Steffen Schaffer*
Necdet Isci
Brigitte Zickner
Peter Dürre

Mikrobiologie und Biotechnologie, Universität Ulm, Ulm, Germany

Changes in protein synthesis and identification of proteins specifically induced during solventogenesis in *Clostridium acetobutylicum*

At the end of the exponential growth phase the Gram-positive bacterium *Clostridium acetobutylicum* performs a metabolic switch from classical sugar fermentation accompanied by the production of acetate and butyrate to reinternalization and oxidation of these acids to acetone and butanol. Protein synthesis in acidogenic and solventogenic *C. acetobutylicum* cells was compared by two-dimensional gel electrophoresis of radioactively labeled proteins. The results show that the switch from acid to solvent production is accompanied by dramatic changes in the protein pattern. During solventogenesis, the synthesis of 52 proteins out of 130 analyzed was increased more than twofold, the synthesis of 34 proteins decreased to less than half as compared with synthesis in acidogenic cells. The changes in protein synthesis were generally reflected by changes in the abundance of the respective proteins, as determined from quantitative analysis of silver-stained second-dimensional gels. Nine proteins induced during solventogenesis were identified by *N*-terminal microsequencing. One of these proteins, the acetoacetate decarboxylase, is directly involved in solventogenesis. Other proteins synthesized in higher amounts during solventogenesis were three general stress proteins (DnaK, GroEL, Hsp 18), two enzymes involved in serine biosynthesis (serine aminotransferase and 3-phosphoglycerate dehydrogenase) as well as a seryl-tRNA synthetase. mRNA analysis provided evidence that the latter three are encoded by genes organized in an operon and are transcriptionally induced at the onset of solventogenesis. The proteins acetoacetate decarboxylase and Hsp 18 occurred in two variants, indicating possible covalent modification of these proteins.

Keywords: *Clostridium acetobytylicum* / *N*-Terminal sequencing / Proteome analysis / Solventogenesis / Two-dimensional electrophoresis EL 4725

1 Introduction

Clostridia are obligately anaerobic, Gram-positive bacteria with the ability to form endospores. Classically, the genus has been divided into proteolytic and saccharolytic clostridia. While the first group contains a number of human pathogens, such as *C. perfringens* and *C. botulinum,* the second group consists of exclusively nonpathogenic species, one of which is *C. acetobutylicum* [1]. The species was isolated at the beginning of this century because of its ability to ferment starch-containing substrates, the principal fermentation products during exponential growth being acetate and butyrate, while mainly acetone and butanol are produced during stationary phase. Later on, the microbial production of acetone and butanol became the second most important biotechnological process, only surpassed in production scale by ethanol fermentation. Due to competition by cheaper chemical synthesis and increasing prices for the fermentation substrates the microbial production of solvents started to decline in the 1960's [2, 3].

Although butanol and acetone are nowadays exclusively produced chemically, the scientific interest in *C. acetobutylicum* has ever increased. The species can now be regarded as being a model organism for the nonpathogenic group of clostridia, a number of genetic tools have been developed, and its genome has recently been sequenced. Although most of the enzymes involved in formation of solvents have been biochemically characterized and their corresponding genes have been cloned and sequenced [4, 5], few studies have analyzed the effects of switching from acidogenesis to solventogenesis at a more global level [6, 7].

Our studies were initiated by attempts to identify proteins synthesized prevalently or exclusively during solventogenesis. This included enzymes not necessarily linked to acetone and butanol production as well as those directly

Correspondence: Prof. Dr. Peter Dürre, Mikrobiologie und Biotechnologie, Universität Ulm, D-89069 Ulm, Germany
E-mail: peter-duerre@biologie.uni-ulm.de
Fax: +49-731-50-22719

* Present address: Institut für Biotechnologie 1, Forschungszentrum Jülich, Jülich, Germany

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involved in solvent formation. In addition, we wanted to establish two-dimensional polyacrylamide gel electrophoresis protocols for this organism to be able to use this powerful technique, combined with MALDI-TOF-mass spectrometry for protein identification purposes, for the analysis of regulatory networks in this organism, now its genome sequence has become publicly available [8].

2 Materials and methods

2.1 Culture conditions, radioactive labeling of proteins and preparation of cell-free extracts

C. acetobutylicum DSM 792 was grown under continuous culture conditions in phosphate-limited mineral medium [9] in a 1-L Biostat B chemostat (B. Braun Biotech International GmbH, Melsungen, Germany). The flow rate was set at 0.08 h^{-1} and the culture incubated at 37°C and pH 6.0 until equilibrium conditions were established. Synthesis of solvents was induced by lowering the pH setpoint to 4.7 or 4.5. Growth parameters and concentration of fermentation products were then monitored [9] for up to 20 h. Samples taken in the course of the growth experiment were analyzed with respect to external pH, external concentration of fermentation products, and expression of genes coding for enzymes known to be directly involved in solventogenesis (butanol dehydrogenases A and B, *bdhA* and *bdhB;* acetoacetate decarboxylase, *adc*; butyraldehyde/butanol dehydrogenase, *adhE;* CoA transferase subunits A and B, *ctfA*/*B*). Based on the resulting data the sample taken immediately before lowering the pH setpoint was regarded as being representative for acidogenic cells. The sample taken 6 h later was regarded as being representative for solventogenic cells. These samples were chosen for further analysis. For labeling of newly synthesized proteins, 5 mL of culture were transferred to anaerobized 10 mL tubes at timepoints t_0 and $t_{6\,h}$ (with respect to lowering the pH setpoint) and incubated for 3 min at 37°C in the presence of 10 μCi/mL L-[^{35}S]methionine (Amersham Pharmacia Biotech GmbH, Freiburg, Germany). Labeling was stopped by addition of 1 mL stop solution (10 mM L-methionine, 1 mg/mL chloramphenicol in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and setting the cells on wet ice. Cells were washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), suspended in 1.5 mL TE with 10 mM PMSF and broken by sonication. Intact cells and cell debris were removed by centrifugation (20 000 × *g* for 30 min at 4°C) and aliquots of the cell-free extract containing 2×10^6 cpm were vacuum-dried and stored at –20°C. For the preparation of unlabeled cell-free extracts, the cells were disrupted by five passages through a French pressure cell (SLM Instruments, Rochester, USA) at 12.5 MPa and the lysate cleared by centrifugation (20 000 × *g* for 30 min at 4°C). Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories, Hercules CA, USA) and bovine serum albumin as standard. Aliquots containing 100 and 500 μg protein, respectively, were vacuum-dried and stored at –20°C.

2.2 2-DE

Aliquots were solubilized in 400 μL rehydration buffer (8 M urea, 2 M thiourea, 1% w/v CHAPS, 20 mM DTT, 0.5% v/v Pharmalyte 3–10; Amersham Pharmacia Biotech) and used to rehydrate Immobiline DryStrips 4–7, 18 cm (Amersham Pharmacia Biotech), for 24 h. The first-dimensional electrophoresis was performed using a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech) according to the recommendations of the manufacturer with the following electrophoresis parameters: 0–500 V gradient for 500 Vh, 500 V for 2500 Vh, 500–3500 V gradient for 10 kVh, 3500 V for 35 kVh. The second-dimensional electrophoresis was performed using a vertical electrophoresis apparatus fitting two homogeneous 12% polyacrylamide gels with dimensions 174 × 240 × 1 mm, the active separation length being reduced by a 4% stacking gel of 2 cm height (the use of a stacking gel increased spot resolution in our hands). To perform the second-dimensional electrophoresis, the Immobiline DryStrips were equilibrated in equilibration solution A (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 4% w/v SDS, 3.5 mg/mL DTT) and B (as equilibration solution A, except for omission of DTT and addition of 45 mg/mL iodoacetamide and a few grains of bromophenol blue) for exactly 15 min each. The equilibrated strips had to be trimmed to 16 cm length to fit the second-dimensional gels (limiting the displayed p*I* range to 4–6.2) and were then placed on top of the second-dimensional gels and held in place with embedding agarose (0.5% w/v agarose in 62.5 mM Tris-HCl, pH 6.8, 4% w/v SDS). After filling the electrophoresis apparatus with cold electrode buffer (50 mM Tris, 190 mM glycine, 0.1% w/v SDS), two gels were run in parallel at 4°C for 16 h with 2.5 W per gel. For determination of molecular masses a Broad Range Protein Marker (New England Biolabs, Schwalbach, Germany) was run at the sides of selected gels. p*I* values were used as indicated by the manufacturer of the Immobiline DryStrips.

2.3 Staining, processing, and analysis of second-dimensional gels

Nonradioactive analytical gels were silver-stained according to the protocol described by Blum *et al.* [10]. Silver-stained gels were scanned with a UMAX Power

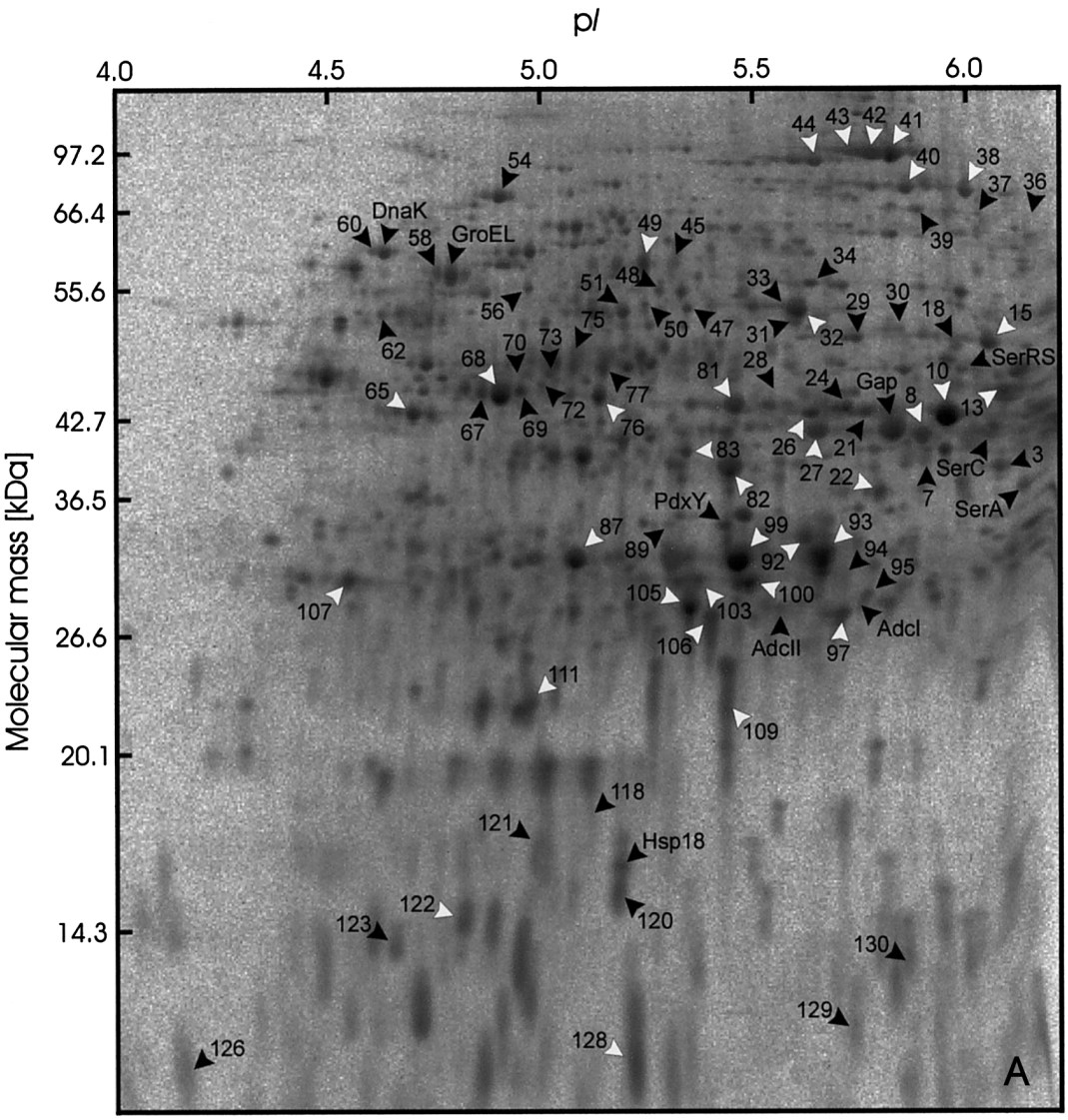

Figure 1. Protein synthesis in acidogenic and solventogenic *C. acetobutylicum* cells. Autoradiographs of second-dimensional gels after separation of radioactively labeled proteins from (A) acidogenic and (B) solventogenic cells are shown. Proteins contributing more than 0.1% to total protein synthesis and having an induction ratio >2 (black arrowheads) or <0.5 (white arrowheads) are labeled. The induction ratio is equivalent to the ratio of synthesis during solventogenesis and acidogenesis.

Look scanner and the UMAX MagicScan software, Version 2.2 (UMAX Data System, Hsinchu, Taiwan) at 300 dpi resolution. Spot matching was performed by eye and protein spots were quantified with the Intelligent Quantifier software, Version 2.1 (BioImage, Ann Arbor, MI, USA). Nonradioactive preparative gels were stained with 0.2% Coomassie Brilliant Blue R-250 in an ethanol/glacial acetic acid/water mixture (70:25:5 v/v/v) and destained with the same solution without Coomassie. Radioactive gels were fixed in a methanol/glacial acetic acid/water mixture 50:10:40 v/v/v) and dried for 90 min at 70°C on a conventional gel dryer. Dried gels were exposed to BASIIIs Imaging Plates and analyzed on a BAS 1000 Phosphoimager (Fuji Photo Film Europe, Düsseldorf, Germany) using the Image Reader software, Version 1.1, and MacBas software, Version 2.3 (Fuji Photo Film). Spot matching was confirmed by eye. Theoretical and observed molecular masses and p*I* values were calculated using GeneWorks, Version 2.4N (IntelliGenetics, Campbell, USA) and Intelligent Quantifier 1D software, Version 2.1 (BioImage, Great Britain), respectively.

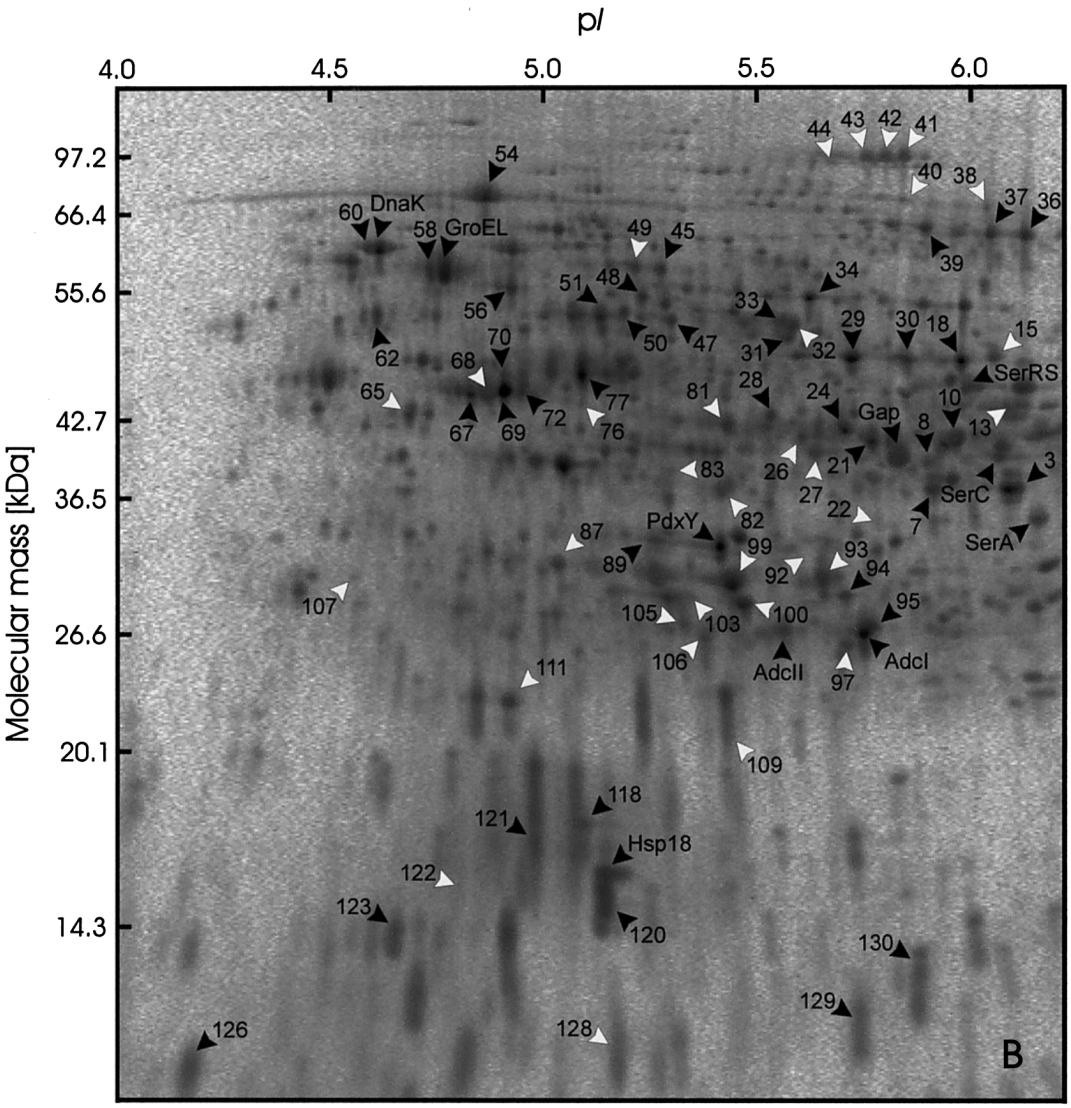

Figure 1. Continued

2.4 Concentration of proteins from second-dimensional gels and *N*-terminal sequencing of proteins

Protein spots from up to 16 second-dimensional gels were concentrated according to the protocol of Rider *et al.* [11], except that the horizontal concentration effect by removing and reinserting spacers was omitted in our experiments. Transfer of concentrated protein bands onto Biotrace PVDF membranes (Gelman Sciences, Ann Arbor, MI, USA) and subsequent Coomassie Brilliant Blue R-250-staining of PVDF membranes was performed following standard protocols [12]. Proteins were *N*-terminally sequenced by the Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany) using either ABI 473A or ABI 494A Procise HT automated sequencers (Applied Biosystems, Foster City, CA., USA). The obtained *N*-terminal sequences were used to search the *C. acetobutylicum* genome sequence at the NCBI BLAST server and the amino acid sequences of the corresponding ORFs were compared with the nonredundant NCBI protein data base.

2.5 RNA analyses

Total RNA was isolated by a modification of the hot phenol-chloroform procedure [13]. Northern blotting and primer extension analysis were performed as described recently [13, 14]. Hybridization probes for Northern experiments were synthesized by PCR [13, 14] using primer pairs adhEI (5'-GCCCTTAAGAGTGGTGCCCC-3')

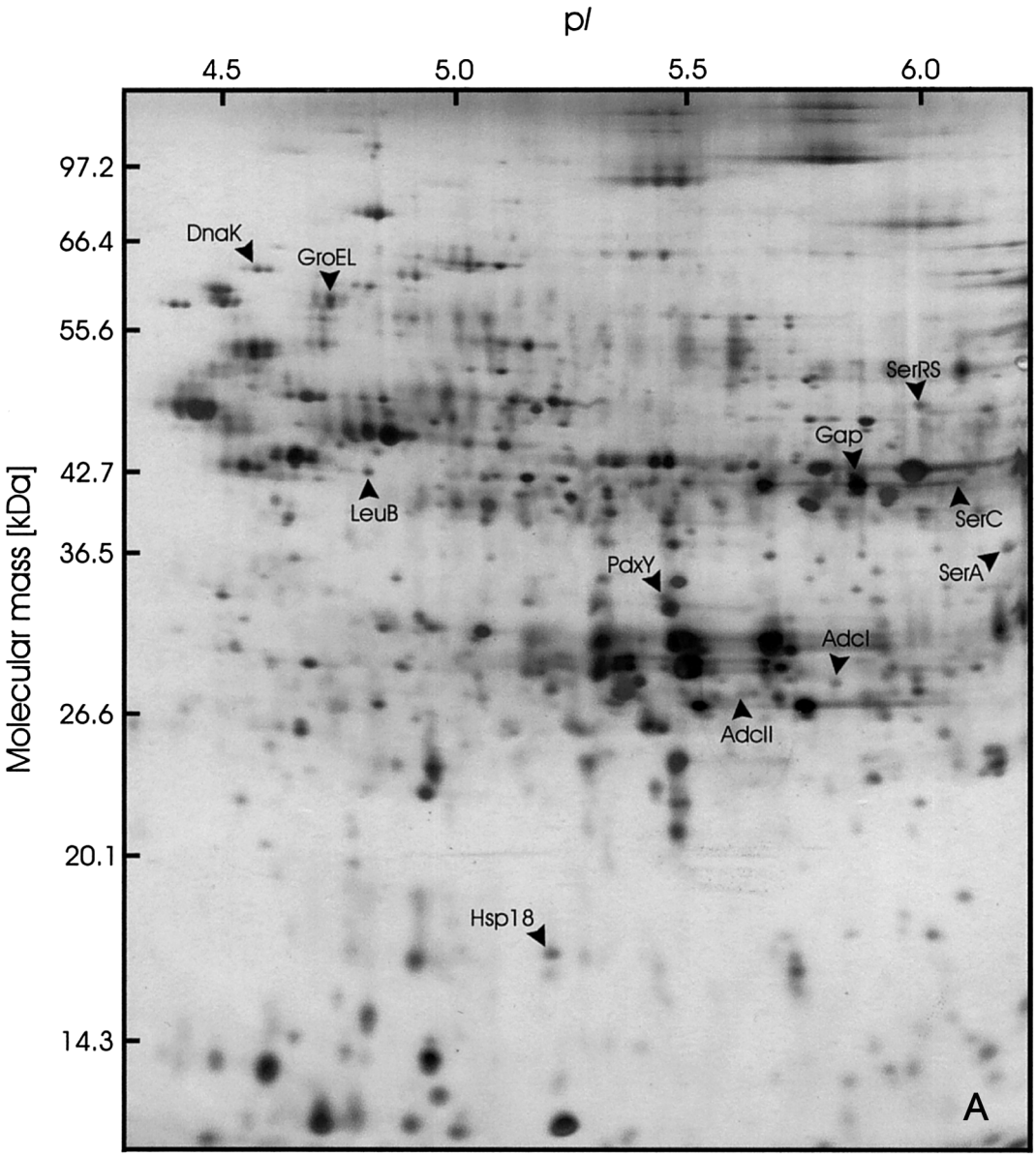

Figure 2. Protein patterns of acidogenic and solventogenic *C. acetobutylicum* cells. Silver-stained second-dimensional gels after separation of proteins from (A) acidogenic and (B) solventogenic cells are shown. Proteins identified in the course of this study or identified previously on 2-D gels are labeled.

and adhE2 (5'-GGGGTGTTACCCGGACCAAC-3') for construction of a 178-bp fragment of the *adhE* gene as a positive control as well as serA 1 (5'-GGACTTTTGAG GGAGGCTG-3') and serA2 (5'-CACATCAAGCGCTGCC CC-3') for construction of a 201-bp *serA* probe. For primer extension experiments, IRD800-labeled primers (MWG-Biotech AG, Ebersberg, Germany) were used. Primer1 (5'-GCTACTTCTACAGGTCCAGGT-3') is complementary to a region 21 to 41 bp downstream of the translation start point of the first structural gene in the operon, Primer2 (5'-GACAATAAAAAAAGCCTCTCA-3') is complementary to a region 149 to 169 bp upstream of that translation start point, and Primer3 (5'-CAGTTTT-CACCAACCACTGAC-3') is complementary to a region 312 to 332 bp upstream of that translation start point.

3 Results

Figure 1 shows the autoradiographs of second-dimensional gels after separation of radioactively labeled cell-free extracts from acidogenic and solventogenic *C. aceto-*

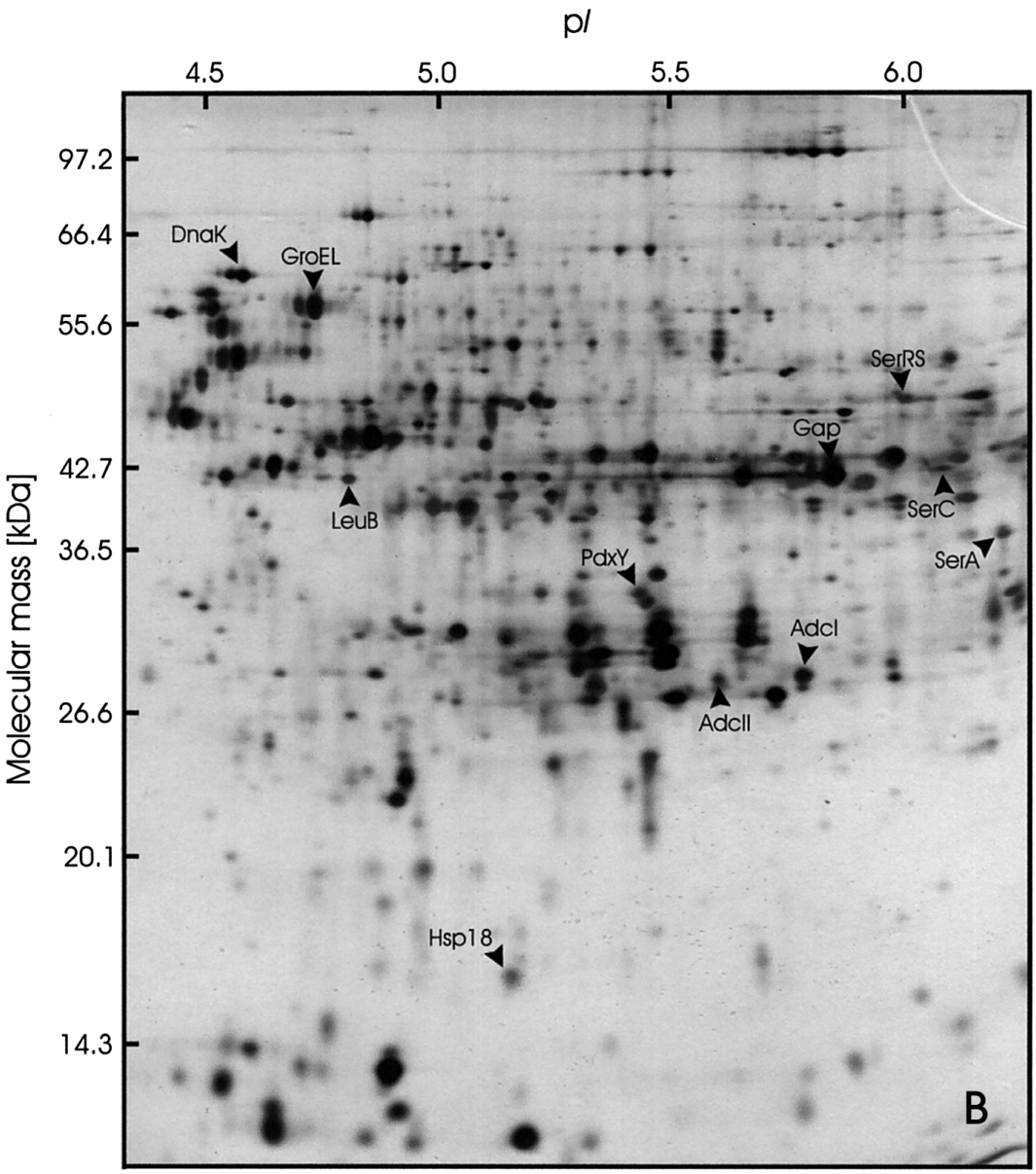

Figure 2. Continued

butylicum cells. There is a significant decrease in spot resolution in the lower section of the autoradiographs. Modifying the equilibration and running conditions or omission of the stacking gel did not improve resolution in this area. However, comparison of protein synthesis data from independent experiments for the affected low-molecular-weight proteins did not reveal an increase in data variability when compared with protein synthesis data for proteins of higher mass, indicating that quantitation in that area is justified. The synthesis rates of those 130 proteins, which individually accounted for more than 0.1% of total protein synthesis within the analysis window in either acidogenic and solventogenic cells, were quantified (data not shown, but available on request from the authors). Of these, 34 were synthesized at a similar rate (induction rate between 0.5 and 2) during acid and solvent production. The synthesis of 52 proteins was induced more than twofold, that of 34 proteins was reduced to less than half during solventogenesis. The induction ratio of proteins which were induced in either one of the two metabolic states was in most cases moderate, ranging from two- to tenfold. A minority of seven (spots 18, 28, 30, 36, 37, Adc I, 118) and nine proteins (spots 22, 40, 44, 76, 87, 92, 93, 105, 107) was induced more than tenfold in solventogenic and acidogenic *C. acetobutylicum* cells as compared to the other metabolic state, respectively. In both cell types,

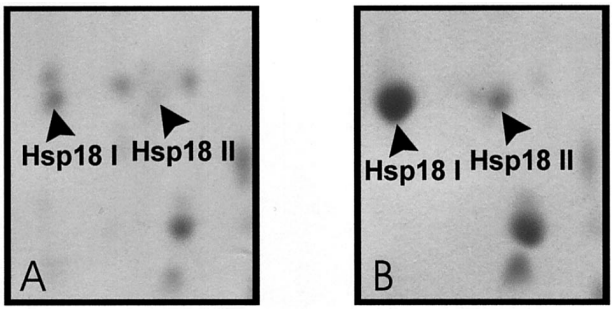

Figure 3. Presence of two Hsp18 variants in solventogenic *C. acetobutylicum* cells. Corresponding sections of preparative Coomassie-stained second-dimensional gels after separation of proteins from (A) acidogenic and (B) solventogenic cells are shown.

we found ten abundantly synthesized proteins, each of which contributed more than 1% to total protein synthesis within the analysis window. With few exceptions, these proteins were induced in the respective metabolic state (spots 3, GroEL, 69, 77, PdxY, Adc I, 99, Hsp 18 I during solventogenesis and spots 8, 10, 68, 82, 87, 93, 99, 100, 105 during acidogenesis).

In order to identify some of the proteins synthesized with higher rate in solventogenic cells (Fig. 2), we performed preparative 2-DE of unlabeled proteins. Only a minority of these proteins could be detected on preparative second-dimensional gels (data not shown). The *N*-terminal sequences of seven of these proteins were determined. Additionally, the *N*-terminal sequences of two proteins more abundant in solventogenic cells, but not detectable on radioactive second-dimensional gels (isopropylmalate dehydrogenase, LeuB, and Hsp18 II) and of one abundant protein with reduced synthesis rate in solventogenic cells (glyceraldehyde-3-phosphate dehydrogenase, Gap) were determined. The *N*-terminal sequences, characteristics and putative functions of these proteins are listed in Table 1. In two cases, excised protein spots contained more than one protein. Spot 3 contained at least three different proteins in approximately equal amounts. Assignment of PTH amino acids to individual *N*-termini was therefore not possible. Spot Adc II contained two proteins, the acetoacetate decarboxylase (Adc II)-derived PTH amino acids being at least one order of magnitude more abundant than those of the putative ABC transporter (Hyp 1), thereby allowing unambiguous assignment to the individual *N*-termini. In two cases (Adc, Hsp18), the same *N*-terminal sequence was obtained from two different protein spots, respectively (although the genome sequence revealed that only one *adc* and one *hsp18* gene are present). Hsp18 II was not visible on silver-stained gels, but appeared after Coomassie staining of preparative second-dimensional gels 0.24 pH units shifted to acidic pH with a molecular mass almost identical to that of Hsp18 I (Fig. 3).

The synthesis rates and abundancies with SD values calculated from three independent experiments as well as synthesis rate and abundance ratios of nine proteins identified in the course of this study and of DnaK and GroEL, which had been identified previously on 2-D gels [7], are listed in Table 2. Except for glyceraldehyde-3-phosphate dehydrogenase (Gap), increased synthesis of these proteins in solventogenic cells is reflected by increased abundance. The induction ratios for synthesis and abundance do not correlate in most cases. However, this is not surprising, as a protein's abundance is not only determined by its synthesis rate, but also by its degradation rate (reflected by its half-life), which has not been determined in this study. While isopropylmalate dehydrogenase (LeuB) is also more abundant during solventogenesis, its synthesis rate was apparently too low to be detectable by autoradiography. This is not due to scarcity of methionine residues, but could be explained by a very long half-life of this protein.

An unexpected result was the increased synthesis during solventogenesis of two enzymes involved in serine biosynthesis (phosphoglycerate dehydrogenase, SerA, and serine aminotransferase, SerC) and of a seryl-tRNA synthetase, SerS (Table 2). The synthesis induction ratio is similar for all three enzymes, and analysis of the genome sequence of *C. acetobutylicum* revealed that the structural genes for these proteins are located adjacently, together with a fourth gene encoding a protein of unknown function. The genes are arranged in the order *serC* (serine aminotransferase), *serA,* (3-phosphoglycerate dehydrogenase), *serX* (unknown function), and *serS* (seryl-tRNA synthetase) with no obvious promoter or termination structures between the genes, but a putative σ^A-dependent promoter upstream of *serC* and a putative rho-independent terminator downstream of *serS* (Fig. 4). Northern blots revealed that transcription of these genes (exemplified for *serA,* but similar patterns were obtained for the other genes) did not take place during acidogenesis but was induced at the onset of solventogenesis, shortly before solvents could be detected (data not shown). Induction starts at the same time as that of *adhE,* whose gene product is directly involved in solventogenesis (Fig. 5). Massive induction of *serA* mRNA can be seen at 7 h after inducing solvent formation by lowering the pH setpoint. This is at the same time when about threefold increased SerA synthesis was determined in the course of the 2-D experiments and parallels also the first appearance of *adhE* transcripts (Fig. 5). Two major mRNA species of approximately 5.5 and 2 kb hybridizing with

Table 1. *N*-terminal sequences, characteristics and putative functions of identified *C. acetobutylicum* proteins

Protein[a]	Experimentally determined (top) and expected (bottom) *N*-terminal sequence	MM[c] (kDa)	p*I*[c]	Function[d]	Similar proteins[a]	Identity [%]
Adc I	MLKDEVIKQISTPLTSP	27.5	5.76	Acetoacetate decarboxylase (Adc)	Adc of *C. acetobutylicum* (298085)	100
(15004868)	MLKDEVIKQISTPLTSP	29.3	5.79			
Adc II[b]	MLKDEVIKQISTPLTSPAFPRGPY	27.5	5.76	Acetoacetate decarboxylase (Adc)	Adc of *C. acetobytylicum* (298085)	100
(15004868)	MLKDEVIKQISTPLTSPAFPRGPY	28.9	5.60			
Gap	AKIAINGFGRIGRLALRRILEVP	35.7	5.84	Glyceraldehyde-3-phosphate dehydrogenase (Gap)	Gap of *C. acetobutylicum* (2829138)	100
(15023589)	AKIAINGFGRIGRLALRRILEVP	41.0	5.92			
Hsp18 I	MFGMVPFRRNNNGLMRREDFFDK	17.7	4.98	General stress protein (Hsp18)	Hsp18 of *C. acetobutylicum* (40363)	100
(15026820)	MFGMVPFRRNNNGLMRREDFFDK	16.5	5.16			
Hsp18 II	MFGMVPFRRNNNGLM?REDFFD	17.7	4.98	General stress protein (Hsp18)	Hsp18 of *C. acetobutylicum* (40363)	100
(15026820)	MFGMVPFRRNNNGLMRREDFFD	16.3	4.98			
Hyp1[b]	?ESL(RL)GPN(EP)(LG)?AEADG?EDL	27.4	5.75	ATP-binding subunit of ABC transporter	*Treponema pallidum* (3322909)	52
(15026366)	GEKL L QIN N I HAEADGKEIL	28.9	5.60		*Bacillus subtilis* (2635767)	49
					Cyanophora paradoxa (1351834)	47
LeuB	MKEYKVAVIPGDGIGVEIVGEA	38.9	4.77	Isopropylmalate dehydrogenase (LeuB)	LeuB of *Clostridium pasteurianum* (400184)	76
(15026239)	MKEYKVAVIPGDGIGVEIVGEA	41.5	4.80		LeuB of *Aquifex aeolicus* (2982949)	61
					LeuB of *Pseudomonas aeruginosa* (3237310)	60
SerA	M(QI)?I(LN)AN(DN)GM?KNAVLGLID(LN)?Y	33.3	6.14	Phosphoglycerate dehydrogenase (SerA)	Ser A of *Methanococcus jannaschii* (3122874)	46
(15022834)	M I RI L AN D GMNKNAVNGLID L GY	37.1	6.09		SerA of *Methanothermobacter thermoautotrophicum* (3122860)	43
					SerA of *Pyrococcus horikoshii* (7431362)	44
SerC	MHKKLFIPGPVEVAEDVLQ	39.9	6.21	Serine amino-transferase (SerC)	Aminotransferase of *Methanococcus jannaschii* (2117761)	40
(15022833)	MHKKLFIPGPVEVAEDVLQ	42.5	6.21		AspC of *Archaeglobus fulgidus* (2649155)	39
					SerC of *Pyrococcus horikoshii* (3257730)	37
SerS	(MQ)LDLDLIR(NQ)DTEKV??AL?K	48.6	5.92	Seryl-tRNA synthetase (SerS)	SerS of *Bacillus subtilis* (586064)	63
(15022836)	M LDLDLIR N DTEKVKKALLK	48.3	6.00		SerS of *Staphylococcus aureus* (2501053)	57
					SerS of *Aquifex aeolicus* (3915066)	56
PdxY	SD??DKNENL(AV)?ML??TV	31.6	5.30	Pyridoxine biosynthesis protein	YaaD of *Bacillus subtilis* (586857)	66
(15023463)	MDRSDMNKNL A QMLKGGV	32.8	5.43		SOR1 of *Cercospora nicotianae* (2979688)	63
					HEVER of *Hevea brasiliensis* (2501578)	65

a) GenBank accession numbers are given in parentheses.
b) Proteins identified in the same protein spot
c) Expected (top) and observed values (bottom) for the molecular mass (MM) and p*I*
d) For proteins which have not been characterized in *C. acetobutylicum* the putative function based on similarity to proteins of known function is indicated.

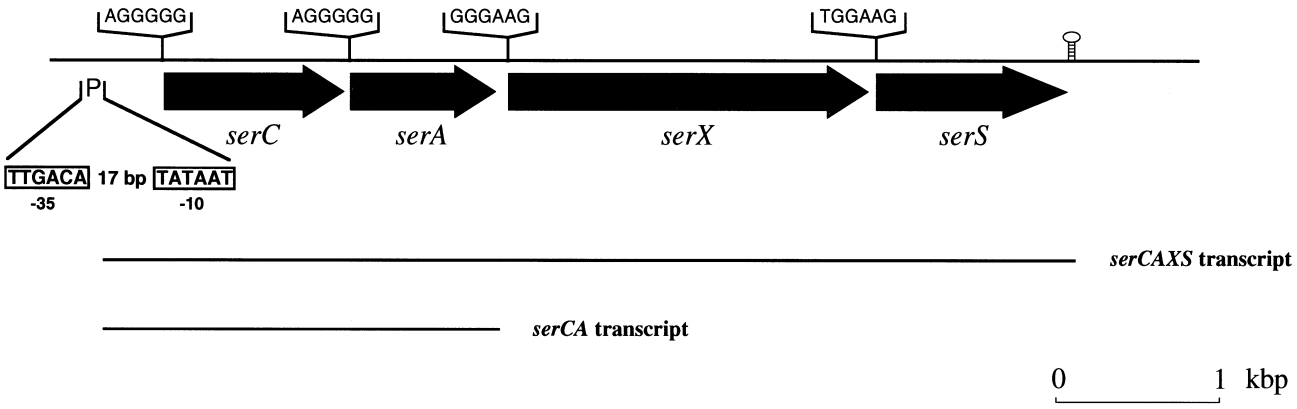

Figure 4. Schematic map of the *serCAXS* region in the chromosomal DNA of *C. acetobutylicum* (drawn to scale). Arrows and arrowheads represent lengths, locations, and orientations of ORFs. Putative ribosome binding sites upstream of the respective genes are indicated above the solid line symbolizing the DNA. The promoter region as deduced from primer extension analysis is shown in boxes, representing the –10 and –35 region. A putative rho-independent transcription terminator at the end of the operon is indicated by a hairpin symbol. Transcript sizes are indicated by a line, representing the mRNA, followed by the designation of the transcript.

the *serA* probe could be detected (Fig. 5). This indicates a common *serCAXS* operon structure, corresponding to the 5.5 kb transcript. The dominant band at 2 kb might indicate processing of the tetracistronic mRNA resulting in a *serCA* (2 kb) transcript. We then performed primer extension experiments to determine the transcription start point of the putative *serCAXS* operon (Fig. 6). The primer extension signals correspond to two adjacent adenine and thymine bases. Upstream of the transcription start point, –10 and –35 boxes identical to the respective consensus sequences for σ^A-dependent promoters of low G+C Gram-positive bacteria can be found, including the typical TG pair just upstream of the –10 region [15]. The spacing of 3 bp between +1 and the –10 box is also in agreement with typical values of such promoters [15].

Table 2. Synthesis and abundance of *C. acetobutylicum* proteins during acidogenesis and solventogenesis

Protein	Synthesis[a]			Abundance[a]		
	Acidogenesis	Solventogenesis	Induction[b]	Acidogenesis	Solventogenesis	Induction[b]
Adc I	243±37	8804±455	36.2	421±41	4589±223	10.9
Adc II	425±32	1423±109	3.3	145±32	1767±156	12.2
DnaK	1221±129	4513±276	3.7	423±120	6068±429	14.3
Gap	11333±1877	5674±393	0.5	11570±401	22597±4010	2.0
GroEL	2901±344	10934±1450	3.8	1705±98	15789±2089	9.3
Hsp18 I	890±93	7680±870	8.6	729±56	980±93	1.3
LeuB	N. A.[c]	N. A.[c]	N. A.[c]	432±44	1403±200	3.2
SerA	555±65	1587±142	2.9	291±45	1089±123	3.7
SerC	760±41	2193±220	2.9	1408±123	2498±320	1.8
SerS	412±51	1323±151	3.2	220±34	1356±111	6.2
PdxY	1885±123	7534±879	4.0	619±87	2030±223	3.3

a) Values are given in arbitrary units and represent mean values with SD from three independent experiments.
b) Induction ratios are equivalent to the ratio of respective mean values during solventogenesis and acidogenesis.
c) N. A., not applicable

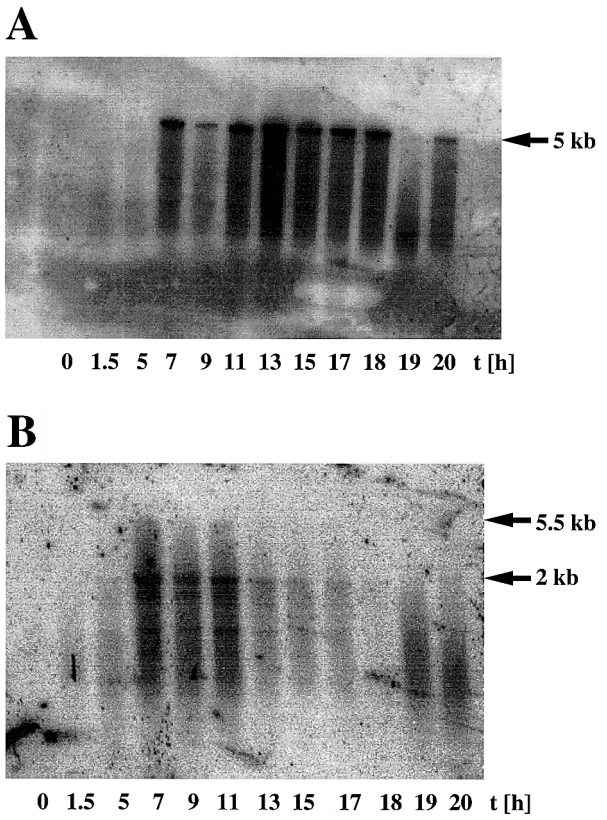

Figure 5. Northern hybridizations using radiolabeled PCR-generated probes complementary to parts of the *adhE* and *serA* genes. Lanes represent the time point of sampling from the continuous culture experiment in hours. Arrows indicate transcript sizes. (A) *adhE* probe, as a representative marker for solventogenesis; (B) *serA* probe.

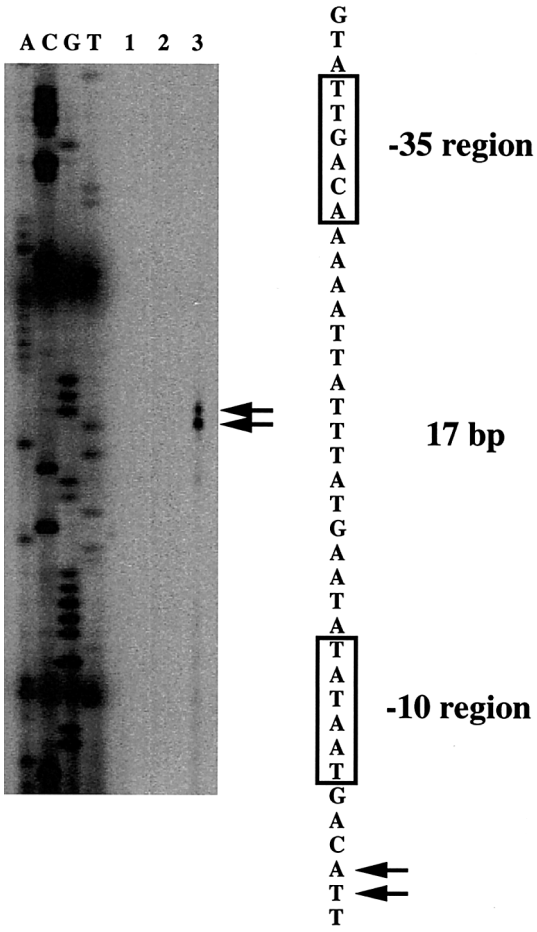

Figure 6. Identification of the transcriptional start point of the *ser* operon by primer extension analysis. Lanes A, C, G, T show a sequencing ladder obtained with DNA from *Synechocystis* sp. PCC 8803 to allow determination of primer-to-signal distance with base-by-base resolution. Lanes 1–3 correspond to the different primers used (see Section 2.5). Arrows indicate two adjacent primer extension signals. To the right, the deduced promoter structure is indicated, with two arrows pointing to the two nucleotides of the transcription start.

4 Discussion

The present study aimed at the identification of proteins directly (*e.g.*, enzymes synthesizing the solvents) or indirectly (*e.g.*, proteins coping with environmental conditions brought about by solventogenesis, such as low pH and high concentration of solvents) involved in solventogenesis by 2-D electrophoresis. Of the identified proteins only the acetoacetate decarboxylase is directly involved in solventogenesis in *C. acetobutylicum* [4]. Acetoacetate decarboxylase is the protein with the highest induction rate of all proteins analyzed. At least two other solventogenic enzymes, the butanol dehydrogenases A and B, have theoretical p*I* values and molecular masses (5.71, 43.0 kDa; 5.65, 43.2 kDa) which should have allowed their identification in the 2-D electrophoresis system employed in our studies. We do not know if their synthesis rates and/or abundancies are below the detection limit or if they have been detected but were not identified, because they did not appear on preparative 2-D gels.

The conditions experienced by *C. acetobutylicum* during solventogenesis, such as low pH and high concentrations of noxious substances, represent significant environmental stress. Consequently, more than 3% of total protein synthesis is dedicated to the synthesis of general stress proteins GroEL, DnaK, and Hsp18, which are known to be synthesized in response to a variety of environmental stresses in *C. acetobutylicum* [11, 16–18] and aid in refolding of misfolded or aggregated proteins.

The induction of proteins involved in serine biosynthesis and its incorporation into proteins together with solventogenesis came as a surprise. There is no indication why *C. acetobutylicum* should have an increased need for

these enzymes during acetone and butanol formation. Comparison of sequences of *C. acetobutylicum* proteins synthesized in either all growth phases or exclusively during solventogenesis did not reveal any significant differences in serine content or serine codon usage. It should be pointed out that *C. acetobutylicum* contains another gene putatively coding for a seryl-tRNA synthetase (*serS2*), which is apparently organized monocistronically and localized on a distant region of the chromosome. There exists at least one other report of differential regulation of expression of genes encoding aminoacyl-tRNA synthetase isozymes. In *Escherichia coli,* the expression of *lysS,* a gene coding for a leucyl-tRNA synthetase isozyme, is induced in response to heat, anaerobiosis, and changes in external pH, while the gene *lysSU* coding for the isozyme is expressed constitutively [19]. The physiological significance of these observations is not known.

The protein PdxY (for pyridoxine biosynthetic protein) of *C. acetobutylicum* is a member of a highly conserved protein family present in all three kingdoms of life. Homologous proteins include SOR1 from *Cercospora fungi,* rendering the organism resistent to singlet oxygen [20], HEVER from *Hevea brasiliensis,* specifically synthesized in response to general stress conditions [21], SNZ1p from yeast synthesized in response to nutrient limitation [22] and YaaD from *Bacillus subtilis* synthesized in response to oxidative stress [23]. It has recently been shown that the PdxY homologues from *Aspergillus nidulans* and *Cercospora fungi* are part of a novel pyridoxine biosynthesis pathway [24, 25]. It is tempting to speculate that *C. acetobutylicum* increases pyridoxine synthesis in order to quench reactive oxygen species increasingly occurring during solventogenesis. Pyridoxine and its derivatives have been shown to be effective quenching agents for singlet oxygen [26].

Two proteins, Hsp18 and acetoacetate decarboxylase, occurred at two positions on second-dimensional gels. In both cases, the existence of only one gene is revealed by the genome sequence data available for *C. acetobutylicum* ATCC 824 and the identity of the *N*-termini from both protein spots. Both variants display identical molecular masses, but differ in their p*I* values by 0.19 (acetoacetate decarboxylase) and 0.24 units (Hsp18), respectively. It is therefore tempting to speculate that one of these variants, possibly the less abundant variant exhibiting a more acidic p*I*, is modified by a charged group, such as phosphorylation, acetylation, deamidation, adenylation, or amino acid substitution. In that context it should be pointed out that in case of the *C. acetobutylicum* DnaK and GroEL proteins, two other protein spots are visible on second-dimensional gels immediately to the left and right of the main spot, which also have identical molecular masses but differ slightly in their observed p*I* values. For both proteins, *in vivo* phosphorylation has been reported in *E. coli* [27, 28] which might account for the observed train of protein spots. However, the p*I* shift is far less pronounced in these cases. The presence of variants with identical molecular mass but different p*I* has also been observed in several other bacteria. In *B. subtilis* SodA, the superoxide dismutase, IlvC, the ketol-acid reductoisomerase, and ClpC, a regulatory subunit of the Clp protease, are present in such variants [29].

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