 ml/min. The elution was monitored with a Waters 486 variable-wavelength spectrophotometer set at 210 nm, and the A_{210} was recorded and integrated with Waters Baseline 810 software running on an Nec 386SX computer with an 80387X math coprocessor. Organic acid standards were prepared from reagent grade chemicals dissolved in HPLC-grade water. Standards were injected under the same conditions as the fermentation samples, and the retention times were compared.

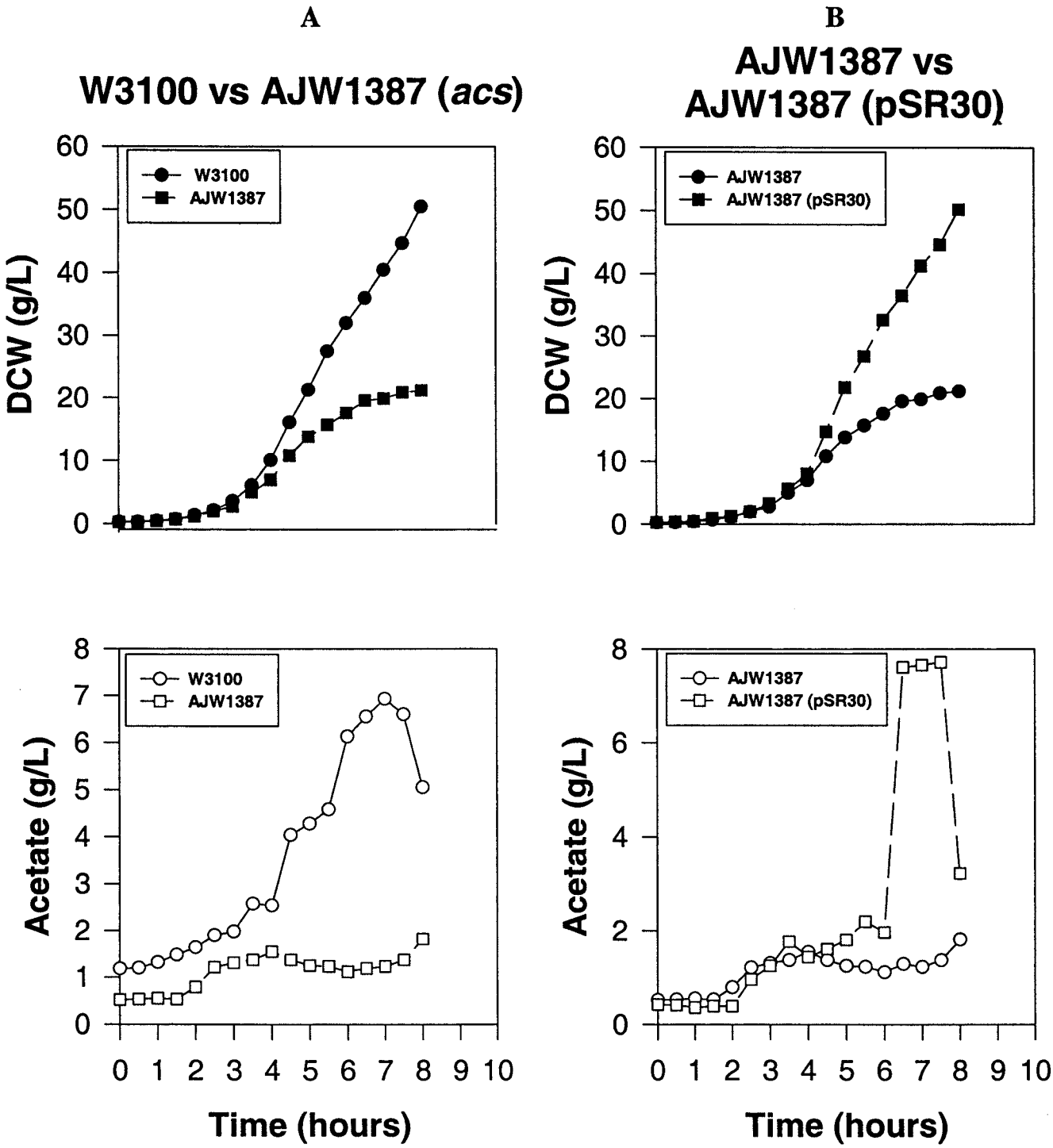

Figure 1 Abbreviations for this and all figures: DCW, dry cell weight; GC-HCDF, glucose-controlled high cell density fermentation. (A) Growth (top) and acetate accumulation (bottom) of cells: wild type (W3100) or deleted for *acs* (strain AJW1387) during growth in GC-HCDF conditions. (B) Growth (top) and acetate accumulation (bottom) of cells deleted for *acs* (strain AJW1387) or (AJW1387) transformed with a plasmid (pSR30) that carries the wild-type *acs* allele during growth in GC-HCDF conditions.

Results and discussion

Growth of the wild-type strain in glucose-controlled high-cell-density fermentation (GC-HCDF)

Growth of the wild-type *E. coli* K-12 strain W3100 during GC-HCDF using the predictive and feedback glucose-control system resulted in a biomass that reached approximately 50 g/l DCW (Figure 1A) and a maximum specific growth rate (μ_{max}) of 0.92 h^{-1} (Table 2). Acetate accumulated to a maximum of 6.9 g/l about 7 h into the fermentation (Figure 1A, Table 2) or a specific production of about 0.14 g of acetate/g DCW. Consistent with our previous studies of *E. coli* K-12-derived strains [19,20], this excreted acetate was resorbed and utilized even though glucose was still present in the feed medium.

Growth by an acs mutant in GC-HCDF

The *acs* deletion strain, AJW1387, grew at the same maximum specific growth rate as the wild-type strain W3100 (μ_{max}, 0.92 h^{-1}; Table 2) for about the first 4 h of the fermentation (Figure 1A). After that, however, it grew more slowly than did its wild-type parent, achieving less than half the final biomass (21.2 g/l). Intriguingly, this mutant produced only 1.8 g/l of acetate. An *acs* mutant grown in batch culture accumulates as much extracellular acetate as its wild-type parent (about 0.1 g/l) because it possesses an intact Pta/AckA pathway. Unlike, its parent, however, the mutant cannot resorb the acetate because it lacks the high-affinity enzyme Acs [22] and because the affinity of AckA for acetate is too low [4]. We find it surprising, therefore, that the specific production of acetate by the *acs* mutant during GC-HCDF was only half that of its wild-type parent (0.08 g/g DCW; Table 2). Although we do not understand this result, it implicates *acs* in the control of carbon flux through the Pta/AckA pathway. Complementation of the *acs* mutant strain AJW1387 by a plasmid (pSR30) that carries the wild-type *acs* allele resulted in growth (final biomass, 50.2 g/l; μmax, 0.92 h^{-1}) and acetate production and utilization (maximum, 7.7 g/l at about 7 h; specific production, 0.15 g/g DCW) that closely resembled those of its wild-type parent (Figure 1B; Table 2). Thus, Acs plays little or no role during normal density growth (up to about 4 h), yet clearly plays a crucial role in reaching optimal growth at higher cell densities.

Growth by a pta mutant in GC-HCDF

Figure 2 shows the growth and acetate profiles for a strain, AJW1388, which lacks Pta. The Pta mutant had a substantially longer lag phase and grew more slowly than its wild-type parent. Also, in contrast to its parent, the final biomass of the Pts mutant reached only 17.9 g/l and its maximum acetate production was 1.5 g/l. Thus, its specific production of acetate was 0.08 g/g DCW or about half that of its wild-type parent and very similar to that produced by the *acs* mutant. Hahm *et al.* [12] reported previously that a *pta* mutant of *E. coli* HB101 reached densities of 75 g DCW/l during HCDF. This mutant strain also produced less acetate than its parent in batch culture, and produced only ca. 1 g/l of acetate in HCDF culture.

In batch culture experiments, various *E. coli pta* mutants produce less acetate [8,12,16] and grow much more slowly than the wild-type parent [4] (AJ Wolfe, B Prüß, and S Kumari, unpublished results). One would expect a *pta* mutant to produce considerably less acetate since the Pta/AckA pathway represents the primary pathway for acetate production from acetyl-SCoA [7,22]. Alternatively, the low levels of acetate excreted by *pta* mutants could result from pyruvate oxidase (PoxB), which catalyses the decarboxylation of pyruvate to acetate [5,7]. This seems unlikely, however, since cells do not induce *poxB* until the stationary phase [5] and because cells that lack Pta, yet retain PoxB, do not accumulate significant amounts of acetate during growth in batch culture [27].

Growth by an acka mutant in GC-HCDF

The *ackA* null mutant, strain AJW1392, grew the poorest of all the acetate metabolism mutant strains. In multiple attempts, final dry weights never exceeded 10 g/l (Figure 3A; Table 2) although the μ_{max} was only slightly depressed (0.85 h^{-1}) relative to the wild-type parent (0.92 h^{-1}). Although the maximum acetate concentration was low like that of the *acs* and *pta* mutants (1.4 g/l), the specific production of acetate (0.18 g/g DCW) resembled that of the wild type. This acetate likely derives from decomposition of the Pta/AckA pathway intermediate, acetyl-phosphate (AcP), which is labile at physiological pH (4). Alternatively, it could originate via PoxB, as mentioned previously.

Complementation of the *ackA* mutant with a plasmid (pKK7) that carries the wild-type *ackA* allele restored mostly wild type phenotype to *ackA*-deficient cells (Figure 3B; Table 2). The

Table 2 Comparison of *E. coli* mutant strains grown in fed-batch fermentations

Strains	Genotype	DCW[a] (g/l)	μmax (1/h)	Acetate produced (max g/l)[b]	Acetate produced per g DCW
W3100	wild-type	50.2	0.92	6.9	0.14
AJW1387	*acs*	21.2	0.92	1.8	0.08
AJW1387 (pSR30)	*acs/acs*$^+$	50.2	0.92	7.7	0.15
AJW1388	*pta*	17.9	0.92	1.5	0.08
AJW1392	*ackA*	8.0	0.85	1.4	0.18
AJW1392 (pKK7)	*ackA/ackA*$^+$	40.3	0.85	7.6	0.19
AJW1483	*rpoS*	44.3	0.99	1.7	0.04
AJW1509	*iclR*	15.6	0.94	2.5	0.16
AJW1507	*fadR*	16.7	nc	7.6	0.45
AJW1508	*poxB*	16.9	nc	6.6	0.39

[a]Abbreviations: DCW, dry cell weight (after 8 h of fed-batch fermentation); nc, not calculated.
[b]Maximum concentration of acetate produced prior to re-utilization (as described by Kleman and Strohl [19]).

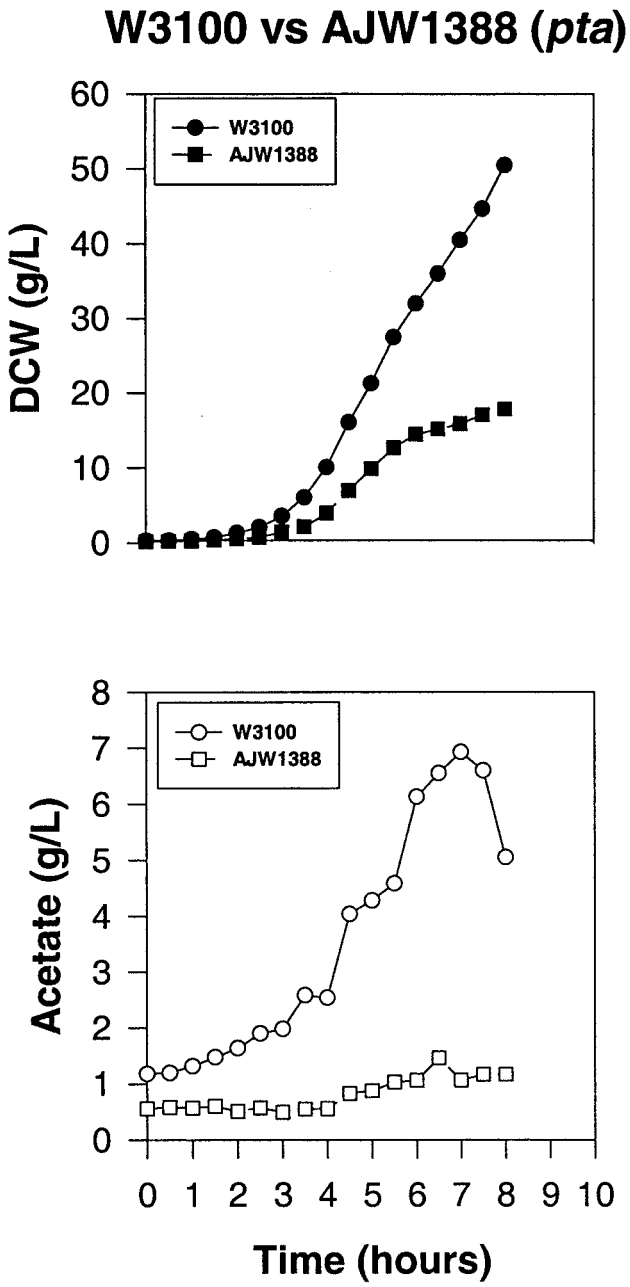

Figure 2 Growth (top) and acetate accumulation (bottom) of cells: wild type (W3100) or deleted for *pta* (strain AJW1388) during growth in GC-HCDF conditions.

final biomass reached 40.3 g/l, the maximum production of acetate was 7.6 g/l, and the specific acetate production was 0.19 g/g DCW. While μ_{max} was not restored to the wild-type level, *ackA*-complemented cells grew for a longer period than did mutant cells.

Hahm *et al.* [12] also observed that an *ackA* mutant grew more slowly during HCDF and produced less acetate than did its parent, *E. coli* HB101. Kakuda *et al.* [16] also noticed that an *ackA* mutant growing in batch culture produced significantly less acetate than did its wild type parent, JM103. However, the growth rates of both strains were similar. In our hands, *ackA* mutants grown in batch culture always produce less acetate and grow more slowly than their wild-type parents (AJ Wolfe, B Prüß, and S Kumari, unpublished results).

On the basis of measurements made previously by Prüß and Wolfe [27], we expect the *ackA* mutant to accumulate considerably more intracellular AcP than do any of the other mutants tested or their wild type parent. AcP acts as a phosphodonor for the autophosphorylation of certain two-component response regulators (e.g., OmpR, which can control flagellar synthesis [27,29] and the expression of outer membrane porins [11], or RssB (also known as SprE and MviA), which modulates the stability of σ^s [3]. The poor growth exhibited during GC-HCDF by the *ackA* mutant, therefore, may result from the simultaneous inappropriate phosphorylation of several response regulators. Since AcP levels peak during exponential growth on glycolytic substrates, e.g., glucose [27], this problem should be most acute during GC-HCDF.

Growth by an rpoS mutant in GC-HCDF

The gene *rpoS* encodes the alternative sigma factor σ^s, which controls the expression of its regulon, a battery of more than fifty genes involved in cellular stress responses, including transition to stationary phase, starvation, osmotic stress, temperature and acid shocks, and oxidative DNA damage [24]. The *rpoS* null mutant, strain AJW1483, exhibited unexpected behavior during HCDF (Figure 4A; Table 2): it grew to high cell density (44.3 g/l DCW) with the fastest μ_{max} (0.99 h^{-1}). Thus, environmental conditions within the fed-batch fermentor, which include high cell density and greater than normal osmotic stresses due to the nutrient input required to achieve those densities [19,23] seemingly do not exert enough stress to require the σ^s regulon. Surprisingly, the *rpoS* mutant produced low levels of acetate (maximum, 1.7 g/l). Thus, σ^s seems to play a significant role in production of acetate during glucose-controlled fed batch fermentation. In its absence, specific acetate production dropped almost fourfold (0.04 g/g DCW *versus* 0.14 g/g DCW for the wild-type parent). Whether this dramatically reduced acetate production results from a direct σ^s effect upon the expression or activity of Pta and/or AckA or to more global changes in carbon flux through glycolysis remains unknown. However, this reduction seems to relate to physiological conditions specific to HCDF. In batch culture, prior to resorption, *rpoS* mutant cells accumulate more extracellular acetate at a slower rate than do cells of their wild type parent. In contrast, the mutant cells resorb acetate just as rapidly as do their parent [30] (AJ Wolfe, B Prüß, and S Kumari, unpublished results).

Growth by an iclR mutant in GC-HCDF

In most respects, the IclR-deficient strain (AJW1509) behaved like the *pta* mutant (Figure 4B; Table 2). It grew to an intermediate final biomass (15.6 g/l DCW) despite a μ_{max} (0.94 h^{-1}) similar to that of its wild type parent (0.92 h^{-1}), while producing reduced levels of acetate. The specific acetate production (0.16 g/g DCW), however, was the same as for the wild type. Acetate was produced early in the fermentation and quickly resorbed.

During GC-HCDF, wild-type cells (strain W3100) increase ICL activity about fourfold as they undergo the acetate-associated metabolic switch [19], indicating that they induce the GS either as a consequence or as a cause of this switch. IclR represses the *aceBAK* operon that encodes the GS enzymes, including ICL [7].

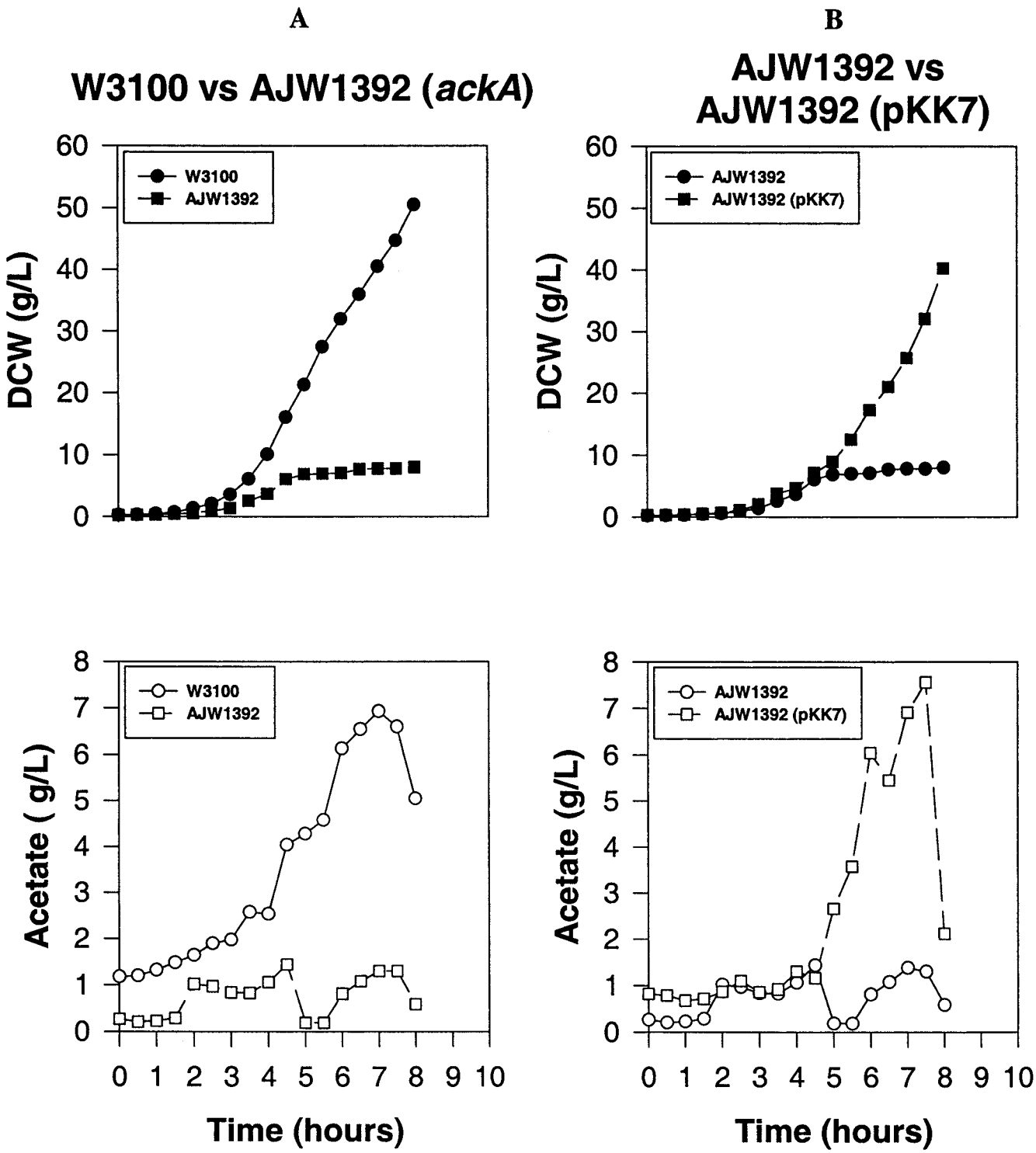

Figure 3 (A) Growth (top) and acetate accumulation (bottom) of cells: wild type (W3100) or deleted for *ackA* (strain AJW1392) during growth in GC-HCDF conditions. (B) Growth (top) and acetate accumulation (bottom) of cells deleted for *ackA* (strain AJW1392) or (AJW1392) transformed with a plasmid (pKK7) that carries the wild-type *ackA* allele during growth in GC-HCDF conditions.

Thus, cells that lack IclR express GS constitutively at elevated levels; i.e., they do not repress GS during exponential growth in batch culture [7]. We infer from our data that high-cell-density growth requires that cells keep GS repressed until they undergo the acetate-associated metabolic switch [19].

Growth by a fadR mutant in GC-HCDF

With respect to growth, the FadR-deficient mutant (strain AJW1507) behaved like the *pta* and *iclR* mutants (final biomass, 16.7 g/l DCW; Table 2). In contrast, the FadR mutant accumulated wild type levels of acetate (>7 g/l) early in

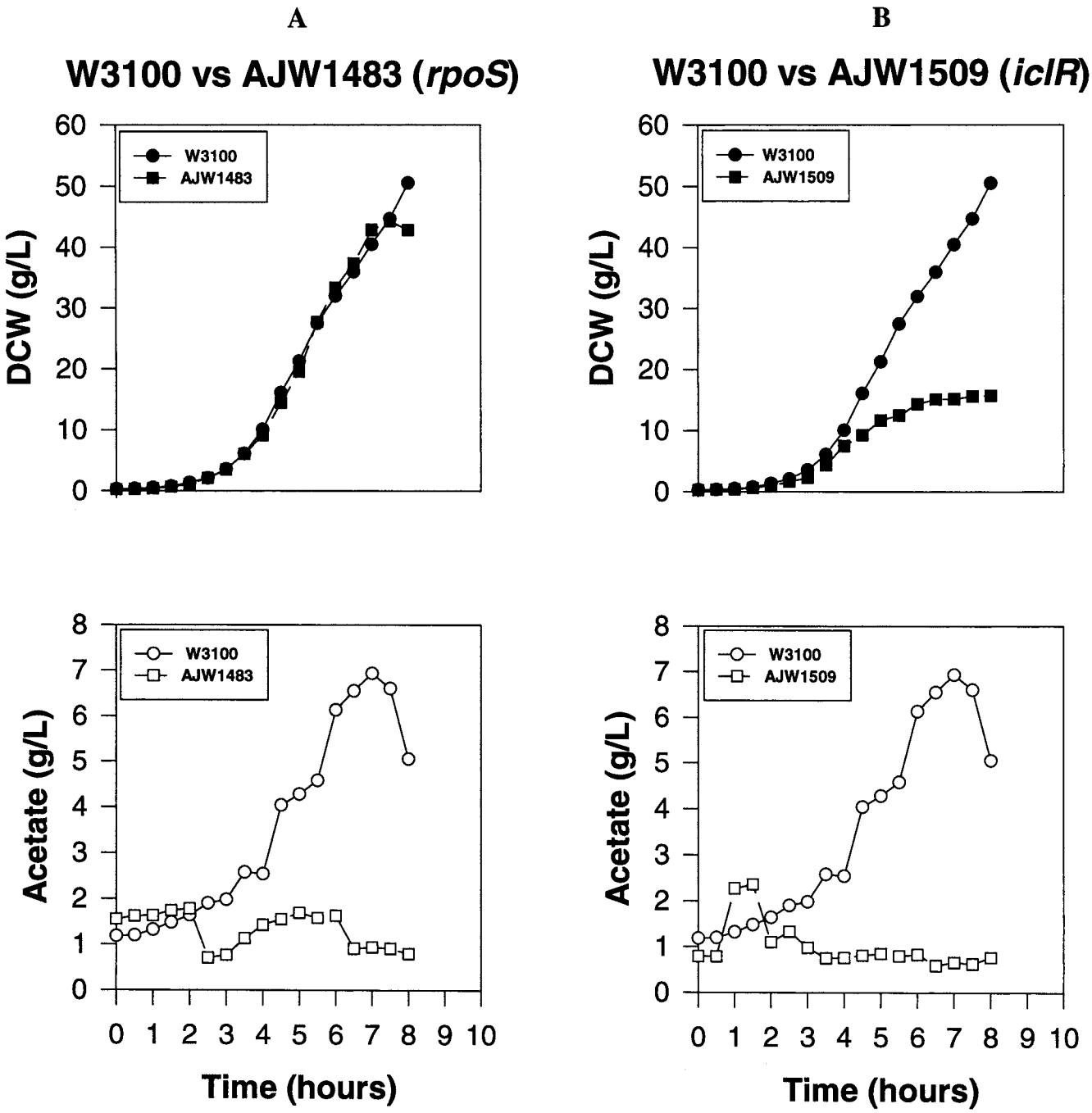

Figure 4 (A) Growth (top) and acetate accumulation (bottom) of cells: wild type (W3100) or deleted for *rpoS* (strain AJW1483) during growth in GC-HCDF conditions. (B) Growth (top) and acetate accumulation (bottom) of cells wild type (W3100) or deleted for *iclR* (strain AJW1509) during growth in GC-HCDF conditions.

fermentation during the period of μ_{max} growth, and then rapidly re-assimilated the acetate (Figure 5A). Interestingly, specific acetate production by this strain was more than threefold higher than that of wild type cells (0.45 g/g DCW *vs.* 0.14 g/g DCW, respectively; Table 2).

The behavioral difference between the *fadR* and *iclR* mutants seems surprising. Both proteins mediate repression of *aceBAK*, the operon that encodes the GS enzymes. Whereas FadR regulates *aceBAK* indirectly by acting as an activator of *iclR* transcription, *IclR* represses *aceBAK* directly [7]. Thus, the *fadR* acetate phenotype must result from some FadR-deficient consequence other than constitutive GS expression. Perhaps the mutant's ability to produce massive amounts of acetate results from the role played by FadR modulating fatty acid degradation [7].

Growth by a poxB mutant in GC-HCDF

With respect to growth, the PoxB-deficient mutant (strain AJW1508) behaved like the *pta*, *iclR*, and *fadR* mutants (final

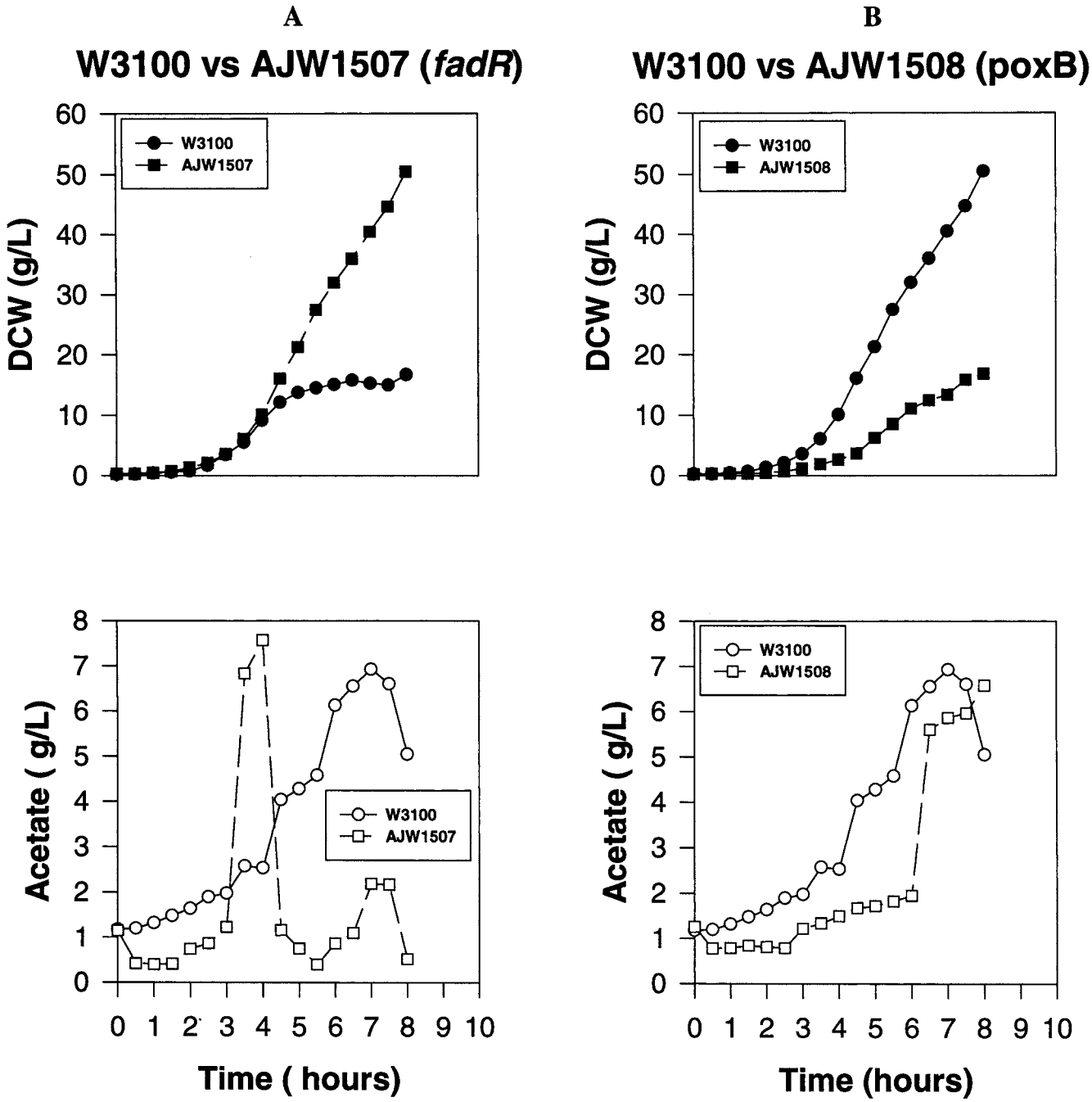

Figure 5 (A) Growth (top) and acetate accumulation (bottom) of cells: wild type (W3100) or deleted for *fadR* (strain AJW1507) during growth in GC-HCDF conditions. (B) Growth (top) and acetate accumulation (bottom) of cells: wild type (W3100) or deleted for *poxB* (strain AJW1508) during growth in GC-HCDF conditions.

biomass, 17.0 g/l DCW; Table 2). Like the *fadR* mutant, it accumulated wild-type levels of acetate (>6 g/l) that correspond to a specific acetate production almost threefold that produced by wild-type cells (0.39 g/g DCW vs. 0.14 g/g DCW, respectively; Table 2). In this case, however, the acetate accumulated toward the end of the fermentation run and was not resorbed within the course of that run (Figure 5B).

PoxB, the flavoprotein that catalyzes the oxidative decarboxylation of pyruvate to acetate and CO_2 with concomitant reduction of the tightly bound flavin adenine dinucleotide (FAD) cofactor to $FADH_2$, is not considered to be the relevant pathway in acetate production [5,7]. From these data, however, it is apparent that in the absence of other modifications, cells require PoxB activity to grow successfully to high cell densities, perhaps because of its role in supplying acetate for fatty acid biosynthesis [7]. Transcription of *poxB* depends upon σ^S [5,24]. Since *poxB* mutants do not grow to high cell density and excrete large amounts of acetate, the *rpoS* mutant phenotype must encompass more than that caused by the

lack of PoxB; i.e., some other member(s) of the σ^s regulon must counterbalance the *poxB* effect.

Conclusions

On the basis of our data, we conclude that cells of *E. coli* W3100 require the entire network of acetate metabolism pathways to reach normal levels of high cell density during glucose-controlled fed-batch fermentation. Considering the central position of the acetate-associated metabolic switch during growth to high cell density [19], perhaps it should not be surprising that such growth requires both the catabolic Pta/AckA pathway that excretes acetate and the anabolic Acs pathway that resorbs it. Indeed, immunoblot analysis using anti-Acs polyclonal antibodies demonstrate that cells growing at high cell density synthesized Acs, even though the medium contained significant amounts of glucose (AJ Wolfe, B Prüß, and S Kumari, unpublished results). This is an intriguing result considering that during batch culture Acs synthesis and activity is repressed during growth on glucose [4] (AJ Wolfe, B Prüß, and S Kumari, unpublished results).

The extreme behavior displayed by the *ackA* mutant likely results from its propensity to accumulate intracellular AcP. This large pool of AcP then would be available to act as the phospho-donor for the inappropriate autophosphorylation of several two-component response regulators responsible for transducing certain key environmental and metabolic signals.

The behavior exhibited by the regulatory mutants, *iclR* and *fadR*, surprised us. Since FadR positively regulates *iclR*, which itself negatively regulates the GS *aceBAK* operon, we expected that perhaps these mutants should yield similar results. In fact, the *fadR* mutant accumulated excessive amounts of acetate, while the *iclR* mutant produced only background levels. It is clear, then, that *fadR* exerts effects during GC-HCDF other than through its regulation of *iclR*.

The behavior of the *rpoS* mutant in GC-HCDF is the most intriguing. Since cells that lack σ^s grow to high cell density without accumulating large concentrations of acetate, the environment within GC-HCDF appears to be less stressful than previously thought [19,23]. Based on our observations, it would be desirable to investigate the suitability of an *E. coli rpoS* mutant for production of foreign recombinant proteins in high-cell-density fermentations.

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