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Evaluation of two proteomics technologies used to screen the membrane proteomes of wild-type *Corynebacterium glutamicum* and an *L*-lysine-producing strain

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Abstract The membrane proteomes of a wild-type *Corynebacterium glutamicum* and an L-lysine-producing strain were quantitatively analyzed by two complementary proteomics techniques—anion exchange chromatography AIEC/SDS-PAGE and 16BAC-PAGE/SDS-PAGE—and the results were compared. Although both techniques allow for the fast screening of differences in protein abundance, AIEC/SDS-PAGE was superior to 16BAC-PAGE/SDS-PAGE with respect to protein separation, it was more suitable for relative protein quantification, and allowed more differentially regulated proteins to be detected (the succinate dehydrogenase complex, an ABC-type cobalamin/Fe^{3+} siderophore transport system, the maltose binding protein, and a subunit of the cytochrome bc-aa_3 supercomplex were upregulated, while a periplasmic component of an ABC-type transporter and an iron-regulated ABC-type transporter were downregulated in the producer). The results indicate the important role of tricarboxylic acid cycle enzymes as well as the adaptation of transport processes in L-lysine-producing cells. Since the only genetic differences between the wild type and the L-lysine producer occur between four central metabolic enzymes in the cytoplasm, our study illustrates the complex effects of metabolic engineering on cell physiology and the power of the new AIEC/SDS-PAGE proteomics approach to detect these effects.

Keywords Membrane proteins · Anion exchange chromatography · Mass spectrometry · Quantitative analysis

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Abbreviations

AIEC	anion exchange chromatography
ASB-14	amidosulfobetaine-14
16BAC	benzyldimethyl-*n*-hexadecylammonium-chloride
TM	transmembrane helix
MALDI-TOF	matrix-assisted laser desorption/ionization
PMF	time-of-flight peptide mass fingerprinting

Introduction

Many methods suitable for the separation and relative quantification of cytoplasmic proteins (including two-dimensional gel electrophoresis, 2-DE) cannot be used for membrane proteins, due to their mainly hydrophobic character (for an overview see [1]). Alternative technologies are MudPIT [2] or a combination of SDS-PAGE and LC–ESI MS/MS [3], but since these methods achieve their high resolution at the peptide level, quantification is only possible at the late experimental stage using mass spectrometry. Common methods for the separation and quantification of intact membrane proteins are blue native (BN)-PAGE and 16BAC-PAGE [4], although only a rather limited proteome resolution is usually obtained in this case, and multiple sample prefractionation steps must be carried out in advance. Hence we developed an alternative technology based on a two-dimensional separation approach consisting of anion exchange chromatography in the first and SDS-PAGE in the second dimension (AIEC/SDS-PAGE) in order to separate the membrane proteome of *C. glutamicum* [5]. In combination with MALDI-TOF PMF, this allowed a fast proteome screening, which was used in this study to quantitatively analyze the

membrane proteome of an L-lysine production strain and then compare it to the wild type. In parallel, a well-established separation procedure for membrane proteins, 16BAC-PAGE in combination with SDS-PAGE [4], was optimized for the membrane proteins of *C. glutamicum*. Separation in the first dimension is achieved by discontinuous electrophoresis, using the cationic detergent 16BAC in an acidic buffer system. The properties of the acidic 16BAC system are sufficiently different from the basic SDS system of Laemmli [6] which is used as the second dimension, and this therefore yields substantial resolution. Proteins are separated around a diagonal in the SDS gels according to the differences in migration. Up to now, this technique has mainly been used for the separation of eukaryotic membrane proteins [4, 7–9].

Corynebacterium glutamicum wild-type and the L-lysine-producing strain appear to be attractive targets for screening and comparing the performance of 16BAC-PAGE and AIEC/SDS-PAGE. Due to its ability to secrete large amounts of amino acids, *Corynebacterium glutamicum* is one of the most important organisms used in industrially applied biotechnology. It belongs to the *Actinomycetes* subphylum of Gram-positive eubacteria [10]. Since many methods for in vivo mutagenesis are available, such as gene disruption and allelic exchange by homologous recombination [11], this organism is ideal for rapid and directed metabolic engineering, and it is an important model organism for basic research.

It was shown that the amino acid production for a *C. glutamicum* L-lysine production strain constructed by repeated random mutation and selection is inefficient due to poor growth and sugar consumption [12]. Whole-cell mutagenesis has the serious disadvantage of accumulating uncharacterized secondary mutations which may impair the performance of the organism. To overcome these problems, a genome-based strain reconstruction that involves identifying mutations by comparative genomic analysis, defining mutations beneficial for production, and assembling them in a single wild-type background was performed [12]. Four genes important for L-lysine overproduction have been identified: 1) a point mutation in aspartate kinase (*lysC*) stops feedback inhibition of this enzyme in the presence of L-lysine; 2) a point mutation in glucose-6-phosphate dehydrogenase (*zwf*) (Degussa AG, personal communication) enhances the activity of the enzyme and increases the flux through the pentose phosphate pathway in order to increase the NADPH supply [13]; 3) a point mutation in pyruvate carboxylase (*pyc*) enhances the supply of oxaloacetate needed as a precursor for L-lysine production, and; 4) a mutation in homoserine dehydrogenase (*hom*) reduces enzyme activity and focuses fluxes towards L-lysine production. Owing to the complexity of the metabolic network, it is still difficult to predict the effect of mutations on amino acid production. For instance, Koffas et al. [14] found that simultaneous overexpression of pyruvate carboxylase and aspartate kinase yielded a 250% increase in productivity in lactate minimal medium, while monocistronic overexpression of each protein was detrimental to production. The completed sequencing of the genome [15], [16] yielded considerable progress in the systemic understanding of *C. glutamicum*. This information is the basis for genomics and proteomics, which allow a much deeper insight into bacterial physiology.

Our results show that (AIEC/SDS-PAGE) is superior to 16BAC-PAGE for the separation of the membrane proteome from *C. glutamicum*. Furthermore, AIEC/SDS-PAGE was more suitable for relative quantification than 16BAC-PAGE and allowed the identification of eight differentially expressed proteins. Since most of these proteins are not related to the metabolically engineered central metabolism, our study clearly shows the power of proteomics in general and the AIEC/SDS-PAGE approach in particular for discovering unanticipated gene regulation processes.

Materials and methods

Bacterial strains

Cells of a *C. glutamicum* L-lysine production strain (DM1730) and a wild type (DM2-1) were grown in a 40L fermenter on CgXII [17] medium without MOPS, pH regulated at 7.0, in a fed-batch process with glucose and ammonia, performed at 30 °C and 20% partial pressure of oxygen (cell material kindly supplied by Degussa AG, Halle, Germany). Cells were harvested in the stationary phase, i.e., after 48 h at an OD of 85 for the wild type and after 60 h and an OD of 44 for the producer.

AIEC/SDS-PAGE (2D-IEC)

For details of the method see [5]. Briefly, cells were disrupted by French pressure treatment and membranes were washed with 2.5 M NaBr to remove membrane-associated proteins. The resulting membrane fraction was solubilized in buffer containing 2 % (w/v) ASB-14 and applied onto an anion exchange column (Poros20 HQ material, Applied Biosystems, Darmstadt, Germany). Fractions were precipitated by trichloroacetic acid [18] and SDS-PAGE was performed according to Laemmli [6] using gels with acrylamide concentrations of 12% T (% acrylamide + % bisacrylamide) and 1% C (% crosslinking). Gels had a separation length of 20 cm.

16BAC/SDS-PAGE (2D-BAC)

The two-dimensional separation of proteins was performed by a combination of 16BAC-PAGE and SDS-PAGE according to Hartinger et al. [4] with some modifications. Proteins (about 300 μg of pelleted membrane proteins) were resuspended in 16BAC sample buffer by thorough vortexing for 10 min followed by 5 min incubation at 60 °C. An acrylamide gradient of 9% T (0.75% C) to 13% T (1.08% C) was found to be optimal for the separation of the membrane fraction from *C. glutamicum*. Electrophoresis was carried out at 6 °C, starting with an initial current of 25 mA and performing the separation at 80 mA/gel- until the dye front started to leave the gel. Gels were fixed for at least 1 h in an isopropanol: acetic acid:water mix (3.5:1:5.5), with three changes of the fixative performed to remove 16BAC. Staining of the gels was done with 0.15% (w/v) Coomassie Blue R-250 in fixative for 30 min, followed by destaining in fixative. After excision, the protein lanes were incubated for re-equilibration three times in 10 ml SDS-stacking gel buffer (0.13 M Tris-HCl pH 6.8, 0.1 % (w/v) SDS) with gentle shaking. SDS-PAGE, used as the second separation dimension, was performed according to Laemmli using 12% T (1% C) to 15% T (1.25% C) acrylamide gradients. The first dimension gel strip was sealed within the stacking gel, overlaid with SDS–sample buffer [10% (w/v) glycerol, 5 % (v/v) β-mercaptoethanol, 3 % (w/v) SDS, 62 mM Tris-HCl, pH 6.8, 0.01 % (w/v) bromophenolblue] and incubated for 5 min before electrophoresis was started at 6 °C. Initially 20 mA/gel was applied until the blue dye front entered the separating gel. Separation was carried out at 60 mA/gel until the dye front had reached the bottom of the gel. The separation length for both dimensions was 15 cm.

Densitometry for the calculation of regulation factors

Proteins were stained with Coomassie according to Neuhoff [19], and gels were scanned on an image scanner with the LabScan software (both Amersham Biosciences, Freiburg, Germany). The scanner was calibrated with a grayscale marker (Kodak), and the same settings were applied for all gels. Scanning was carried out at 300 dpi and 8-bit grayscale. Gel bands were quantified relative to each other by densitometry using the software Scion Image (version beta 4.0.2; Scion Corporation, http://www.scioncorp.com).

In-gel tryptic digestion and MALDI-MS protein identification

Proteins were tryptically digested and characterized by MALDI-ToF PMF using a Voyager DE-Pro Instrument [5]; identification was performed by a MASCOT (http://www.matrixscience.com/search_form_select.html) search of the NCBInr (National Center for Biotechnology Information) database, where the search was restricted to “other actinobacteria” entries. Search parameters were set to: oxidation of methionine; one missed cleavage of trypsin; and 100 ppm mass accuracy. Identifications were considered significant if the MASCOT score exceeded 63 with a minimum of five matched peptides.

Results

Although it has been realized that membrane processes, such as the L-lysine export [20], are important for amino acid production in *C. glutamicum*, little is known about the secondary mechanisms located in the plasma membrane that allow the production of enormous amounts of L-lysine. In this report, the membrane proteomes of a large-scale L-lysine production strain and a wild type are analyzed by the newly developed two-dimensional separation method AIEC/SDS-PAGE [5], whose quantification performance could not be judged until now due to the lack of a systematic comparison with a well-established technique. Therefore, the two-dimensional separation is performed via 16BAC/SDS-PAGE (2D-BAC) in parallel as an alternative method in order to compare protein quantifications and separation capabilities.

2D-BAC

16BAC-PAGE/SDS-PAGE (2D-BAC), which has previously been applied for the separation of mostly eukaryotic proteins [4, 7], was optimized for the separation of the membrane proteome of *C. glutamicum* in this work and used to analyze both strains. Since a washing procedure with 2.5 M NaBr was considered most effective in previous tests [5], it was used for the membrane fraction before applying the proteins to 16BAC-PAGE. About 50 protein spots could be detected in the SDS-PAGE used as the second separation dimension (Fig. 1); they were analyzed by MALDI-ToF PMF. Table 1 shows 29 identified proteins, of which only four are integral membrane proteins (1–3 TMHs), while 15 are cytoplasmic, nine are membrane-associated, and one is a secreted protein. Thus only 14% of all identified proteins are predicted to be integral membrane proteins; in contrast, the percentage of IMPs in a master gel after 2D-IEC was 29%, i.e., more than twice as high (out of 50 identified IMPs in total) [5]. Although we employed an optimized prefractionation procedure consisting of membrane isolation and washes [5], a high percentage of the identified cytosolic proteins persisted. This is a general problem with membrane proteomics and one that has been observed for related bacteria, too [21]; possibly due to the special cell wall compositions of actinomycetes. Eight identified cytoplasmic or membrane-associated proteins

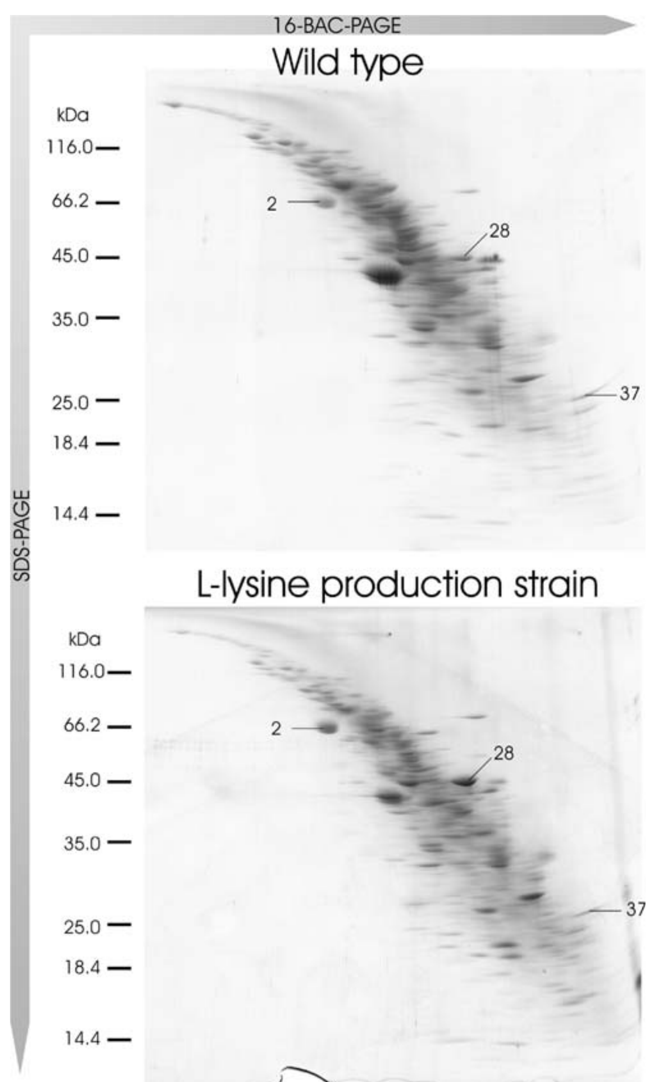

Fig. 1 16BAC/SDS-PAGE (2D-BAC) of the 2.5 M-NaBr-washed membrane fractions from the wild-type *C. glutamicum* strain and the L-lysine production strain. Spots were excised from the Coomassie-stained gel and identified by MALDI-ToF PMF. Regulated proteins are marked by numbers and shown in Table 1

were exclusively found in 2D-BAC. It is worth noting that these proteins all had masses above 67 kDa, indicating either that large proteins are better resolved, or that only a few proteins are present in this range, i.e., there are fewer problems with overlaying protein spots in this region. For three of the identified proteins, a reproducible regulation was found in three independent separations (Table 1). Considering the calculated standard deviations and p-values (a p-value below 0.1 is considered statistically significant), AmyE, the maltose binding protein of an ABC-type transporter, is 2.6-fold up-regulated. The observed regulations are discussed in the next section in relation to the results obtained from 2D-IEC.

2D-IEC

The separations of the NaBr-washed membrane fraction of the wild type and that of the producer strain by the 2D-IEC method resulted in about 150 protein bands (Fig. 2). For most of the proteins no differences in expression were visible by eye. At the present time there is no satisfactory software for the detection of gel bands, and therefore the first screening of the gels was performed by eye since it was considered to be the most effective and accurate method. The relatively low number of differently expressed proteins that were detected was not astonishing, as the producer strain differs from the wild type in only four point mutations, and no differences in mRNA abundance were found for a similar strain [22]. About 14 proteins from the Coomassie-stained gel that showed different abundances by eye were identified, and reproducible and significant quantifications by densitometry were possible for eight of these (see Table 1). The separation ability of the system was satisfactory; no more than one protein was identified in each gel band when MALDI-Tof PMF was used for protein identification. Three independent separations were performed, and the elution profiles obtained after AIEC and SDS-PAGE were highly reproducible and showed only minor gel-to-gel variations. The proteins identified were always eluted in the same or the adjacent AIEC fraction.

Regulated proteins: comparison of 2D-IEC and 2D-BAC

The washed and solubilized membrane fractions of the L-lysine production strain and the wild type were separated by 2D-BAC and in parallel by 2D-IEC. Several differences become apparent upon comparing the two protein patterns; the proteins in these bands were also identified by MALDI-ToF PMF. After separation by 2D-IEC or 2D-BAC, the proteins were quantified by densitometry with the regulation factor of three independent separations being averaged (Table 1). Eight proteins showed different abundances in the L-lysine production strain compared to the wild type by 2D-IEC. Three of them were also detected in the 2D-BAC analysis with the same regulation tendency. The MASCOT scores obtained when identifying the regulated proteins are given in Table 2.

Aside from the eight regulated proteins listed in Table 1, some other protein bands also showed different expression levels and were analyzed in this study; however, they either turned out to be cytoplasmatic contaminations still present in the membrane fraction to some extent, or their regulation was not reproducible.

The following proteins are significantly up-regulated in the production strain according to the 2D-IEC approach: a putative secreted or binding protein (cg0737), all three subunits of the succinate dehydrogenase, two uncharacter-

Table 1 Proteins that could be identified in the membrane fractions of either the L-lysine production strain or the wild type

Spot No.	cg-Identifier	Identified protein	Localization[a]	2D-IEC Fraction No.	2D-IEC MW [kDa]	2D-BAC MW [kDa]	Regulation 2D-IEC PS/WT	S.D.	p-value·10²	Regulation 2D-BAC PS/WT	S.D.	p-value·10²
1	cg0445	(sdhCD) succinate dehydrogenase CD	5	17	23	–	**4.1**	***0.4***	**0.25**			
2	cg0446	(sdhA) succinate dehydrogenase A	a	17, 18	66	66	**3.9**	***1.7***	**4.74**	1.5	*0.3*	10.7
3	cg0447	(sdhB) succinate dehydrogenase B	a	17	27	–	**4.3**	***1.1***	**1.50**			
4	cg0577	DNA-directed RNA polymerase beta subunit / 160 kDa subunit	c	–	–	120						
5	cg0583	(fusA) elongation factor G	c	15, 16	80	75						
6	cg0587	(tuf) elongation factor TU	c	12–18	43	40						
7	cg0610	(rplE) 50S ribosomal protein L5	c	7, 8	20	22						
8	cg0654	(rpsD) ribosomal protein S4	c	5,6	19	23						
9	cg0737	ABC-type transporter, periplasmic component	s	35	19, 20	–	**0.5**	***0.1***	**9.10**			
10	cg0791	(pyc) pyruvate carboxylase	c	14	115	110						
11	cg0924	ABC-type cobalamin/Fe^{3+}-siderophores transport system	s	17	47	–	**1.5**	***0.1***	**1.22**			
12	cgl0836	3-oxoacyl-(acyl-carrier-protein) synthase (EC 2.3.1.85)	a	–	–	150						
13	cg1280	(odhA) 2-oxoglutarate dehydrogenase	c	12, 14	116	116						
14	cg1656	(ndh) NADH dehydrogenase	1	13	45	45						
15	cg1762	(sufC) iron-regulated ABC transporter ATPase subunit	a	8, 9	27	–	**0.8**	***0.0***	**4.61**			
16	cg1790	(pgk) phosphoglycerate kinase	c	14, 15	43	42						
17	cg1838	alanyl-tRNA synthase	c	–	–	108						
18	cg2181	ABC-type transporter, periplasmic component	a	18,19	58	–						
19	cg2196	putative secreted or membrane protein	2	15	15	–						
20	cg2359	isoleucyl-tRNA synthase	c	–	–	115						
21	cg2404	(qcrA1) Rieske iron-sulfur protein	3	8, 9	44	43						
22	cg2408	(ctaC) cytochrome c oxidase	3	19	37	36						
23	cg2409	(ctaC) cytochrome c oxidase chain II	a	–	–	68						
24	cg2466	pyruvate dehydrogenase	c	–	–	110						
25	cg2492	probable glucosamine-fructose-6-phosphate aminotransferase	c	iP	70	65						
26	cg2662	aminopeptidase N	a	–	–	110						
27	cg2675	ATPase component of ABC-type transport system	a	13, 14	68	–						
28	cg2705	(amyE) maltose binding protein	a	19	47	45	**2.0**	***0.1***	**0.25**	**2.6**	***0.5***	***1.61***
29	cg2833	(cysK) O-acetylserine (thiol)-lyase	c	8-11	36	35						
30	cg2958	(butA) L-2,3-butanediol dehydrogenase/ acetoin reductase	c	10-12	29	29						
31	cg2963	(clpC) probable ATP-dependent protease (heat shock protein)	a	15, 16	116	100						
32	cg2964	(guaB1) inositol-monophosphate	c	11	44	45						

Table 1 (continued)

Spot No.	cg-Identifier	Identified protein	Locali-zation[a]	2D-IEC Fraction No.	2D-IEC MW [kDa]	2D-BAC MW [kDa]	Regulation 2D-IEC PS/WT	S.D.	p-value·10^2	Regulation 2D-BAC PS/WT	S.D.	p-value·10^2
		dehydrogenase										
33	cg3079	(clpB) probable ATP-dependent protease (heat shock protein)	a	12, 13	90	85						
34	cg3100	(dnaK) heat shock protein 70	a	11-13	67	66						
35	cg3138	membrane protease subunit, stomatin/ prohibitin homolog	2	13, 14	32	32						
36	cg3178	polyketide synthase modules and related proteins	a	–	–	140						
37	cg2444	subunit of the cytochrome bc-aa_3 supercomplex	s	16, 17	26	26	**1.9**	*0.1*	**0.46**	1.6	*0.2*	*85.8*

Proteins were separated by either 2D-IEC or 2D-BAC and three independent separations were performed with every strain. *PS*, production strain; *WT*, wild type. Regulation factors are only given for proteins which showed significant regulation.

[a] *c*, cytoplasmic; *s*, secreted; *a*, membrane-associated; digits indicate numbers of transmembrane helices

iP, protein identified in insoluble pellet (not shown)

S.D., standard deviation; n=3; *p*-value for paired *t*-test (two-tailed) for regulation ratio

ized ABC-transport systems (cg0924, cg1762), the maltose binding protein AmyE (part of an ABC-type transport system), and a subunit of the cytochrome bc-aa_3 supercomplex (cg2444) [23]. For succinate dehydrogenase subunit A, AmyE and the hypothetical protein, the regulation tendency was verified with the 16BAC system, although with less significance. It should be emphasized that only the membrane-associated subunit A of the succinate dehydrogenase complex was identified with the 16BAC system, whereas after the 2D-IEC separation the whole complex was found, including the membrane integral subunit cd; all three with a comparable regulation factor of about four. These results show the high applicability of the 2D-IEC system for fast and easy densitometric quantification, which is underlined by the observed regulation of a subunit from the cytochrome bc-aa_3 supercomplex. A significant difference between the 2D-IEC system and the 2D-BAC approach can be demonstrated via the following example: in Fig. 2 (2D-IEC system), the protein for spot no. 37 is clearly resolved and separated in the gels and a significant upregulation can be observed. In contrast, spot no. 37 in Fig. 1 (2D-BAC approach) is not clearly resolved due to horizontal striking, and therefore the upregulation detected with this system is not statistically significant. Indeed, the 2D-BAC approach did not provide superior performance for any of the regulated protein spots.

Discussion

Due to the problems associated with membrane proteomics, the dynamics of the membrane proteome or transcriptome have not been analyzed in detail for any organism. Because of the limited space in membranes and their essential functions as filters or barriers, sophisticated regulation mechanisms are expected for membrane proteins. This should also be the case for the membrane proteome of the industrial L-lysine production strain, which was analyzed in this study. Differences between the membrane proteomes of this strain and the wild type were not obvious, since no gene coding for a membrane protein was affected by the four selective point mutations. For this reason it is very important to develop new proteomics methods such as anion exchange chromatography in combination with SDS-PAGE [5] that can comprehend these subtle differences. Another established two-dimensional separation procedure for membrane proteins is 16BAC-PAGE [4] combined with SDS-PAGE. The 16BAC/SDS-PAGE approach was adapted for the separation of the membrane proteome of *C. glutamicum* in this work, and the separation capacity and the applicability of both systems for quantitative proteomics was evaluated. Furthermore, the differences between both *C. glutamicum* strains discovered here will be discussed in

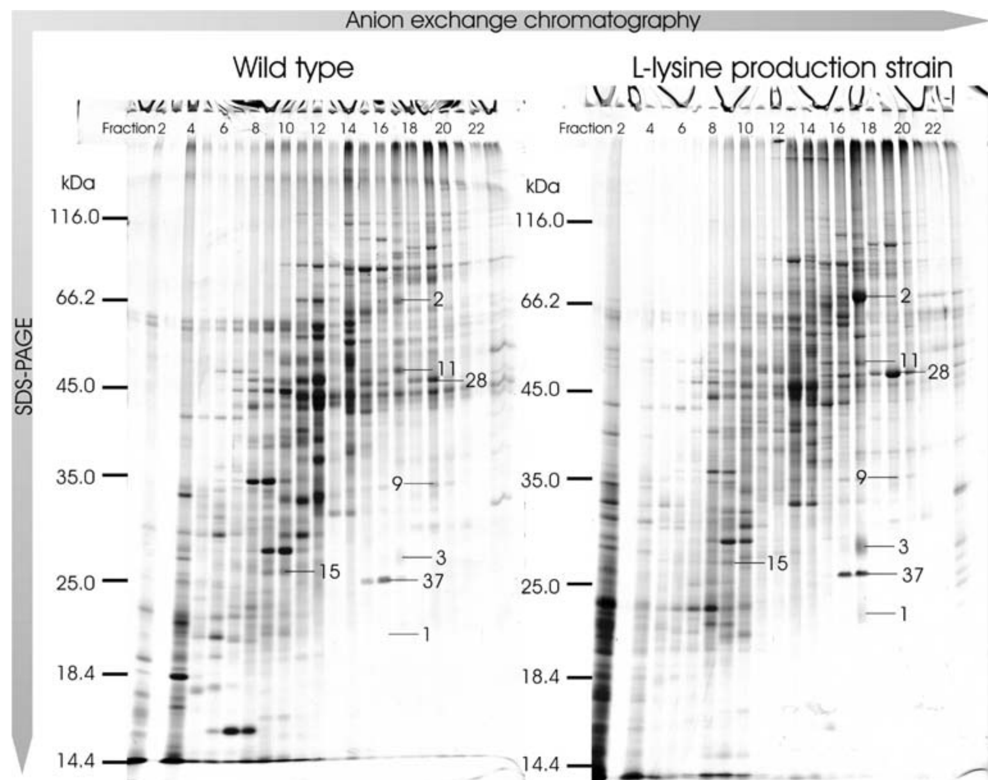

Fig. 2 Separation of the solubilized membrane fractions from the wild-type *C. glutamicum* strain and the L-lysine production strain using AIEC as the first and SDS-PAGE as the second separation dimension (2D-IEC). Before being applied to a 1.5 ml column packed with Poros20 HQ, the membranes were washed twice with 2.5 M NaBr and solubilized in buffer containing 2 % (w/v) ASB-14. 1.7 mg protein was applied onto the column. The numbers of the precipitated fractions are given at the tops of the gels (SDS-PAGE, 12% T, 20 cm separation length). For the sake of clarity, only significantly regulated proteins are marked by numbers

order to highlight the potential of proteomics for metabolic engineering.

Regulation mechanisms in the L-lysine producer

Proteomics technologies can provide invaluable information, e.g., on posttranslational modifications [24], but up to now this potential has not been used for comparative proteomic studies on L-lysine-producing *C. glutamicum* strains. Characterization of such a strain by DNA microarray analysis involved the use of a small array of 52 genes and revealed downregulation of the genes for citrate synthase and 2-oxoglutarate synthase, both part of the citrate acid cycle [25]. Another study combined transcriptome, metabolome and fluxome data, resulting in a good correlation between gene expression and in vivo activity for many enzymes [26]. One drawback of these studies is the use of strains generated by random mutagenesis, which implies a less well-defined genetic background. We therefore decided to examine in more detail an L-lysine producing strain containing only mutations in the four previously described genes *lysc*, *hom*, *zwf*, and *pyc*. Although this strain differs from the wild type in only these four mutated cytoplasmic proteins, it shows significantly different features, including the physical properties of the membrane. In order to analyze these secondary effects of metabolic engineering and obtain better insights into the process of L-lysine production and excretion, various approaches have been applied. Previously published studies focused mainly on data from transcriptome analysis [25], [22] or a combination of metabolome and fluxome analysis [26] for rational strain improvement of *C. glutamicum*. Remarkably, the expression of most genes encoding enzymes of the primary metabolism changed by less than 1.3-fold upon the shift to L-lysine production for one strain [25]; in contrast, several enzymes of the industrial strain B-6 show a much stronger regulation, with factors of up to 54. Among the membrane proteins, especially transcription of *lysE*, which encodes a lysine transporter, was strongly induced under L-lysine production conditions [25, 26], in agreement with the expectation that decisive regulation mechanisms particularly effect proteins of the cytoplasma membrane. On the other hand, no regulation of central metabolic enzymes was observed for a strain obtained by genome-based strain reconstruction, i.e., similar to ours [12]. The present 2D-IEC study of the L-lysine-producing *C. glutamicum* cells clearly shows that a number of membrane proteins are regulated, though some of them displayed only minor changes. Among them are the upregulation of a maltose-binding protein (AmyE) and an ABC-type cobalamin/Fe^{3+}-siderophore transport system (cg0924) in combination with the downregulation of other unknown ABC-type transport systems (cg0737, cg1762), as evidenced by the 2D-IEC studies. This may point to the consumption of alternative carbon sources of the L-lysine-producing cells, which has not been found in previous studies; instead, they showed

Table 2 Identification scores of the regulated proteins identified in this study by MALDI-TOF PMF

Cg-Identifier	Identified protein	MASCOT score	Coverage [%]	Matched peptides
cg0446	(sdhA) succinate dehydrogenase A	82	20	15
cg0447	(sdhB) succinate dehydrogenase B	67	19	5
cg0445	(sdhCD) succinate dehydrogenase CD	68	20	5
cg0577	DNA-directed RNA polymerase beta subunit / 160 kDa subunit	192	28	30
cg0583	(fusA) elongation factor G	157	36	22
cg0587	(tuf) elongation factor TU	139	59	17
cg0610	(rplE) 50S ribosomal protein L5	71	53	10
cg0654	(rpsD) ribosomal protein S4	61	39	8
cg0737	secreted lipoprotein	68	38	7
cg0791	(pyc) pyruvate carboxylase	137	24	23
cg0924	ABC-type cobalamin/Fe^{3+}-siderophores transport system	93	30	6
cg0957	fatty acid synthase	185	11	31
cg1280	(odhA) 2-oxoglutarate dehydrogenase	100	19	19
cg1656	(ndh) NADH dehydrogenase	94	36	13
cg1762	(sufC) iron-regulated ABC transporter ATPase subunit	129	65	15
cg1790	(pgk) phosphoglycerate kinase	68	12	6
cg1838	alanyl-tRNA synthase	82	22	16
cg2181	ABC-type peptide transport system, secreted component	67	16	5
cg2359	isoleucyl-tRNA synthase	101	24	18
cg2404	(qcrA1) Rieske iron-sulfur protein	76	42	12
cg2408	(ctaC) cytochrome c oxidase	80	25	5
cg2409	(ctaC) cytochrome c oxidase chain II	69	25	8
cg2466	pyruvate dehydrogenase	140	31	21
cg2492	probable glucosamine-fructose-6-phosphate aminotransferase	109	33	15
cg2662	aminopeptidase N	142	31	22
cg2675	ATPase component of ABC-type transport system	134	40	18
cg2705	(amyE) maltose binding protein	72	39	10
cg2833	(cysK) O-acetylserine (thiol)-lyase	192	72	19
cg2958	(butA) L-2,3-butanediol dehydrogenase/acetoin reductase	67	43	8
cg2963	(clpC) probable ATP-dependent protease (heat shock protein)	82	23	17
cg2964	(guaB1) inositol-monophosphate dehydrogenase	154	48	17
cg3079	(clpB) probable ATP-dependent protease (heat shock protein)	121	32	20
cg3100	(dnaK) heat shock protein hsp 70	135	40	17
cg3138	membrane protease subunit, stomatin/prohibitin homolog	104	44	13
cg3178	polyketide synthase modules and related proteins	76	15	20
g2444	subunit of the cytochrome bc-aa_3 supercomplex	80	45	5

Proteins were identified using the MASCOT algorithm http://www.matrixscience.com/search_form_select.html) by searching in the NCBInr (National Center for Biotechnology Information) database or using Sequest [5]

decreasing expression of genes which code for PTS proteins (responsible for sugar uptake) at the end of the exponential growth phase [26]. Upregulation of a subunit of the cytochrome bc-aa_3 supercomplex points to the increased activity of oxidative phosphorylation. This finding agrees well with the increased abundance of succinate-DH, which was detected in L-lysine-producing cells by 2D-IEC. Previous DNA microarray results did not show changes in succinate-DH [26], and metabolic network analysis has revealed decreased flux through the tricarboxylic acid (TCA) cycle accompanied by increased flux through the pentose phosphate pathway. Succinate itself was measured as a byproduct accumulating in L-lysine-producing cells [27], so an increased expression of succinate-DH would be coherent. Both strains analyzed in this work were grown via large-scale fermentation and harvested in the stationary phase. Future analysis should focus on the analysis of the production strain during different growth phases, especially since it was shown that the growth phase has a stronger impact on transcriptional regulation than the shift to L-lysine production conditions [26], and thus calculated regulations may result from a couple of effects, such as different culture conditions.

In summary, our study indicates that TCA cycle enzymes and members of the respiratory chain play an important role in amino acid production, besides their previously known impact on the pentose phosphate pathway. More studies are required in order to increase the amino acid production with

C. glutamicum, especially as the majority of the identified regulated proteins are not directly linked to the L-lysine production pathways (as summarized in Fig. 3) or to the mutated genes in the L-lysine production strain, i.e., secondary effects seem to influence the regulation of these proteins. While these secondary effects may be triggered by the differences in metabolic activity between producer and wild type, they may also be caused by the different conditions of the two cultures, such as cell density or the accumulation of L-lysine and other metabolites. Comparison of more samples under various culture conditions should allow these effects to be differentiated in the future. Since the observed differences in the membrane proteome could not have been predicted, our study impressively demonstrates the importance of proteomics screening technologies for enhancing our understanding of bacterial physiology and that this systemic approach can be used to further improve production strains [28, 29].

Evaluation and comparison of the 2D-IEC and 2D-BAC methods used for quantitative membrane proteomics

A special advantage of 2D-BAC is its two-step electrophoretic protein separation that circumvents the limitations of

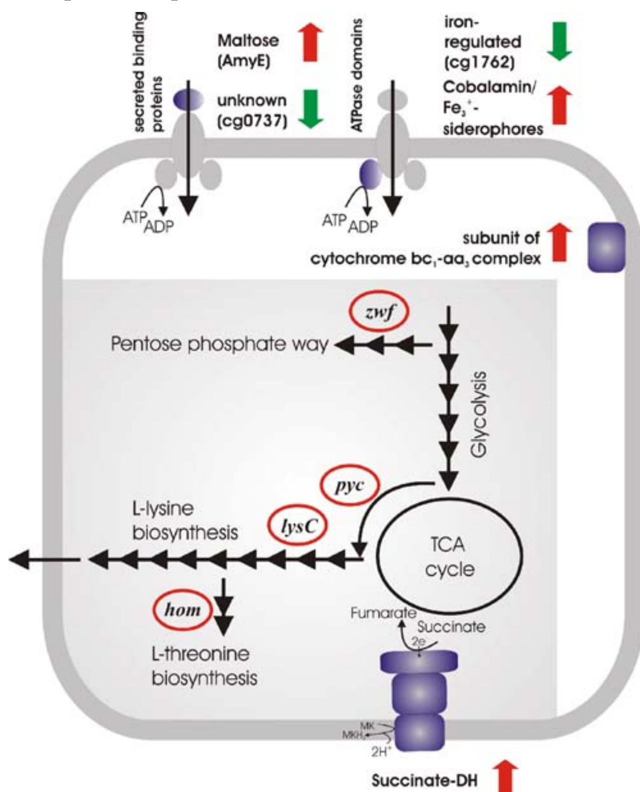

Fig. 3 Pathways involved in L-lysine production in *C. glutamicum*. The four mutated genes in the L-lysine production strain are marked by *red circles*. Regulations of membrane-integral or membrane-associated proteins identified in this study are indicated by arrows. Besides succinate-DH, no obvious relation was found between the regulated proteins and the pathways involved in L-lysine biosynthesis

common 2-DE. Sample prefractionation (i.e., high-salt wash with NaBr) was performed according to the optimized protocol for the AIEC/SDS-PAGE method (2D-IEC), and samples were analyzed in parallel, allowing a direct comparison of both methods.

Remarkably, only 4 out of the 29 proteins identified using the 2D-BAC technique contained integral membrane domains. This result is disappointing, as the analysis of a comparable sample using 2D-IEC identified 170 proteins [5], including 50 integral membrane proteins. Only eight proteins (three membrane-associated and five cytoplasmic proteins) were detected exclusively by the 2D-BAC technique. This is in contrast to other studies which achieved a distinct increase in unique proteins by using alternative separation techniques in parallel (e.g., [30]). The solubilization and separation procedures are markedly different between the 2D-IEC and 2D-BAC approaches. The 2D-BAC technique itself has already been demonstrated to work well for hydrophobic membrane proteins, and even proteins with 12 TMHs and GRAVY scores of up to 0.742 have been identified [4, 7, 31]. It should be emphasized, however, that studies published so far have mainly focused on the separation of eukaryotic membrane proteins by 2D-BAC (i.e., synaptic vesicles or purified mitochondria from *S. cerevisiae*, brain proteins from mice). Prokaryotic membranes, especially the unique actinomycetes membrane, may require a quite different treatment, and 2D-BAC does not seem to be the best strategy for analyzing prokaryotic membrane proteomes. A major reason for this may be the limited resolution and loading capacity of the system. In agreement with this, a recent study that used different proteomic techniques to profile the mammalian mitochondrial proteome showed the limited resolution of the 2D-BAC technique in comparison with 2-DE [9]. While a standard 2D-IEC gel like that used in our study separates proteins in different lanes on an area of 300 cm^2, proteins along the characteristic diagonal in the 2D-BAC gels are concentrated into an area five times smaller (60 cm^2). This may be no problem for enriched samples with low complexity [4], nor for the identification of proteins by additional fractionation techniques at the peptide level (e.g., by LC-MS/MS [7]), but it prevents the analysis of the complex *C. glutamicum* membrane fraction by MALDI-ToF PMF. Although much effort was made to improve the acrylamide gradients for the first and second separation dimensions of 2D-BAC, the resolution power could only be improved to the level shown here. The unsatisfactory separation of the 2D-BAC system also prevents reliable quantification of proteins by densitometric approaches. Only clearly resolved spots containing just one protein can be used for quantitative studies. Nevertheless, 2D-BAC gels were highly reproducible and could be used for the relative quantification of proteins from the L-lysine

production strain in comparison to the wild type provided that the analysis was restricted to clearly isolated protein spots. Regulation factors identified by 2D-BAC or 2D-IEC using the same sample corresponded very well. Comparison of the regulation factors calculated from the two methods, including their standard deviations, shows the high potential of the 2D-IEC method, which is highly reproducible and superior to 2D-BAC. Although the 2D-IEC method is not as comprehensive as other approaches (e.g., MudPIT [2]), its main advantage is that it is very fast and simple. As shown in the present study, the utilization of densitometry of the Coomassie-stained gels for quantification is appropriate for the identification of regulated proteins that are interesting candidates for further, more extensive research. The use of Coomassie-stained gels for quantification is, however, limited to a small linear range. Additionally, gel-to-gel variations may cause problems with high standard deviations of the calculated regulations. Recently published techniques that circumvent these problems are the parallel labeling of the samples with fluorescent dyes prior to their separation (difference in gel electrophoresis, DIGE). This detection method was successfully used for the quantitative profiling of the membrane proteome in a halophilic archeon by 16BAC/SDS-PAGE [32]. The applicability of the fluorescent labeling and the subsequent separation by liquid chromatography was shown previously [33].

Adapting this system to a more elaborate study that used fluorescent labeling of the proteins in combination with the good separation capacity of the 2D-IEC system could specify the regulation tendency of the candidates found in a first screen. This would result in a promising strategy for membrane proteomics in the future.

In conclusion, our study clearly shows that the 2D-IEC approach is very simple, fast and effective for a first screening of interesting mutants and/or growth conditions in *C. glutamicum* and probably other bacteria; it is altogether more suitable than the established 16BAC/SDS-PAGE. With the identification of regulated membrane proteins, a first step towards understanding the membrane physiology of this organism during amino acid production has been made. This in turn should enable the detailed analysis of key proteins in this fermentation process of great biotechnological relevance in the future.

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References

1. Santoni V, Molloy M, Rabilloud T (2000) Electrophoresis 21:1054–1070
2. Washburn MP, Wolters D, Yates JR 3rd (2001) Nat Biotechnol 19:242–247
3. Taylor SW, Fahy E, Zhang B, Glenn GM, et al. (2003) Nat Biotechnol 21:281–286
4. Hartinger J, Stenius K, Hogemann D, Jahn R (1996) Anal Biochem 240:126–133
5. Schluesener D, Fischer F, Kruip J, Rogner M, Poetsch A (2005) Proteomics 5:1317–1330
6. Laemmli UK (1970) Nature 227:680–685
7. Zahedi RP, Meisinger C, Sickmann A (2005) Proteomics 5:3581–3588
8. Dreger M, Bengtsson L, Schoneberg T, Otto H, Hucho F (2001) Proc Natl Acad Sci USA 98:11943–11948
9. Hunzinger C, Wozny W, Schwall GP, Poznanovic S, et al. (2006) J Proteome Res 5:625–633
10. Stackebrandt E, Rainey FA, Ward-Rainey NL (1997) Int J Syst Bacteriol 47:479–491
11. Schafer A, Tauch A, Jager W, Kalinowski J, et al. (1994) Gene 145:69–73
12. Ohnishi J, Mitsuhashi S, Hayashi M, Ando S, et al. (2002) Appl Microbiol Biotechnol 58:217–223
13. Moritz B, Striegel K, De Graaf AA, Sahm H (2000) Eur J Biochem 267:3442–3452
14. Koffas MA, Jung GY, Stephanopoulos G (2003) Metab Eng 5:32–41
15. Kalinowski J, Bathe B, Bartels D, Bischoff N, et al. (2003) J Biotechnol 104:5–25
16. Ikeda M, Nakagawa S (2003) Appl Microbiol Biotechnol 62:99–109
17. Keilhauer C, Eggeling L, Sahm H (1993) J Bacteriol 175:5595–5603
18. Peterson GL (1979) Anal Biochem 100:201–220
19. Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Electrophoresis 9:255–262
20. Vrljic M, Sahm H, Eggeling L (1996) Mol Microbiol 22:815–826
21. Gu S, Chen J, Dobos KM, Bradbury EM, et al. (2003) Mol Cell Proteomics 2:1284–1296
22. Hayashi M, Ohnishi J, Mitsuhashi S, Yonetani Y, et al. (2006) Biosci Biotechnol Biochem 70:546–550
23. Niebisch A, Bott M (2003) J Biol Chem 278:4339–4346
24. Bendt AK, Burkovski A, Schaffer S, Bott M, et al. (2003) Proteomics 3:1637–1646
25. Loos A, Glanemann C, Willis LB, O'Brien XM, et al. (2001) Appl Environ Microbiol 67:2310–2318
26. Kromer JO, Sorgenfrei O, Klopprogge K, Heinzle E, Wittmann C (2004) J Bacteriol 186:1769–1784
27. Wittmann C, Heinzle E (2002) Appl Environ Microbiol 68:5843–5859
28. Wendisch VF (2005) Chem Today 23(1):49–52
29. Koffas M, Stephanopoulos G (2005) Curr Opin Biotechnol 16:361–366
30. Froehlich JE, Wilkerson CG, Ray WK, McAndrew RS, et al. (2003) J Proteome Res 2:413–425
31. Bierczynska-Krzysik A, Kang SU, Silberrring J, Lubec G (2006) Neurochem Int 49:245–255
32. Bisle B, Schmidt A, Scheibe B, Klein C, et al. (2006) Mol Cell Proteomics 5:1543–1558
33. Wang H, Clouthier SG, Galchev V, Misek DE, et al. (2005) Mol Cell Proteomics 4:618–625