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Mapping the membrane proteome of *Corynebacterium glutamicum*

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In order to avoid the specific problems with intrinsic membrane proteins in proteome analysis, a new procedure was developed which is superior to the classical two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) method in terms of intrinsic membrane proteins. For analysis of the membrane proteome from *Corynebacterium glutamicum*, we replaced the first separation dimension, *i.e.*, the isoelectric focusing step, by anion-exchange chromatography, followed by sodium dodecyl sulfate (SDS)-PAGE in the second separation dimension. Enrichment of the membrane intrinsic subproteome was achieved by washing with 2.5 M NaBr which removed more than 35% of the membrane-associated soluble proteins. For the extraction and solubilization of membrane proteins, the detergent amidosulfobetaine 14 (ASB-14) was most efficient in a detailed screening procedure and proved also suitable for chromatography. 356 gel bands were spotted, and out of 170 different identified proteins, 50 were membrane-integral. Membrane proteins with one up to 13 transmembrane helices were found. Careful analysis revealed that this new procedure covers proteins from a wide p*I* range (3.7–10.6) and a wide mass range of 10–120 kDa. About 50% of the identified membrane proteins belong to various functional categories like energy metabolism, transport, signal transduction, protein translocation, and proteolysis while for the others a function is not yet known, indicating the potential of the developed method for elucidation of membrane proteomes in general.

Keywords:
Anion exchange chromatography / *Corynebacterium glutamicum* / Intrinsic membrane protein / Mass spectrometry

1 Introduction

Corynebacterium glutamicum is one of the most important organisms for industrial microbiology. Since the discovery in 1957 by Kinoshita *et al*. [1], it has been used for the production of an increasing amount of the amino acids L-lysine (560 000 tons per year) and L-glutamate (1 000 000 tons per year). Other amino acids obtained by fermentation are L-alanine, L-isoleucine, and L-proline. *C. glutamicum* is a Gram-positive, aerobic, coryneform, nonsporing, nonmotile bacterium with a high GC content [2]. It belongs to the group of mycolic acid containing actinomycetes. Whereas this group also comprises pathogenic bacteria like *Mycobacterium tuberculosis* and *Corynebacterium diphteriae, C. glutamicum* is generally recognized as harmless for humans. The cell envelope of this group is unique among the Gram-positive bacteria being composed of the following layers [3]: the plasma membrane, peptidoglycan covalently linked to arabinogalactan (esterified with mycolic acid), free mycolic acids, and a crystalline protein layer, the S-layer. The cell envelope of *C. glutamicum* thus resembles very much the cell wall of Gram-negative bacteria. The

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Abbreviations: AIEC, anion-exchange chromatography; **ASB-14**, amidosulfobetaine 14; **β-DM**, *N*-dodecyl β-D-maltoside; **MIP**, membrane integral proteins; **TMH**, transmembrane helix

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chain length of the mycolic acids is shorter ($C_{22–36}$) in corynebacteria than in mycobacteria ($C_{60–90}$) [4]. The plasma membrane is mainly composed of phosphatidyl glycerol [5]. The major identified protein components of the cell wall are porins [6, 7], mycoltransferases [8], and the S-layer forming protein PS2 [9]. It has been shown that the cell envelope of mycobacteria presents a major transport barrier [10] which is relevant for the antibiotic resistance of pathogenic mycobacteria [11] and for the amino acid production with *C. glutamicum* [12]. The permeability of the cell envelope is affected by the lipid composition [13] with specific import and export systems existing for different compounds. Examples are the L-lysine permease *lysI* [14], the L-glutamate import system *gluABCD* [15], and the import systems for ammonium *amt* and *amtB* [16]. Exporters for basic amino acids, *lysE*, for L-threonine and L-serine, *thrE*, and other amino acids have been reported [17] and their identification led to the discovery of new translocator families. However, the L-glutamate export system has not yet been identified and it is only known that the L-glutamate excretion is energy-dependent [18].

The biotechnological importance of *C. glutamicum* stimulated groups from industry and academics to determine the complete genome sequence independently [19, 20]. In the sequenced genomes of *C. glutamicum* between 2900 (GenBank NC_003450) and 3099 proteins were identified of which about 660 (22% of the total) were annotated as integral membrane proteins [19]. The availability of the genome sequence now enables more rational approaches for the strain engineering in order to obtain better amino acid producers. In the past, several cycles of undirected mutagenesis combined with small-scale fermentation were carried out in order to increase the productivity, while presently mutations are introduced selectively in the process of metabolic engineering. It was found that five times more L-lysine is exported compared to the wild type if the L-lysine exporter *lysE* is overexpressed [21]. However, the overexpression of a single gene in a product pathway can lead to flux imbalances thereby yielding unexpected results. This can be circumvented by the coordinated overexpression of more than one gene per pathway [22].

Prerequisite for a successful approach is to monitor such pathways as precisely as possible. The required technologies for such a purpose, transcriptomics and proteomics, can now yield qualitative and quantitative information about a large ensemble of gene products in one experiment. These technologies provide the opportunity to monitor several pathways at the transcript/protein level in parallel and to discover global interactions. DNA microarray analysis has been carried out for *C. glutamicum* under growth on acetate or glucose and under heat shock [23], as well as under phosphate starvation conditions [24]. These conditions effect both the expression of soluble and membrane proteins. Under starvation, the increased expression of a phosphate ABC-type uptake system and 24 other genes related to phosphate uptake and metabolism was observed. Also, a putative permease was induced after heat shock. Despite these interesting results, transcriptome data alone are not sufficient to establish pathway models and can lead to false conclusions [25]. So far, the proteome of *C. glutamicum* has been analyzed exclusively by the 2-D electrophoresis technique [26, 27], and preliminary results for a phosphoproteome map of the cytoplasmic fraction have been obtained [28]. However, the number of proteins identified in the membrane fractions was very low in these studies and no integral membrane proteins have been detected. In this report we show that a much higher yield of membrane proteins can be achieved if IEF, *i.e.*, the first step in 2-D PAGE, is replaced by ion-exchange chromatography. While maintaining SDS-PAGE in the second dimension, we show that integral membrane proteins can be successfully separated by this approach and identified by MALDI-TOF-MS. We also show that, prior to chromatography, membrane proteins could be enriched by the development of an optimized prefractionation protocol. We identified 50 membrane integral proteins in this study, which is 7.5% of the predicted membrane proteome. This newly developed separation technique enables us to study the membrane proteome of *C. glutamicum* under different cultivation conditions. It will also be useful for the collection of membrane proteomes from various microorganisms in general.

2 Materials and methods

2.1 Bacterial strains, growth, and cell lysis

Cells of *C. glutamicum* strain DM 1698 (derived from ATCC 21527) were grown in a large-scale fermenter (cell material kindly supplied by Degussa, Halle, Germany) and harvested by centrifugation for 15 min at 4500 × *g*; cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and resuspended at a concentration of 4 mL buffer/g wet cells in disintegration buffer (PBS containing additional 20 mM $MgCl_2$, 10 mM $MnCl_2$, 200 U/mL DNaseI, protease inhibitor mix for bacterial cells (Sigma, St. Louis, MO, USA)). Disruption of the cells was done by a French Pressure Cell (40K cell with a volume of 35 mL, Thermo Spectronic, Rochester, USA) with 4 passages at 20 000 psi. Unbroken cells and cell debris were sedimented twice by centrifugation at 5000 × *g* and 4°C. Membranes were enriched by ultracentrifugation at 100 000 × *g* and 4°C for 30 min. The resulting pellet was resuspended gently with ice-cold PBS buffer and ultracentrifugation was repeated.

2.2 Washing and solubilization of membranes

To optimize removal of membrane-associated proteins, membranes were washed with several salts, chaotropic compounds or a neutral buffer. Membranes (about 300 μg protein) were incubated in 500 μL of the different solutions

(20 mM Tris·HCl, pH 8.0; 100 mM sodium carbonate; 6 M urea; 4 M guanidine thiocyanate; 0.5, 1, 2, and 2.5 M NaBr) at 4°C and mixed on a rotary shaker set to 15 rpm (Rotamix RM1, ELMI, Tartu, Estonia) for 30 min. Membrane integral proteins (MIPs) and membrane-associated proteins (MAPs) were separated by centrifugation at 100 000 × *g* for 30 min. This washing step was repeated twice. The amount of protein in the membrane fraction and in the supernatant was estimated according to Lowry [29]. Membranes were incubated for 1 h at room temperature in solubilization buffer (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 2% w/v detergent at a ratio 10:1 to protein w/w) under agitation by a rotary shaker at 15 rpm. Unsolubilized proteins were sedimented at 100 000 × *g* and 4°C for 30 min. SDS was obtained from GERBU (Gaiberg, Germany), *N*-dodecyl β-D-maltoside (β-DM) from Biomol (Hamburg, Germany), all other detergents were obtained from Calbiochem (Bad Soden, Germany).

2.3 Chromatography

Proteins were separated by anion-exchange chromatography (AIEC) using a column (4.6 mm diameter/100 mm length) packed with Poros 20 HQ material (Applied Biosystems, Darmstadt, Germany). The washed and solubilized membrane fraction was applied in 2 mL solubilization buffer (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 2% w/v detergent at a ratio 10:1 to protein w/w). After rinsing the column with 1.5 column volumes starting buffer (20 mM Tris·HCl, pH 8.0, 0.03% w/v amidosulfobetaine 14 (ASB-14)), the concentration of NaCl was increased from 0.2 M to 0.65 M by 38 column volumes followed by a sharp increase to 1 M NaCl. Finally, the column was washed with 6.5 column volumes of elution buffer and fractions of 1.5 mL were collected. HPLC was performed on a Waters system (two pumps, model 510 fitted with preparative pump heads and Rheodyne injector, Model 9125i) coupled to a diode array detector (PDA 996; Waters, Milford, MA, USA) and a conductivity monitor (Amersham Biosciences, Freiburg, Germany). All buffers were cooled to 4°C and columns were operated at 10°C by using a column oven (Jasco, Gross-Umstadt, Germany).

2.4 SDS-PAGE

For concentration and desalting, proteins were precipitated by either TCA [30] or a modified method employing sodium deoxycholate [31], which is compatible with high concentrations of chaotropic compounds. SDS-PAGE was performed according to Laemmli [32] with gels containing 1 M urea. Precipitated proteins were dissolved in sample buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v SDS, 62 mM Tris·HCl, pH 6.8, 0.01% bromophenol blue) and incubated for 30 min at 60°C. Proteins were stained by colloidal Coomassie according to Neuhoff [33].

2.5 In-gel tryptic digestion followed by CNBr cleavage

After visualization with Coomassie blue, protein bands were excised from the SDS-polyacrylamide gel and completely destained with 100 µL 25 mM ammonium hydrogencarbonate and 50% v/v acetonitrile (three times for 20 min at 37°C following the protocol of Hellman [34]). Subsequently, the gel pieces were completely dried in a SpeedVac. Tryptic digest was started by the addition of 10 µL from a 12.5 ng/µL trypsin solution (sequencing grade, Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 8.0, and the protein was digested overnight at 37°C. After tryptic digest the gel pieces were completely dried in the SpeedVac and a CNBr cleavage was carried out in the dark for at least 12 h at room temperature. For this purpose one small crystal of CNBr was dissolved in 200–300 µL of 70% TFA and added to the dried gel pieces followed by several washing steps according to the protocol of Van Montfort [35].

2.6 MALDI-MS and protein identification

The dried samples were dissolved in 5 µL of 50% v/v acetonitrile, 0.5% TFA and sonicated for 5 min. Aliquots of 0.6 µL were applied onto the target plate and immediately mixed with an equal volume of α-cyano-hydroxycinnamic acid in 60% v/v acetonitrile, 1% v/v TFA. MALDI-TOF mass spectra were recorded with an Applied Biosystems Voyager-DE Pro system in reflector mode. The spectra were first precalibrated externally, followed by an internal recalibration using trypsin autoproteolytic products. Monoisotopic peptide masses obtained from MALDI-TOF-MS were used to search the *C. glutamicum* protein database with GPMAW (Lighthouse data, Ver. 6.01, Denmark) and in addition the actinobacteria database with the MASCOT (www.matrixscience.com, Ver. 2.0) algorithm. Protein identification was accepted if there were more than six peptides matched by GPMAW or if a significant MASCOT score was achieved. The range of molecular masses for protein search was set between 1000 and 200 000 Da with a peptide ion mass tolerance of $\leq$ 100 ppm.

2.7 LC-ESI and protein identification

Samples that could not be identified by MALDI-TOF-MS (see protocol above) were analyzed by a LC-ESI-MS system described previously [36]. CNBr-trypsin fragments were dissolved in 10 µL 25 mM ammonium carbonate buffer and loaded onto a 100 × 365 µm fused-silica capillary (J&W Science, Folsom, CA, USA) which was packed with 10 cm of 5 µm Zorbax Eclipse XDB C_{18} (Hewlett-Packard, Palo Alto, CA, USA). An Ultimate Dual Gradient pump (LC-Packings, Amsterdam, NL) was interfaced with a Finnigan LTQ ion trap mass spectrometer to generate an effective flow rate of 0.2 µL/min and supply a spray voltage of 1.8 kV. After the column was equilibrated for 5 min with buffer A (5% aceto-

nitrile, 95% H_2O, and 0.5% acetic acid) a linear gradient from 0% up to 100% buffer B (80% acetonitrile, 20% H_2O, and 0.5% acetic acid) was generated. The LTQ was operated *via* Instrument Method files in the Sequence Setup window Xcalibur. The LTQ was set to acquire a full MS scan between 400 and 2000 *m/z* followed by full MS/MS scans (between 400 and 2000 *m/z)* of the top three ions from the preceding MS scan. Dynamic exclusion was enabled with a repeat count of 2. The repeat duration was set to 30 sec and the exclusion duration window to 3 min. The SEQUEST algorithm was used to interpret MS/MS spectra. Results were interpreted on the basis of a conservative criteria set, *i.e.*, only results with ΔCn scores greater than 0.1 were accepted, all fragments had to be tryptic or fragments of a CNBr cleavage and the cross-correlation scores (Xcorr) of single charged, double charged or triple charged ions had to be greater than 1.5, 2.5, or 3.5. Two peptides for each protein needed to be identified for the identification to be considered legitimate. Spectra were manually evaluated to match the following criteria: distinct peaks with signals clearly above noise levels, differences of fragment ion masses in the mass range of amino acids, and fulfilment of consecutive b and y ion series.

3 Results

3.1 Washing and solubilization of membranes

The aim of the present work was to develop an efficient method for the mapping of membrane proteins from *C. glutamicum* using LC in the first and SDS-PAGE in the second dimension. For this purpose, the membrane fraction was separated from the cytosolic proteins. A preliminary MALDI-TOF analysis revealed that even after lysis in PBS buffer a lot of soluble and membrane-associated proteins remain in the membrane fraction (for example, the ribosomal proteins L1/L2/L3/L4/L5 or the elongation factor Tu). These proteins are often highly abundant and may therefore obscure membrane proteins in the SDS-PAGE. Our prior aim was to separate and identify integral membrane proteins, and therefore it was necessary to further reduce the complexity of the membrane fraction by removing cytosolic and membrane-associated proteins.

To obtain a fraction enriched in membrane integral proteins, different washing conditions were tested (Fig. 1). If membranes were washed with a neutral buffer (Tris·HCl, pH 8.0) or sodium carbonate (pH 11), between 18% and 26% of the total protein amount was removed from the membranes. To estimate the ratio of soluble and membrane integral proteins in the membrane and wash fraction, proteins were separated by SDS-PAGE and the most intense bands analyzed with MALDI-TOF-peptide mass fingerprinting (PMF). We found that many soluble and membrane-associated proteins remain in the membrane fraction, for example, the ribosomal proteins S3, L3, and others. These proteins are removed from the membrane fraction by treatment

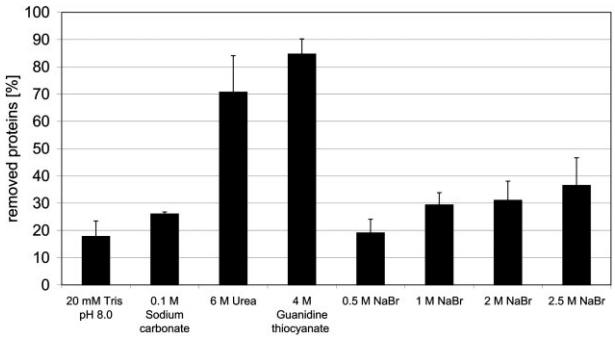

Figure 1. Amount of proteins removed from *C. glutamicum* membranes by treatment with different washing solutions. Black bars indicate the standard deviation ($n = 3$). The total amount of protein (washed membranes + supernatant) was set to 100%. The membranes were washed with various salts, chaotropic compounds, and a neutral buffer to remove membrane-associated proteins as completely as possible. Membranes were separated from the soluble proteins by ultracentrifugation at 100 000 g. The amount of protein in each fraction was estimated according to Lowry [29].

with 2.5 M NaBr, but not with 20 mM Tris. Using the chaotropic reagents urea or guanidine thiocyanate, between 70% and 90% of these proteins could be removed from the membranes. After the guanidine thiocyanate wash, followed by ion-exchange chromatography (IEC) and SDS-PAGE (see above) we could detect 20 bands and identify 15 proteins: 3 soluble, 2 membrane-associated, 4 secreted, 2 membrane integral (1 and 3 TMH), and 4 with lipid anchor. Of these identified proteins 3 were not detected in the master gel (cg0040 putative secreted protein, cg3237 manganese superoxide dismutase (soluble) and cg0044 probable solute-binding lipoprotein (membrane-associated)). In comparison to milder washing procedures, not only bands from soluble proteins, but also integral membrane proteins are missing.

Performing the IEC and SDS-PAGE with urea washed membranes leads to about 150 gel bands; all are also present in the master gel. In the supernatant of the urea washed membrane fraction we identified several membrane proteins *via* ESI-MS, among them the putative membrane proteins cg2196 and cg2657, which are found in the membrane fraction after a milder wash with 2.5 M NaBr. We therefore concluded that milder procedures have to be used. The best results were obtained by washing the membranes with 2.5 M NaBr, which removes about 40% of the proteins.

3.2 Protein solubilization

The washed membrane fraction has to be solubilized by a detergent buffer which is compatible with AIEC. Therefore, beside SDS as control, only nonionic and zwitterionic detergents were tested (Fig. 2). The highest amount of protein was solubilized by a buffer containing 2% w/v of the zwitterionic detergent ASB-14 (Fig. 2C); only 5–12% of the whole-membrane proteins remained in the insoluble pellet according to

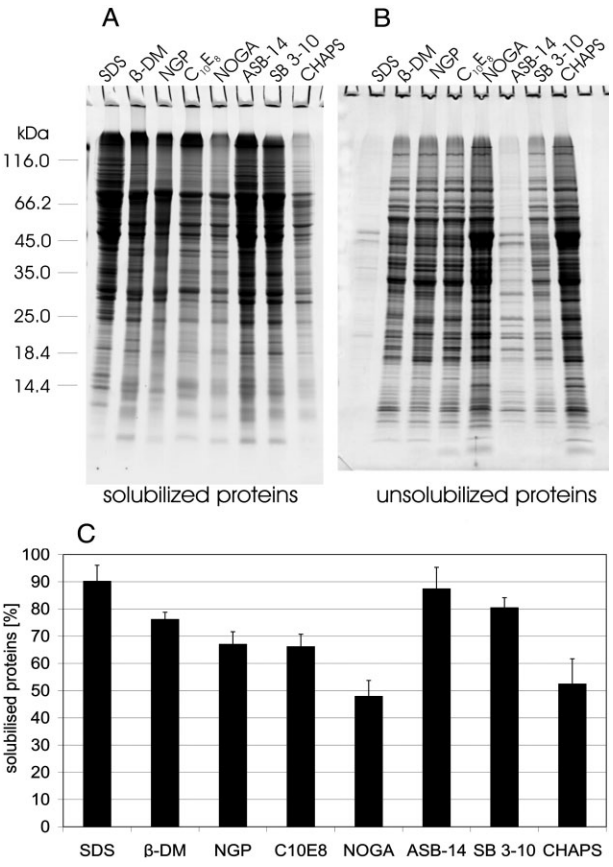

Figure 2. Effect of different detergents on the solubilization of *C. glutamicum* membrane proteins. A defined amount of protein (300 μg) was solubilized in solubilization buffer at room temperature for 1 h. Solubilized and not solubilized proteins were separated by ultracentrifugation at 100 000 × *g*. Among the detergents which are compatible with anion-exchange chromatography, ASB-14 yielded the best solubilization. (A) SDS-PAGE of membrane proteins solubilized with various detergents; 12% T (1% C) to 15% T (1.3% C) gradient gel, 15 cm. After solubilization, the supernatant was precipitated [31] and completely applied onto the gel. (B) SDS-PAGE of unsolubilized proteins; 12% T (1% C) to 15% T (1.3% C) gradient gel, 15 cm. After solubilization, the pellet was dissolved in SDS-sample buffer and completely applied onto the gel. (C) Amount of membrane proteins solubilized with different detergents. Error bars indicate the standard deviation (n = 3). The total amount of protein was set to 100% (solubilized + unsolubilized proteins). The amount of protein in each fraction was estimated according to Lowry [29].

SDS-PAGE. Surprisingly, MALDI-TOF analysis revealed that the most abundant proteins found in this pellet are cytosolic proteins: out of the 13 proteins in the pellet (see Table 1) 12 were cytosolic, and one was a membrane integral protein with two transmembrane helices (TMHs), which was absent in the master gel. The solubilization efficiency of different detergents was analyzed by SDS-PAGE (Fig. 2A). The best result in respect to amount of solubilized protein and solubilization selectivity was obtained with the detergent ASB-14 (Fig. 2B).

Table 1. Proteins identified in the unsoluble pellet after solubilization of the membrane fraction with 2% w/v ASB-14

cg identifier	Function	Apparent MW (kDa)	Localization[a)]
cg0991	(rpmB) 50S ribosomal protein L28	14	c
cg2099	Putative membrane protein	24	2
cg0654	(rpsd) Ribosomal protein S4	25	c
cg0631	(rpsE) 30S ribosomal protein S5	26	c
cg0596	(rplD) 50S ribosomal protein L4	27	c
cg0601	(rpsC) 30S ribosomal protein S3	30	c
cg0598	(rplB) 50S ribosomal protein L2	33	c
cg0587	(tuf) Elongation factor Tu	45	c
cg2499	(glyS) Glycyl-tRNA synthetase (glycine-tRNA ligase) (EC 6.1.1.14)	50	c
cg2492	(glmS) Probable glucosamine-fructose-6-phosphate aminotransferase	70	c
cg2523	(malQ) 4-α-Glucanotransferase (EC 2.4.1.25)	80	c
cg0583	(fusA) – Elongation factor G	105	c
cg1787	(ppc) Phosphoenolpyruvate carboxylase (EC 4.1.1.31)	110	c

c, cytosolic
Digits indicate number of TMHs.

In addition to the listed detergents, also mixtures of two detergents (besides SDS) have been tested. After mixing of two detergents in a 1:1 ratio w/w they were added to the solubilization buffer at a final concentration of 2%. For the tested strain *C. glutamicum* DM 1698, no mixture was more efficient in protein solubilization than ASB-14 alone. However, we observed an increase in solubilized protein with a detergent mixture, if membranes of the wild-type cultivated in shaker flasks were used (data not shown).

Figure 3 summarizes the standard membrane preparation. After cell lysis, about 1/10 of the total amount of protein is found in the membrane fraction. Washing with 2.5 M NaBr removes about 45% of the proteins from the membrane and 80%–95% of the protein in the washed membranes can be solubilized with the method given above. In summary, starting with 6 mg of the unwashed membrane fraction results in ~2.6 to ~3.1 mg of solubilized proteins (Fig. 3B). The protein composition of the cytosolic, membrane-associated, and membrane integral fraction is markedly different as revealed by 1-D SDS-PAGE in Fig. 3A, but the sample is still too complex for protein identification by MALDI-TOF-PMF analysis. Comparing the washed membranes before and after solubilization it can bee seen that the protein pattern is identical; there is no specific loss of any high-abundant pro-

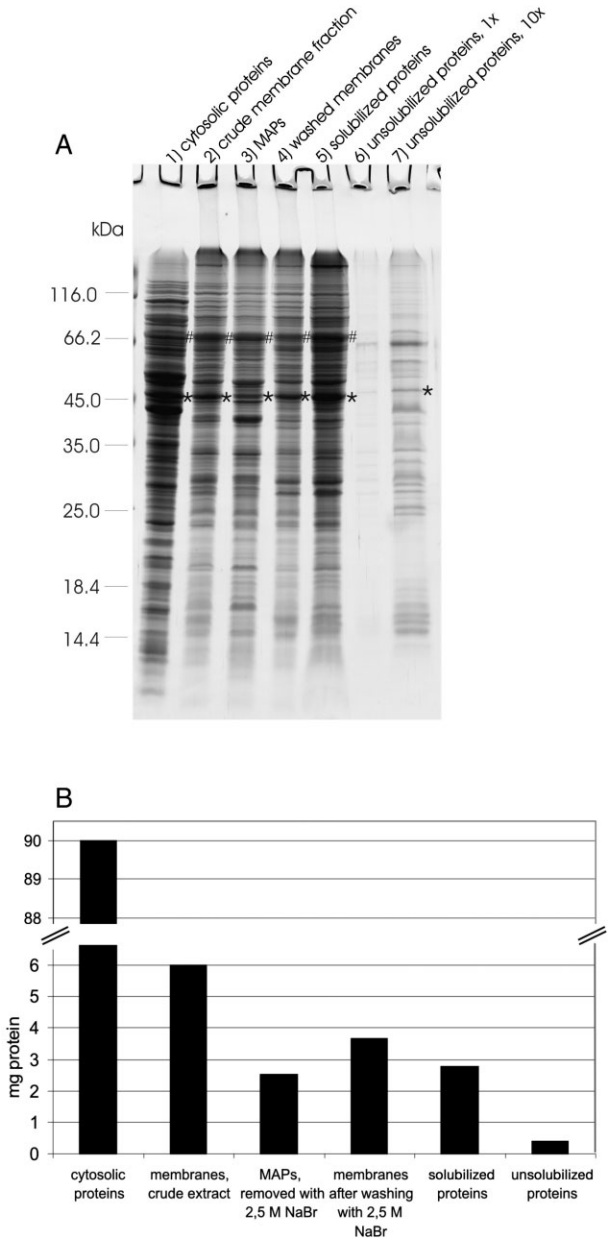

Figure 3. Survey on the standard membrane preparation and solubilization. Membranes were washed twice with 2.5 M NaBr, followed by solubilization in buffer containing 2% ASB-14. (A) SDS-PAGE of the different fractions obtained during the membrane preparation and solubilization; fractions were precipitated according to [31], gradient gel 12% T (1% C) to 15% T (1.3% C), 15 cm. Lane 1, cytosolic proteins, 100 μg; lane 2, membrane proteins, crude extract, 100 μg; lane 3, membrane-associated proteins removed by 2.5 M NaBr, 100 μg; lane 4, membranes after washing with 2.5 M NaBr, 100 μg; lane 5, membrane proteins solubilized with 2% ASB-14, 100 μg; lane 6, unsolubilized proteins, 8 μg, not concentrated (in comparison to the solubilized proteins); lane 7, unsolubilized proteins, 80 μg, 10 times concentrated (in comparison to the solubilized proteins). * Elongation factor Tu; # (sdhA) succinate dehydrogenase A. (B) Amount of protein in the different fractions estimated according to Lowry [32].

tein during solubilization. Few similarities exist between the cytosolic fraction and the solubilized membrane fraction; exceptions are very high-abundant soluble proteins or membrane-associated proteins such as succinate dehydrogenase subunit A or elongation factor Tu (marked in Fig. 3A: # = dehydrogenase subunit A; * = elongation factor Tu).

3.3 Protein separation by IEC and SDS-PAGE and MS analysis

After washing the membranes, the solubilized proteins were separated by chromatography. While preliminary experiments indicated that AIEC is the most promising method for such a separation, other methods, such as cation-exchange chromatography and hydrophobic interaction chromatography, were also tested with less success (data not shown). Figure 4 shows an AIEC separation of the *C. glutamicum* membrane fraction. As shown in Table 2, integral membrane proteins with variable amounts of TMHs are distributed evenly among the seven main elution peaks. The proportion of MIPs in the elution fractions was between 14% and 38%.

In order to further resolve the still complex fractions, the second dimension, *i.e.*, SDS-PAGE, was optimized (Fig. 4A). The best separation occurred on a 20 cm gel (12% T and 1% C); gels with 15 cm separating length and various concentration gradients were also tested, but showed no sufficient separation of the complex protein mixture (data not shown).

For protein identification, gel bands were excised and most of the separated proteins were identified by MALDI-TOF-MS. Proteins were digested by trypsin followed by CNBr; although more time-consuming in comparison to the digestion with trypsin alone, this method was reported to yield better MALDI-TOF-PMF results for integral membrane proteins smaller than ~30 kDa [35]. Indeed, our experiments showed that upon trypsin digestion alone, for example, no significant MASCOT score was achieved for the CD subunit of the succinate dehydrogenase.

Protein spots without a result or a significant score were further analyzed by ESI-MS/MS. From 356 excised bands, we identified 170 different proteins by the combination of MALDI and ESI (see Table 3, Addendum). 49 proteins were found in more than one gel band. We further categorized the

Table 2. Elution of integral membrane proteins with variable amounts of TMH was evenly distributed during the AIEC

Fractions	Number of MIPs identified	% MIPs of totally identified proteins
1– 3	1	33
4– 6	7	38
7– 9	13	38
10–12	20	32
13–15	13	25
16–18	4	14

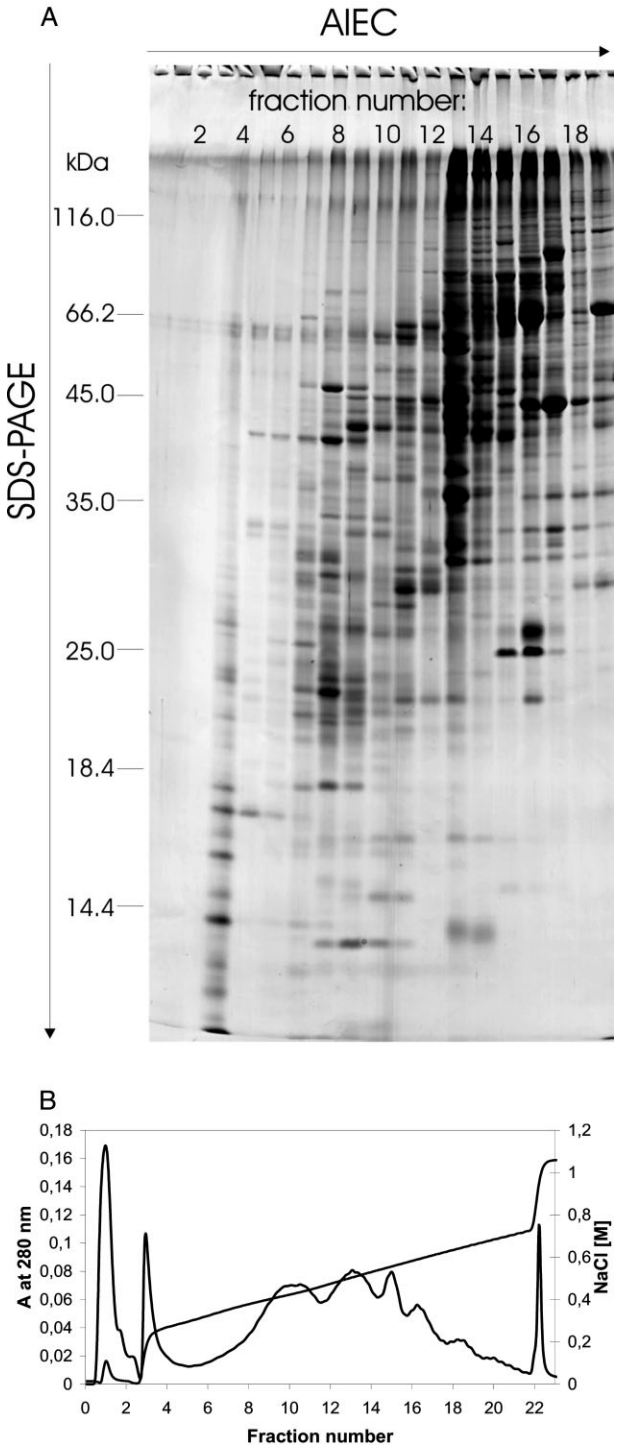

Figure 4. Separation of a solubilized *C. glutamicum* membrane fraction using AIEC as first and SDS-PAGE as second dimension. Before application (1.5 mL column packed with Poros 20 HQ) the membranes were washed with 2.5 M NaBr twice and solubilized in solubilization buffer containing 2% w/v ASB-14. (A) SDS-PAGE of AIEC fractions after TCA precipitation; 20 cm gel (12% T, 1% C). (B) Chromatogram of the separation by AIEC. Two mg protein was applied to the column. Grey line: A 280 nm; black line: salt concentration.

proteins according to their predicted subcellular location: 44% cytosolic, 9% secreted, 18% membrane-associated, and 29% membrane integral. Our analysis was not limited to integral membrane proteins with only one or two transmembrane TMHs. We identified several membrane proteins with more than two TMHs up to proteins with 13 TMHs (proline transport system). Several components of the respiratory chain were identified: the NADH-dehydrogenase, all subunits of the succinate dehydrogenase, subunits A and B of the cytochrome bc1 complex, the subunits A and D of the cytochrome aa_3 oxidase, and the subunits α, β, and b of the ATP-synthase. Subunits of the alternative bd-type menaquinol oxidase could not be identified; this may be due to a suppression under our cultivation conditions, since expression of the oxidase is induced under microaerobic or copper-deficient conditions [37].

Several ABC-transport systems were identified: the ATPase and permease components of the cobalt transporter, several sugar transport systems, two multidrug transport systems, two peptide transporters, all components of the glutamate uptake system, and two ABC transporters without a known substrate. Regarding the phosphotransferase systems, the membrane integral subunits specific for fructose and specific for glucose, and a soluble subunit specific for ribitol have been found. Among the identified membrane proteins, two types of membrane integral proteases were detected: the protease ftsH which degrades misfolded or misassembled membrane proteins, and a stomatin/prohibitin homolog type protease (weakly homologous to HflK from *E. coli*, a protein which regulates ftsH activity). Also, several soluble and membrane integral proteins for cell wall synthesis were found, among them the trehalose corynomycolyl transferases cop1, cmt1, and cmt2. Other identified integral membrane proteins are the mechanosensitive channel, a subunit of the Na^+/H^+ antiporter, an adenylate-cyclase, two serine/threonin protein kinases, a DTDP-glucose 4,6-dehydratase, and members of the SEC-translocation machinery (secE and secF). Out of the 50 identified integral membrane proteins, 24 are hypothetical proteins without known function. Since we only identified 37 hypothetical proteins in total, membrane proteins are clearly overrepresented in this class.

4 Discussion

In contrast to the proteomic analysis of cytoplasmic proteins from *C. glutamicum*, by which 152 soluble proteins have been identified [26, 27], all attempts to also display the membrane proteome of this organism by 2-D PAGE were of rather limited success in these studies: for prefractionation soluble proteins were separated from the plasma membrane, the cell wall fraction, and the secreted proteins. Among them, the analysis of the membrane fraction with only about a dozen proteins identified yielded poor results different to all other fractions. Moreover, none of the proteins from the membrane fraction was really membrane-integral. Our results

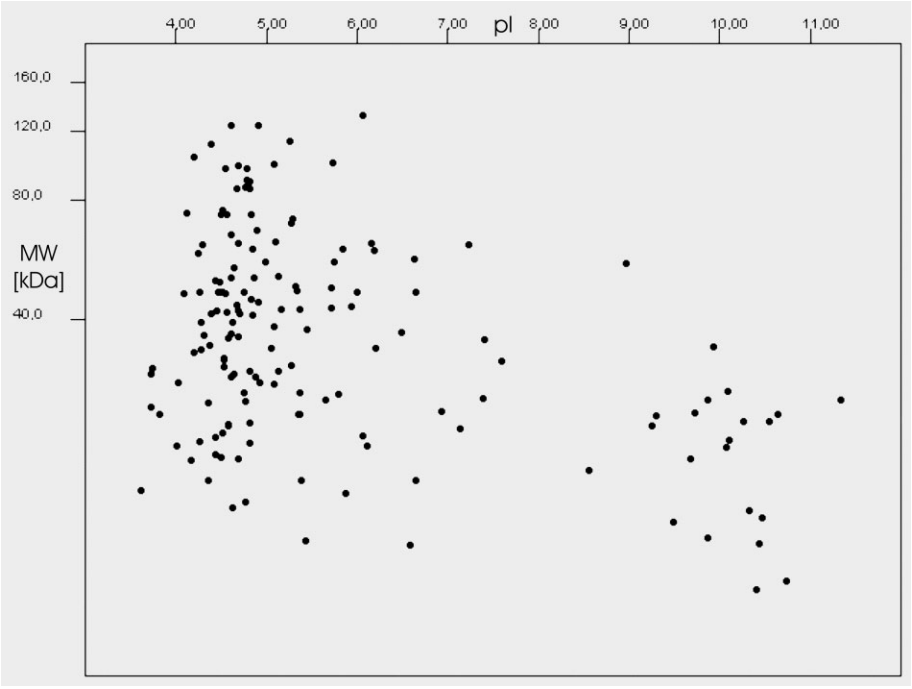

Figure 5. Virtual 2-D map of the proteins identified in this study. Calculation was done by JVirGel [46].

show that prefractionation by AIEC followed by SDS-PAGE yiels much better result with 50 out of 170 identified proteins being clearly integral membrane proteins. It is generally agreed that integral membrane proteins are highly underrepresented in 2-D gels [38]. There are three main reasons for this observation: (i) they are often low abundant, (ii) most of them have an alkalic p*I* which hampers separation, (iii) most importantly, they are poorly soluble in the buffer used for IEF or become insoluble during the focusing process. In view of the relevance of membrane proteins in pathways of *C. glutamicum*, and the poor performance of the 2-D electrophoresis technique for membrane proteins, we decided to develop an alternative approach for the analysis of the membrane proteome. Chromatographic protein separation techniques have already been shown to be extremely useful as fractionation steps for the proteomics of soluble proteins [39], and recently also for membrane proteins [40]. The generally low amount of membrane proteins relative to water-soluble cytosolic proteins may be due to the limited space in the plasma membrane. In order to enrich membrane proteins prior to chromatography and thereby increase the probability of their detection, the development of a prefractionation protocol was mandatory. While the separation of the bacterial membrane from cytosolic proteins was basically done as previously published [27], various washing buffers had to be tested for their ability to remove soluble proteins which are associated with the plasma membrane. Although we could obtain quite good results with sodium carbonate, a washing agent often used for subcellular organelles [41] and bacterial membranes [42, 43] NaBr turned out to be superior. The unique cell wall composition of actinomycetes may be the major reason for this observation. The fact that 44% of the identified proteins in the membrane fraction are cytosolic proteins indicates that the NaBr-wash does not completely dissociate soluble subunits of membrane protein complexes as well as membrane-associated proteins from the membrane. Also, proteins of the protein synthesis machinery may have cofractionated because they were still attached to nascent membrane or secreted proteins. Another reason for cofractionation with the membrane may be the high abundance of some proteins.

The test of the solubilization efficiency revealed striking differences between various detergents; these findings stress the necessity to perform a thorough detergent screening for optimal protein solubilization, which in turn is relevant to prepare the sample for the chromatographic separation. Finally, ASB-14 proved to be the best choice with the optimized solubilization conditions being nearly as effective as solubilization with SDS. However, the outcome of such a screening is in our opinion difficult to predict and will have to be optimized individually from case to case. For instance, we did not expect the zwitterionic detergent CHAPS, which is widely and successfully used for proteomics, to perform much worse than the milder detergent β-DM. One reason may be that we did not use a denaturing solubilization buffer – in contrast to solubilization conditions generally used for 2-D PAGE. We consider our milder solubilization conditions rather as an advantage, since it should allow us to separate not only single membrane proteins, but also membrane protein complexes by chromatography, if the conditions are slightly modified.

To check whether the separation protocol showed a bias with respect to protein size and protein p*I*, we used the software JVirGel to visualize the identified proteins on a virtual 2-D gel (Fig. 5). While protein masses from 10 kDa to 120 kDa can be found, the p*I* range extends from 3.7 to 10.6. These data are in good agreement with the previously depicted virtual 2-D gel for the complete *C. glutamicum* proteome [27] although we did not identify extremely basic proteins (p*I* 11–13). In general, our study shows a higher ratio of acidic/basic proteins than predicted for the whole proteome which may be explained by the fact that the anion-exchanger of our first dimension did not bind extremely basic proteins. A lack of

strongly basic proteins after AIEC was also reported by Szponarski *et al.* [40] which is further supported by the observation that increasingly acidic proteins are eluted with increasing salt concentration. We therefore tested cation-exchange chromatography as an additional separation step for the basic proteins; however, the only moderate increase in the number of identified proteins in our opinion does not justify the introduction of this additional separation step. We also examined, whether the relative proportions of proteins classified in the various functional categories of Table 1 changed markedly. Overall, the proportions in our study comply with the complete proteome, except for two distinct differences: In this study, the proportion of proteins involved in amino acid synthesis is higher and the proportion of hypothetical proteins is lower than in the complete proteome. In contrast, the amount of integral membrane proteins reflects their status in the proteome, with more than 50% of the identified integral membrane proteins being hypothetical or unclassified.

The *C. glutamicum* strain used for this study is a model production strain and was grown in a fermenter, whereas in the previous proteome studies, strain ATCC 13032 was cultivated aerobically in shaker flasks [26, 27]. It is tempting to examine, whether these differences in the genome and in the cultivation conditions have an impact on the membrane proteome, although the amount of data is very limited up to now. The presence of highly abundant proteins (ATP-synthase, succinate dehydrogenase) from the respiratory chain in both studies is quite obvious, although their expression level may differ. In agreement with previous studies, we could also identify the presence of the maltose binding protein, the glutamate ABC transporter, the glucose specific phosphotransferase system and a protein with similarity to a phage shock protein. However, we did not observe subunits of the glutamine ABC transporter. A comparison of our data with previous studies is summarized in the Supplementary table 4.

In order to overcome the problems with the IEF step of 2-D electrophoresis, several alternative techniques for the analysis of integral membrane proteins have been reported. These include a combination of SDS-PAGE and reverse-phase chromatography [44] or a 2-D chromatography approach [45]. In most cases, these approaches could identify more proteins than our approach which may be mainly due to the fact that they separate at least in one dimension on the peptide level and also use powerful MS/MS technologies for protein identification. In contrast, our method separates intact proteins in two dimensions, which allows the identification of most proteins by simple MALDI-TOF-PMF. This separation of intact proteins will also allow us to screen for protein modifications, such as phosphorylation by immunoblot analysis. Additionally, global changes of protein expression under different cultivation conditions can be easily probed by densitometric analysis of the gel bands. Mapping of the membrane proteome of *C. glutamicum* is important to elucidate the regulation of transport processes and to monitor the energy metabolism. The first step towards a map of the membrane proteome as presented in this work should help to discover new regulatory networks and bottlenecks for amino acid production processes in future.

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6 Addendum

Table 3. Proteins identified in this study

cg Identifier	Function	Fraction No.	Apparent MW (kDa)	Localization[a)]	Identification[b)]
	Amino acid biosynthesis				
cg1436	(ilvN) – Acetohydroxy acid synthase small subunit	4	29	c	m
cg2833	(cysK) – *O*-Acetylserine (thiol)-lyase	8, 10, 11	36	c	m
cg3079	(clpB) – Probable ATP-dependent protease (heat shock protein)	10, 13	85	a	m
cg1129	(aroF) – Probable phospho-2-dehydro-3-deoxyheptonate aldolase	11	38	c	m
cg1698	(hisG) – ATP phosphoribosyltransferase	11	31	c	m
cg2120	(ptsF) – Sugar-specific PTS system, fructose/mannitol-specific transport protein	11	64	9	m
cg1586	(argG) Argininosuccinate synthase (EC 6.3.4.5)	12	44	c	e
cg2304	(hisC) Probable histidinol-phosphate aminotransferase (EC 2.6.1.9)	12	41	c	e
cg0754	(metX) – Homoserine *O*-acetyltransferase	13	44	c	m
cg1133	(glyA) – Serine hydroxymethyltransferase	13	45	c	m
cg1451	(serA) – Phosphoglycerate dehydrogenase	13, 14	50	c	m
cg1437	(ilvC) – Ketol-acid reductoisomerase	14	37	c	m
cg2963	(clpC) – Probable ATP-dependent protease (heat shock protein)	15, 16	116	a	m
cg1290	(metE) – Homocysteine methyltransferase	18	85	c	m
	Biosynthesis of cofactors, prosthetic groups, and carriers				
cg0559	(ispB) Putative octaprenyl-diphosphate synthase protein (EC 2.5.1.)	15	33	c	e
cg1203	Mg-chelatase subunit ChlI	16	50	c	m
cg1027	(dld) – D-Lactate dehydrogenase	10	66	a	m
cg1672	(ppmC) Polyprenol-phosphate-mannose synthase domain 1	12	30	c	e

Table 3. Continued

cg Identifier	Function	Fraction No.	Apparent MW (kDa)	Localization[a)]	Identification[b)]
cg0417	(capD) – Probable dtdp-glucose 4,6-dehydratase transmembrane protein	13	60	5	m
cg2368	(murC) – Probable UDP-*N*-acetylmuramate–alanine ligase protein	13	49	c	m
cg2157	(terC) Tellurium resistance membrane protein	14	34	9	e
cg0951	(accDA) – Acetyl-coenzyme a carboxylase carboxyl transferase	14	44	c	m
cg2470	Secreted ABC transporter substrate-binding protein	18	35	a	e
	Cellular processes				
cg3366	(rmpA) – Putative ribitol-specific enzyme II of PTS system	7	31	a	m
cg1001	(mscL) – Large conductance mechanosensitive channel	10	13	2	m
cg3255	(uspA3) Universal stress protein family	11	34	c	e
cg0310	(katA) – Catalase	11	51	c	m
cg0951	(accDA) – Acetyl-coenzyme a carboxylase carboxyl transferase	14	44	c	m
	Central intermediary metabolism				
cg3182	(cop1) – Trehalose corynomycolyl transferase	10, 11, 12	65	s	m
cg3227	(lldA) – Putative l-lactate dehdrogenase	12	43	a	m
cg3186	(cmt2) Trehalose corynomycolyl transferase (EC 2.3.1.122)	12, 13	30	s	e
cg0413	(cmt1) Trehalose corynomycolyl transferase (EC 2.3.1.122)	13	36	s	e
cg1656	(ndh) – NADH dehydrogenase	13	45	1	m
cg0951	(accDA) – Acetyl-coenzyme a carboxylase carboxyl transferase	14	44	c	m
cg0445	(sdhCD) – Succinate dehydrogenase CD	9, 11, 16	21	5	m, e
cg0447	(sdhB) Succinate dehydrogenase B	16	27	a	m
cg0446	(sdhA) – Succinate dehydrogenase A	16, 17	66	a	m
	DNA metabolism				
cg0007	(gyrB) – DNA gyrase subunit B	14	80	c	m
cg1560	(uvrA) – Excinuclease ATPase subunit	14	114	c	m
cg2141	(recA) DNA recombination/repair (EC 3.4.21.88)	14	41	c	e
cg1525	(polA) – DNA polymerase I	16	85	c	m
	Energy metabolism				
cg1364	(atpF) – ATP synthase B chain	7	17	a	m
cg2404	(qcrA1) – Rieske iron-sulfur protein	9	42	3	m
cg2403	(qcrB) – Cytochrome b, membrane protein	9, 10, 11	45	9	m
cg2291	(pyk) – Pyruvate kinase	11	48	c	m
cg2120	(ptsF) – Sugar-specific PTS system, fructose/mannitol-specific transport protein	11	64	9	m
cg0414	(wzz) – Cell surface polysaccharide biosynthesis/chain length determinant protein	12	60	2	m
cg3227	(lldа) – Putative L-lactate dehydrogenase	12	43	a	m
cg1366	(atpA) – ATP synthase α-subunit	12, 13	52	a	m
cg1280	(odhA) – 2-Oxoglutarate dehydrogenase	12, 14	116	c	m
cg1368	(atpD) – ATP synthase β-subunit	13	50	a	m
cg0791	(pyc) – Pyruvate carboxylase	14	115	c	m
cg1111	(eno) – Enolase	14	45	c	m
cg1337	(hom) – Homoserine dehydrogenase	14, 15	46	c	m
cg1790	(pgk) – Phosphoglycerate kinase	14, 15	43	c	m, e
cg2323	(treY) – Maltooligosyl trehalose synthase	16	80	c	m
cg2333	(treZ) – Malto-oligosyltrehalose trehalohydrolase	16	60	c	m
cg2523	(malQ) – 4-α-Glucanotransferase	16	75	c	m
cg2780	(ctaD) – Probable cytochrome *c* oxidase polypeptide I	17	44	12	m
cg1787	(ppc) – Probable phosphoenolpyruvate carboxylase protein	17, 18	95	c	m, e
cg2408	(ctaC) – Cytochrome *c* oxidase	12, 17, 18, 19	38	3	m, e
	Fatty acid and phospholipid metabolism				
cg2154	(pgsA2) CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (EC 2.7.8.5)	11	18	4	e
cg3182	(cop1) – Trehalose corynomycolyl transferase	10, 11, 12	65	s	m

Table 3. Continued

cg Identifier	Function	Fraction No.	Apparent MW (kDa)	Localization[a)]	Identification[b)]
	Hypothetical proteins				
cg1128	Similar to ribosomal protein S2	4	12	c	e
cg1408	Putative membrane protein	4	10	2	e
cg2331	Putative membrane protein	4	11	2	e
cg1322	Conserved hypothetical protein	5	20	c	m
cg2211	Putative membrane protein	5	16	2	e
cg2151	Similar to phage shock protein A	5, 6, 14	34	c	m, e
cg0781	Membrane protein	6, 7	34	3	m
cg0240	Membrane protein	7	16	4	m
cg0952	Putative integral membrane protein	7	10	2	m
cg0359	Putative membrane protein	8, 9	17	2	m
cg1466	Putative secreted protein	9	19	s	e
cg1859	Putative secreted protein	9	19	s	e
cg2134	Putative membrane protein	9	19	4	e
cg2691	Conserved hypothetical protein	9	20	c	e
cg3014	Hypothetical protein predicted by glimmer/critica	9	37	c	m
cg3017	Putative membrane protein	9, 11	110	2	m
cg2087	Putative membrane protein	10	18	2	m
cg0528	Putative secreted protein	11	15	s	m
cg1635	Putative membrane protein	11	19	2	e
cg1275	Conserved hypothetical membrane protein	11, 12	19	2	m
cg2498	Conserved hypothetical protein	11	20	1	e
cg0451	Putative membrane protein	12	41	2	e
cg0575	Secreted protein	12	35	2	m
cg1662	Putative secreted protein	12	17	s	e
cg3317	Putative membrane protein	12	17	3	e
cg4005	Putative secreted protein	12	15	s	e
cg1603	Conserved membrane protein	12, 13	40	1	m
cg1238	Putative membrane protein	13	34	4	e
cg0765	Secreted protein	13, 14	24	s	e
cg2994	Putative secreted or membrane protein	13, 14	24	s	e
cg1312	Putative membrane protein	14	15	4	e
cg2196	Putative secreted or membrane protein	14	13	2	e
cg1840	Conserved hypothetical protein	15	43	c	m
cg3192	Putative secreted or membrane protein	15	46	s	m
cg2444	Hypothetical protein predicted by Glimmer	15, 16	25	c	m
cg3018	Hypothetical protein predicted by Glimmer	16	116	c	m
cg2799	Putative secreted protein	17	33	s	m
cg2657	Putative membrane protein – fragment	18	32	1	m
cg3195	Flavin-containing monooxygenase (FMO)	18	64	c	e
cg0173	Conserved hypothetical protein	18	64	c	e
cg0896	Membrane protein	18, 19	116	7	m
cg2644	(clpP2) ATP-dependent clp protease proteolytic subunit clpp2 (EC 3.4.21.92)	9	20	a	e
cg3079	(clpB) – Probable ATP-dependent protease (heat shock protein)	10, 13	85	a	m
cg0814	(birA) – Bifunctional biotin ligase/biotin operon repressor	11	27	c	m
cg1865	(secF) – Preprotein translocase subunit SecF	11	50	6	m
cg3100	(dnaK) – Heat shock protein hsp 70	11,12, 13	67	a	m
cg1826	(pepQ) – XAA-PRO aminopeptidase	12	40	c	m
cg2984	(ftsH) – Cell-division protein (ATP-dependent Zn metallopeptidase)	12	116	2	m
cg1868	(secN) : Preprotein translocase subunit YajC homolog	14	16	c	e
cg0868	(secA) – Preprotein translocase	15	80	c	m
cg2963	(clpC) – Probable ATP-dependent protease (heat shock protein)	15, 16	116	a	m

Table 3. Continued

cg Identifier	Function	Fraction No.	Apparent MW (kDa)	Localization[a)]	Identification[b)]
	Protein synthesis				
cg0608	(rplN) – 50S Ribosomal protein L14	2	14	c	m
cg0629	(rplF) – 50S Ribosomal protein L6	2	18	c	m
cg3308	(rpsF) Ribosomal protein S6	4	11	c	e
cg0564	(rplA) – 50S Ribosomal protein L1	5	26	c	m
cg0572	(rplJ) – 50S Ribosomal protein L10	5	17	c	m
cg0654	(rpsD) – Ribosomal protein S4	5, 6	19	c	m
cg0596	(rplD) – 50S Ribosomal protein L4	6, 7	24	c	m
cg0610	(rplE) – 50S Ribosomal protein L5	7, 8	20	c	m
cg2404	(qcrA1) – Rieske iron-sulfur protein	9	42	3	m
cg2609	(valS) – Putative valine-TRNA ligase	11	95	c	m
cg0587	(tuf) – Elongation factor TU	12, 13, 14, 15, 17, 18	43	c	m, e
cg1841	(aspS) – Probable aspartyl-TRNA synthetase protein	13	68	c	m
cg1880	(thrS) – Threonyl-TRNA synthetase	14	75	c	m
cg2499	(glyS) – Glycyl-TRNA synthetase	14	47	c	m, e
cg0583	(fusA) – Elongation factor G	15, 16	80	a	m
	Purines, pyrimidines, nucleosides, and nucleotides				
cg1817	(pyrR) – Pyrimidine operon attenuation protein/uracil	9	22	c	m
cg0375	(cyaB) – Putative adenylate cyclase	11	40	6	m
cg2603	(ndk) Nucleoside diphosphate kinase (EC 2.7.4.6)	11	15	c	e
cg3079	(clpB) – Probable ATP-dependent protease (heat shock protein)	10, 13	85	a	m
cg0414	(wzz) – Cell surface polysaccharide biosynthesis/chain length determinant protein	12	60	2	m
cg2963	(clpC) – Probable ATP-dependent protease (heat shock protein)	15, 16	116	a	m
cg1813	(carB) – Putative carbamoyl-phosphate synthase subunit	18	117	c	m
	Regulatory functions				
cg0350	Transcriptional regulator, crp/fnr family	4, 5	22	c	m
cg0059	(pknA) – Serine/threonine protein kinase	9	70	1	m
cg2404	(qcrA1) – Rieske iron-sulfur protein	9	42	3	m
cg0375	(cyaB) – Putative adenylate cyclase	11	40	6	m
	Signal transduction				
cg1537	(ptsG) – Glucose-specific enzyme II bc component of pts	13,14,15, 16	70/120	10	m
cg0867	Ribosome-associated protein Y (PSrp-1)	8	29	c	m
cg0780	Membrane protein ribonuclease BN-like family	9	33	6	m
cg0576	(rpoB) – DNA-directed RNA polymerase beta chain	13, 14	113	c	m, e
	Transport and binding proteins				
cg2138	(gluC) – Glutamate permease	2, 4	19	6	m
cg0561	(secE) – SecE subunit of protein translocation complex	4	14	1	m
cg3029	(mrpG) Multisubunit Na+/H+ antiporter, G subunit	4	13	3	e
cg2708	(msiK1) – ABC-type sugar transport system, ATPase component	5, 6, 9	43	a	m
cg0914	(ftsE) – Cell division ATP-binding protein	7	25	a	m
cg2845	(pstC) – ABC-type phosphate transport system, permease component	7	30	6	m
cg3366	(rmpA) – Putative ribitol-specific enzyme II of PTS system	7	31	a	m
cg2912	ABC-type cobalamin/Fe3+-siderophores transport system, ATPase component	8, 9	20	a	m
cg1229	ABC-type cobalt transport system, permease component CbiQ	9	18	4	e
cg0736	ABC-type transport system ATPase component	9	40	a	m
cg1228	ABC-type cobalt transport system, ATPase component	9, 10, 11	45	a	m, e
cg1027	(dld) – D-Lactate dehydrogenase	10	66	a	m
cg0046	Probable ABC transport protein, ATP-binding component	10, 11	27	a	m
cg1762	(sufC) – Iron-regulated ABC transporter ATPase subunit	11	28	a	m
cg2136	(gluA) – Glutamate uptake system ATP-binding protein	11	27	a	m

Table 3. Continued

cg Identifier	Function	Fraction No.	Apparent MW (kDa)	Localization[a)]	Identification[b)]
cg2675	ATPase component of ABC-type transport system, contains duplicated ATPase domains	11	60	a	m
cg2120	(ptsF) – Sugar-specific PTS system, fructose/mannitol-specific transport protein	11	64	9	m
cg2184	ATPase component of peptide ABC-type transport system, contains duplicated ATPase domains	11, 12	66	a	m
cg2213	ABC-type multidrug transport sytem, ATPase component	12	32	a	m
cg1081	ABC-type multidrug transport system, ATPase component	13, 14	34	a	m
cg1537	(ptsG) – Glucose-specific enzyme II BC component of PTS	13,14,15, 16	70/120	10	m
cg0953	Na+/proline, Na+/panthothenate symporter or related permease	14	41	13	e
cg1314	(putP) Proline transport system	14	41	13	e
cg0915	(ftsX) Putative cell division protein	15	33	4	e
cg2705	(amyE) – Maltose-binding protein	16, 17, 18	45	a	signal_p
cg2678	ABC-type dipeptide/oligopept	18	64	a	m
cg2137	(gluB) – Glutamate-secreted binding protein	18, 19	30	a	m
cg2181	ABC-type peptide transport system, secreted component	18, 19	58	a	e
	Unclassified				
cg2840	(actA) – Butyryl-CoA:acetate coenzyme a transferase	7	46	c	m
cg3396	Membrane protease subunit, stomatin/prohibitin homologs	7	26	a	m
cg1794	Uncharacterized P-loop ATPase protein	8	31	c	m
cg0752	Putative secreted or membrane protein	8, 9, 10	48	s	m, e
cg1764	(sufB) – Component of an uncharacterized iron-regulated ABC-transporter	11	40	a	m
cg2091	(ppgK) – Polyphosphate glucokinase	11	26	c	m
cg0418	Putative aminotransferase	14	41	c	e
cg2388	(pknL) – Putative serine/threonin protein kinase	14	116	1	m
cg3138	Membrane protease subunit, stomatin/prohibitin homolog	14, 15, 16, 17,	32	2	m, e
cg0737	Secreted lipoprotein	15, 17, 18	35	s	m
cg1730	Secreted protease subunit, stomatin/prohibitin homolog	17, 18	50	s	m
cg2342	Dehydrogenase (related to short-chain alcohol dehydrogenases)	10	35	c	m
cg2958	(butA) – L-2,3-Butanediol dehydrogenase/acetoin reductase	10, 11, 12	29	c	m
cg2964	(guaB1) – Inositol-monophosphate dehydrogenase	11	44	c	m
cg1839	Uncharacterized ATPase related to the helicase subunit of the Holliday junction resolvase	12	44	c	e

a) c = cytosolic; s = secreted; a = membrane-associated; digits indicate numbers of TMHs.

b) e = identified by ESI-MS/MS; m = identified by MALDI-TOF-PMF

Protein location was predicted as follows: the number of TMHs was determined with the software TMHMM v2.0 [47], secreted proteins were predicted with the software SignalP v1.1 [48]. Membrane-associated proteins were predicted by homology searches on the SWALL database (Nov 11, 2003) using BLAST v2.2.6 [49] with default parameters. Retrieved entries were filtered according to the following criteria: Eubacteria, *E*-value cutoff <0.001, keywords in the "subcellular location" section of the SWALL database entry: lipid anchor, outer membrane, membrane-associated, membrane-associated, membrane bound, or membrane-bound. This automatic annotation was manually revised as follows: soluble subunits (including secreted) of membrane protein complexes and clp proteins were annotated as membrane-associated, since they may bind to integral membrane proteins. Assignment of functional categories is based on the genome annotation deposited at DDBJ (http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032).

