

Genetic dissection of trehalose biosynthesis in *Corynebacterium glutamicum*: inactivation of trehalose production leads to impaired growth and an altered cell wall lipid composition

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The analysis of the available *Corynebacterium* genome sequence data led to the proposal of the presence of all three known pathways for trehalose biosynthesis in bacteria, i.e. trehalose synthesis from UDP-glucose and glucose 6-phosphate (OtsA-OtsB pathway), from malto-oligosaccharides or α -1,4-glucans (TreY-TreZ pathway), or from maltose (TreS pathway). Inactivation of only one of the three pathways by chromosomal deletion did not have a severe impact on *C. glutamicum* growth, while the simultaneous inactivation of the OtsA-OtsB and TreY-TreZ pathway or of all three pathways resulted in the inability of the corresponding mutants to synthesize trehalose and to grow efficiently on various sugar substrates in minimal media. This growth defect was largely reversed by the addition of trehalose to the culture broth. In addition, a possible pathway for glycogen synthesis from ADP-glucose involving glycogen synthase (GlgA) was discovered. *C. glutamicum* was found to accumulate significant amounts of glycogen when grown under conditions of sugar excess. Insertional inactivation of the chromosomal *glgA* gene led to the failure of *C. glutamicum* cells to accumulate glycogen and to the abolition of trehalose production in a Δ *otsAB* background, demonstrating that trehalose production via the TreY-TreZ pathway is dependent on a functional glycogen biosynthetic route. The trehalose-non-producing mutant with inactivated OtsA-OtsB and TreY-TreZ pathways displayed an altered cell wall lipid composition when grown in minimal broth in the absence of trehalose. Under these conditions, the mutant lacked both major trehalose-containing glycolipids, i.e. trehalose monocorynomycolate and trehalose dicorynomycolate, in its cell wall lipid fraction. The results suggest that a dramatically altered cell wall lipid bilayer of trehalose-less *C. glutamicum* mutants may be responsible for the observed growth deficiency of such strains in minimal medium. The results of the genetic and physiological dissection of trehalose biosynthesis in *C. glutamicum* reported here may be of general relevance for the whole phylogenetic group of mycolic-acid-containing coryneform bacteria.

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INTRODUCTION

Corynebacterium glutamicum is a Gram-positive soil bacterium that was originally isolated by its ability to produce and excrete glutamic acid (Kinoshita *et al.*, 1957). Today, industrial amino acid production processes using genetically improved strains of this micro-organism are used to satisfy the growing world market for amino acids, in particular L-glutamate and L-lysine (Leuchtenberger, 1996). In the classification system of bacteria, the genus

Corynebacterium, together with mycobacteria, nocardia, rhodococci and some other phylogenetically related taxa, belongs the group of mycolic-acid-containing actinomycetes (see Liebl, 2001). Unusually for Gram-positive bacteria, their cell walls contain a characteristic hydrophobic layer (Minnikin & O'Donnell, 1984; Nikaido *et al.*, 1993). It was shown that this layer plays an important role in drug and substrate permeability (Jarlier & Nikaido, 1990; Puech *et al.*, 2000). In contrast to the Gram-negative bacteria, where the outer membrane is composed of phospholipids and lipopolysaccharides, the predominant constituents of the outer lipid layer of corynebacteria and related taxa are the mycolic acid esters. Recently it was shown that the

Abbreviations: TDCM, trehalose dicorynomycolate; TMCM, trehalose monocorynomycolate.

outer hydrophobic barrier of corynebacterial cells represents a lipid bilayer composed of both covalently cell wall-linked mycolates and non-covalently bound glycolipids (Puech *et al.*, 2001). Two trehalose-containing corynomycolic acid esters, i.e. trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM), were shown to be the major free lipid fractions of this lipid bilayer (Puech *et al.*, 2000). The presence of trehalose in *C. glutamicum* is not restricted only to these two structural components. Significant amounts of free trehalose are observed in *C. glutamicum* cells as a response to hyperosmotic stress (Skjerdal *et al.*, 1996). Also, it is notable that trehalose was found as one of the by-products excreted into the growth medium during lysine overproduction by *C. glutamicum* (Vallino & Stephanopoulos, 1993; Wittmann & Heinzle, 2001).

Trehalose (α -D-glucopyranosyl α -D-glucopyranoside) serves different biological roles in different organisms (for a review, see Argüelles, 2000). In bacteria it can be used as

a carbon source (*Escherichia coli*, *Bacillus subtilis*), or is synthesized as a compatible solute under osmotic-shock conditions (*E. coli*), or plays a structural role (*Corynebacteriaceae*). In yeast and filamentous fungi trehalose is stored intracellularly primarily as a reserve carbohydrate or as a protector against different stress factors.

Several possible pathways for trehalose biosynthesis are known. The most abundant pathway, i.e. trehalose synthesis from UDP-glucose and glucose 6-phosphate (OtsA-OtsB pathway; Fig. 1a), is widely represented in the prokaryotes and the only one known in the eukaryotes. The first step of this pathway is the condensation of glucose 6-phosphate with UDP-glucose, resulting in the formation of trehalose 6-phosphate and release of UDP. Trehalose is then formed by dephosphorylation of trehalose 6-phosphate. This biosynthetic reaction mechanism has been found in bacteria like *E. coli* (Kaasen *et al.*, 1994) and in yeast (De Virgilio *et al.*, 1993; Londesborough & Vuorio, 1993). In *E. coli*, the reactions are catalysed by the enzymes

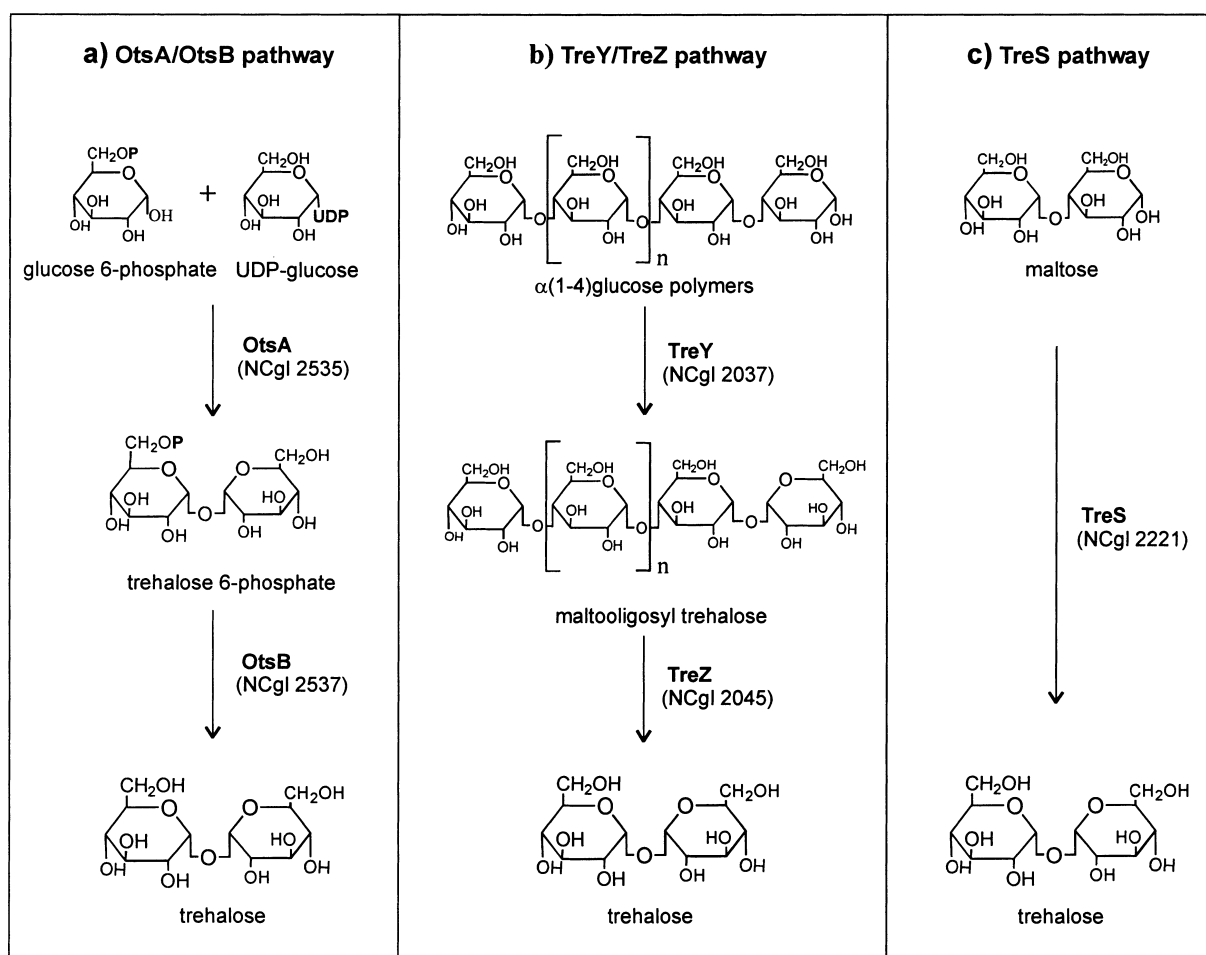


Fig. 1. Trehalose biosynthesis pathways found in bacteria. The three known pathways leading to trehalose from the substrates glucose 6-phosphate and UDP-glucose (a), malto-oligosaccharides or α -1,4-glucan polysaccharides (b) and maltose (c) are shown. The *C. glutamicum* ORFs showing high similarity to trehalose synthesis genes are shown in parentheses.

trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB). The transcription of both enzymes is induced by osmotic shock or upon entry into the stationary growth phase (Kaasen *et al.*, 1994). In *Saccharomyces cerevisiae*, both reactions are catalysed by an enzyme complex which consists of two catalytic polypeptides, TPS1 and TPS2, and one regulatory subunit responsible for activation of the complex under stress conditions (Reinders *et al.*, 1997). Coding regions for corresponding enzymes were also identified in the genomes of higher eukaryotes (*Arabidopsis* Genome Initiative, 2000; Adams *et al.*, 2000). An alternative pathway for trehalose synthesis that uses glycogen as the initial substrate (TreY-TreZ pathway; Fig. 1b) was discovered in some bacteria (Maruta *et al.*, 1996a, b) and archaea (Maruta *et al.*, 1996c). In this case, first the terminal $\alpha(1\rightarrow4)$ glycosidic bond at the reducing end of the α -glucan polymer is transformed into an $\alpha(1\rightarrow1)$ glycosidic bond via transglycosylation, resulting in the formation of a terminal trehalosyl unit. Subsequently, trehalose is released from the polymer's end via hydrolysis. The enzymes involved in this pathway are maltooligosyltrehalose synthase (TreY) and maltooligosyltrehalose hydrolase (TreZ). An additional pathway for trehalose synthesis, which is based on trehalose production from maltose, was discovered in some bacteria (Tsusaki *et al.*, 1996, 1997). In this case, trehalose is synthesized by a single reaction catalysed by trehalose synthase (TreS), which converts the $\alpha(1\rightarrow4)$ glycosidic bond of maltose into an $\alpha(1\rightarrow1)$ bond to form trehalose (TreS pathway; Fig. 1c). It was shown (Nishimoto *et al.*, 1996; Nakada *et al.*, 1995) that, although close in their intramolecular transglycosylation activity, TreY and TreS cannot substitute for each other *in vivo* because of the differences in their substrate specificities.

In most bacteria studied, only one of the three biosynthesis

pathways was found, with the exception of *Mycobacterium* species. Strains of this genus have been shown by *in vitro* assays to possess all three pathways for trehalose synthesis (De Smet *et al.*, 2000). The question arises as to what biological role trehalose has in these bacteria that makes necessary a threefold coverage of its biosynthesis. Also, it is of interest to analyse if *Corynebacterium*, which is phylogenetically related to *Mycobacterium* (Liebl, 2001), contains a similarly rich outfit of trehalose biosynthetic pathways. To answer these questions we have scoured the available genome data in order to identify the pathways used for trehalose biosynthesis in *C. glutamicum*. By inactivation of chromosomal genes encoding enzymes of the identified pathways we intended to probe the role of the different pathways in the *in vivo* synthesis of trehalose. Also, by inactivation of these genes we intended to reduce or even abolish trehalose synthesis in order to reveal the physiological role of this sugar in *C. glutamicum*.

METHODS

Strains, media and cultivation. The *C. glutamicum* strains and plasmids which were used in this study are listed in Table 1. Additionally, the *E. coli* strains XL-1 Blue (Bullock *et al.*, 1987) and S17-1 (Simon *et al.*, 1983) were used for plasmid construction and mobilization of integration vectors into *C. glutamicum*, respectively. The restriction-deficient *C. glutamicum* strain R163 (Liebl *et al.*, 1989a) was used for preparation of plasmid constructs before their electroporation into the *C. glutamicum* type strain. The strains were maintained on LB plates supplemented with antibiotics as required.

For investigation of trehalose synthesis, *C. glutamicum* strains were grown on defined BMC medium (Liebl *et al.*, 1989b) supplemented with various carbon sources as specified in the text. Cells inoculated from LB plates in 5 ml LB and grown overnight (30 °C; 210 r.p.m.) were used for the inoculation of 5 ml or 30 ml BMC cultures at OD₆₀₀ 0.1–0.2. When required, kanamycin was added at 20 µg ml⁻¹. All cultures were grown on a rotary shaker (30 °C; 210 r.p.m.). Rapid

Table 1. *C. glutamicum* strains used

Strain	Description*
DSM 20300	Type strain; obtained from DSMZ (Braunschweig, Germany); same as ATCC 13032
Δ otsAB	DSM 20300 with deletion in the <i>otsA</i> and <i>otsB</i> genes
Δ treZ	DSM 20300 with deletion in the <i>treZ</i> gene
Δ treS	DSM 20300 with deletion in the <i>treS</i> gene
Δ otsAB/ Δ treZ	DSM 20300 with deletion in the <i>otsA</i> , <i>otsB</i> and <i>treZ</i> genes
Δ otsAB/ Δ treS	DSM 20300 with deletion in the <i>otsA</i> , <i>otsB</i> and <i>treS</i> genes
Δ treZ/ Δ treS	DSM 20300 with deletion in the <i>treZ</i> and <i>treS</i> genes
Δ otsAB/ Δ treZ/ Δ treS	DSM 20300 with deletion in the <i>otsA</i> , <i>otsB</i> , <i>treZ</i> and <i>treS</i> genes
<i>glgA</i> ::Km	DSM 20300 with insertionally inactivated <i>glgA</i>
<i>glgA</i> ::Km/ Δ otsAB	Δ otsAB with insertionally inactivated <i>glgA</i>
<i>glgA</i> ::Km/ Δ otsAB/ Δ treS	Δ otsAB/ Δ treS with insertionally inactivated <i>glgA</i>
Δ otsAB/ Δ treZ pWLQ2:: <i>otsA</i>	Δ otsAB/ Δ treZ, complemented with the expression plasmid pWLQ2 carrying <i>otsA</i>
Δ otsAB/ Δ treZ pWLQ2:: <i>otsAB</i>	Δ otsAB/ Δ treZ, complemented with the expression plasmid pWLQ2 carrying <i>otsAB</i>
Δ otsAB/ Δ treZ pWLQ2:: <i>treZ</i>	Δ otsAB/ Δ treZ, complemented with the expression plasmid pWLQ2 carrying <i>treZ</i>
Δ otsAB/ Δ treZ/ Δ treS pWLQ2:: <i>treS</i>	Δ otsAB/ Δ treZ/ Δ treS, complemented with the expression plasmid pWLQ2 carrying <i>treS</i>

*Construction of mutant strains, and plasmids, is described in the text.

shaking at more than 200 r.p.m. was found to be important for growth of trehalose-non-producing mutants (see text). The growth of cultures was monitored by OD₆₀₀ measurements.

Recombinant DNA techniques. Basic methods such as plasmid isolation, DNA restriction and ligation were performed according to Sambrook *et al.* (1989). *C. glutamicum* plasmid DNA was isolated by alkaline extraction (Birnboim & Doly, 1979) after previous treatment of the cells with 10 µg lysozyme ml⁻¹ for 30 min at 37 °C. Genomic DNA from *C. glutamicum* was isolated as described by Lewington *et al.* (1987). PCR reactions were carried out using *Pfu* polymerase (Promega). Some of the PCR products were cloned directly into the vector pCR4 using the TOPO Cloning Kit (Invitrogen).

The following primers were used in this study (regions that are not homologous to the original gene sequences are in italics; regions that are present only in the original sequence but not in the primer are in parentheses; restriction sites used for cloning purposes are underlined): tre351_f, *GGG GAT CCA* AAA GAC CAC CGC AAA GAA GAC; tre351_r, *CCT CTA GAG* CAG TAA AGC AAG CGG AAG AA; otsAB_f, *GGG CAT GC(A)* GTA TGC GGA AAG CGT GCG ATT G; otsAB_r, *GGA AGCTTG* CCC CAA ATA ACC GCA AAG CCA; treZ_f, *GGT CTA GAG CGT* TGG TGT AGG CAT TAA C; treZ_r, *GGT CTA GAC* GCA AAA GCC TGG TCA GTT G; treS_f, *GGT CTA GAT GAG* GCG AAA GTG GTG AAA GT; treS_r, *GGT CTA GAC* ATT CGC GGG ACA ACA CAA T; glg_f, *GGG TCT AGA* GTA TCC ACC AGA GGT TTA CG; glg_r, *GGG TCT AGA* TTA AAT CTT CCG CGT CAT CGA AAG; otsB_f, *GGG GAT CCA* AGG TGC CAG GGC TTT AAA G; otsB_r, *GGG GAT CCG* GAA CCA GAA GTG GAA TTG G; treZ_f2, *GGG GAT CCCGGG* TGA CTT GCA AAA CCT C; treZ_r2, *GGG GAT CCG* CAA AAG CCT GGT CAG TTG; treS_f3, *GGG TCG ACA* TGA GGC GAA AGT GGT GAA AG; treS_r3, *GGG TCG ACA* CAT TCG CGG GAC AAC ACA A.

Construction of ΔotsAB, ΔtreZ, ΔtreS and glgA::Km mutants of *C. glutamicum* DSM 20300. The two-step recombination system (Schäfer *et al.*, 1994), based on the inability of *C. glutamicum* carrying the *sacB* gene to grow in media with high sucrose concentrations, was used for the chromosomal inactivation of the trehalose and glycogen biosynthesis genes of *C. glutamicum*. For each planned inactivation experiment, a mobilizable *C. glutamicum* integration vector was constructed which contained the gene of interest but with an internal deletion, thus providing two homology regions for recombination.

For inactivation of the *otsA-otsB* genes, a fragment of 1.5 kb carrying the entire *otsA* ORF was amplified using the primers tre351_f and tre351_r and cloned into the *EcoRV* restriction site of pBlueScript KS, resulting in pBlueKS::*otsA*. Then, a 0.65 kb region carrying part of *otsB* was amplified with the primers otsAB_f and otsAB_r. The PCR product, cut with *HindIII* and *SphI*, served to replace a 0.90 kb *HindIII-SphI* fragment of the *otsA*-carrying plasmid, resulting in the in-frame fusion of the 5'-part of *otsA* with the 3'-part of *otsB*. Using *XbaI*, the resulting Δ*otsAB* ORF was cloned into the mobilizable integration vector pCLiK8.2. A mobilizable *treZ* inactivation plasmid was constructed as follows. A 2.5 kb *treZ* fragment was amplified with the primers treZ_f and treZ_r. The PCR product was cut with *XbaI* and cloned into pCLiK3, before introduction of an internal 0.65 kb in-frame deletion into *treZ* with *Sall*. The Δ*treZ* gene was cloned via *XbaI* into pCLiK8.2. For chromosomal inactivation of *treS*, the gene was cloned in pBlueScriptKS after amplification with the PCR primers treS_f and treS_r. Upon digestion of the resulting plasmid with *EcoRV* and *StyI*, and treatment with Klenow enzyme, the plasmid was religated, resulting in an 0.65 kb in-frame deletion in the cloned *treS* ORF. The truncated gene was cloned into the mobilizable plasmid pK18mobsac (Schäfer *et al.*, 1994) using *XbaI*.

The three final constructs for inactivation of the *OtsA-OtsB*, *TreY-TreZ* and *TreS* pathways, designated pCLiK8.2::Δ*otsAB*, pCLiK8.2::Δ*treZ* and pK18ms::Δ*treS*, respectively, were transformed into *E. coli* S17-1 and mobilized into heat-stressed *C. glutamicum* according to the procedure described by Schäfer *et al.* (1990). Successful first recombinants (chromosomal integration mutants) were selected by plating on LB plates with 20 µg kanamycin ml⁻¹. For selection of the second recombination event, the integration mutants were plated on agar containing 5–10% (w/v) sucrose. In some cases, trehalose was added at 2% (w/v).

A putative glycogen synthase gene (*glgA*) was inactivated by single-step chromosomal integration. For this purpose, a 0.6 kb internal fragment of *glgA* was amplified using *glg_f* and *glg_r* as the PCR primers. The PCR product was cloned into the integration vector pCLiK6 using its unique *XbaI* site. The resulting plasmid was mobilized using *E. coli* S17-1 as described above. The integration mutants were selected on LB medium supplemented with kanamycin.

The genotype of the mutants obtained was verified by Southern blot analysis and with specific PCR reactions. During the preparation of this work, mutants in the genes *otsA*, *treY* and *treS* (but not *otsB*, *treZ* and *glgA*) were reported by Wolf *et al.* (2002).

Construction of pWLQ2::otsAB, pWLQ2::otsA, pWLQ2::treZ and pWLQ2::treS. Expression plasmids carrying the various trehalose biosynthesis genes were constructed using the *C. glutamicum-E. coli* shuttle expression vector pWLQ2 (Liebl *et al.*, 1992). A 1.6 kb *BamHI-Sall* fragment of pBlueKS::*otsA* (see above) was ligated with pWLQ2 opened with the same enzymes. In the resulting plasmid (pWLQ2::*otsA*) the *otsA* gene is under the control of the *P_{tac}* promoter. For construction of pWLQ2::*otsAB*, the *otsB* gene was amplified from the *C. glutamicum* chromosome using the primers otsB_f and otsB_r. After cloning the PCR product in pCR4-TOPO, the 1 kb *BamHI* fragment was excised and inserted into the *BamHI* site of pWLQ2::*otsA*, yielding pWLQ2::*otsAB* with both *ots* genes under regulation of the *P_{tac}* promoter.

For construction of pWLQ2::*treZ*, a 2.5 kb PCR product generated with the primers treZ_f2 and treZ_r2 was cloned into pCR4-TOPO. The *treZ* gene was excised with *BamHI* and recloned in the *BamHI* site downstream of the *P_{tac}* promoter of pWLQ2. For the construction of pWLQ2::*treS*, the chromosomal *C. glutamicum* *treS* gene was amplified as a 2 kb fragment using the primers treS_f3 and treS_r3. After initial cloning into pCR4-TOPO, the *treS* gene was excised and recloned into pWLQ2 using artificially added *Sall* sites. The plasmid pWLQ2::*treS* was isolated, in which *treS* is orientated collinearly to the *P_{tac}* promoter. All plasmids were transformed into *C. glutamicum* strains by electroporation (Liebl *et al.*, 1989a). The strains were grown with kanamycin selection at 20 µg ml⁻¹. *P_{tac}*-driven gene expression was induced by addition of IPTG at a final concentration of 1 mM.

Isolation and analysis of lipids. Cell lipids were isolated as described by Puech *et al.* (2000). The cells were harvested and washed after approximately 10 h incubation (growth at 210 r.p.m. at 30 °C) as described below (see **Sample preparation**). For lipid extraction the wet cells were suspended in CHCl₃/CH₃OH [1:1 (v/v)] and shaken at room temperature for 16 h. Remaining bacterial residues were re-extracted twice with CHCl₃/CH₃OH [2:1 (v/v)] and the organic phases were pooled and concentrated in a vacuum centrifuge. Water-soluble contaminants were removed by additional extraction with water [2:1 (v/v)] and the organic phases were freeze-dried, yielding the crude lipid extracts. Lipid extracts were dissolved in chloroform at a final concentration of 50 µg µl⁻¹ and analysed by TLC. Samples were applied to silica-gel-coated aluminium plates (type G-60, 5 × 10 cm, Merck) and developed with CHCl₃/CH₃OH/H₂O [30:8:1 (by vol.)] in a tightly sealed chamber at 4 °C.

Glycolipids were visualized by spraying with a 0.2% (w/v) anthrone solution in conc. H₂SO₄ followed by heating (at 100 °C for 10–15 min).

The trehalose content of the lipid extracts was quantified after saponification of the crude lipid extract according to Liu & Nikaido (1999), with modifications: aliquots of the samples were taken before the water extraction, freeze-dried and dissolved in 5% (w/v) potassium hydroxide. The samples were incubated for 1 h at 100 °C, cooled, and aliquots were directly used for trehalose determination by high-pH HPLC (see below).

Sample preparation for trehalose and glycogen determination. Samples of cultures (1.5 ml) were rapidly cooled on ice and centrifuged (13 000 r.p.m., 4 °C, 15 min). All subsequent manipulations were done at 4 °C. The supernatant was collected and frozen at –20 °C for subsequent extracellular trehalose determination. The cells were washed with BMC medium and also stored as a pellet at –20 °C. In order to minimize changes in the extracellular osmotic conditions, ice-cold medium with the same salt and sugar composition as the growth medium was used for washing. Aliquots of the washed cells were used for determination of cell dry weight.

Cells were opened by sonication (40% amplitude, 0.5 s cycle) in 500 µl 10 mM sodium/potassium phosphate buffer pH 6. Cellular debris was removed by centrifugation (13 000 r.p.m., 4 °C, 15 min) and the supernatant was used for trehalose and/or glycogen determination.

Trehalose determination. An enzymic trehalose determination assay was used which was based on the quantitative hydrolysis of trehalose to two molecules of glucose, using recombinant trehalase from *E. coli*. For this purpose, the *E. coli* trehalase TreA was over-expressed and partially purified as described by De Smet *et al.* (2000). Samples of 5–20 µl were incubated with or without recombinant trehalase (5 U) in 90 µl 10 mM sodium/potassium phosphate buffer pH 6.0 for 1 h at 37 °C. The glucose liberated was assayed by the addition of 900 µl freshly prepared enzyme-colour reagent solution from an oxidase/peroxidase glucose detection kit (Sigma 510-DA). Trehalose was calculated from the difference of the glucose amounts in the samples with and without trehalase treatment. A significant background was observed during the measurement of extracellular trehalose at a high concentration of maltose, i.e. in culture supernatants of 10% (w/v) maltose-containing BMC broth, which was caused either by contamination of the maltose with trehalose or by non-specific interference of maltose with the enzymic trehalase assay. The background was determined by the enzymic assay of samples of sterile maltose BMC and subtracted from the values obtained from culture supernatants.

For more complex samples such as crude cell extracts where a high background of glucose was observed, trehalose was measured with high-pH ion chromatography (HPIC) at room temperature using a Carbo-Pak PA1 column installed in a DX500-HPLC system (DIONEX) supplied with a pulsed amperometric detector ED40. Samples of 25 µl of 10-fold diluted crude extracts were applied to the column and eluted with a linear gradient from 0 to 80 mM sodium acetate in 150 mM sodium hydroxide. The column was regenerated by a 10 min wash with 500 mM sodium acetate followed by 10 min equilibration with 150 mM sodium hydroxide. Trehalose was detected as a single peak with a retention time of approximately 3.3 min. Quantification was based on calibration with defined amounts of a trehalose standard solution.

Glycogen determination. The amount of intracellular glycogen in *C. glutamicum* was assayed by hydrolysis with amyloglucosidase (Branan *et al.*, 1982). For this purpose, samples (200 µl) of crude cell extracts (prepared as described above) were mixed with 2 vols 97% (v/v) ethanol, pelleted and redissolved with heating in the same

volume of 10 mM sodium/potassium phosphate buffer pH 6.0. Samples of 5–50 µl were incubated with amyloglucosidase (60 mU; Boehringer Mannheim) in 90 µl 100 mM sodium acetate buffer pH 4.5 for 1 h at 37 °C. The amount of glucose liberated was determined enzymically as described above. The amount of glycogen was calculated from the difference in glucose concentration between the amyloglucosidase-treated samples and control samples without amyloglucosidase.

RESULTS

Analysis of *C. glutamicum* genome sequence data

The available sequences from the raw *C. glutamicum* genome data (<http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html>; accession no. NC_003450) were screened for the presence of ORFs with similarity to genes known to be involved in trehalose metabolism. For the initial identification of potential candidates the suggested genome annotations were used. In addition, a BLAST search was made that was based on the enzymes for trehalose synthesis of *Mycobacterium tuberculosis*, a human pathogen phylogenetically related to *Corynebacterium* bacteria, which possesses all three known pathways for trehalose biosynthesis (De Smet *et al.*, 2000). ORFs with high similarity to all five genes involved in the different pathways were also found in *C. glutamicum* (Fig. 1).

The ORFs NCgl2535 and NCgl2537 were designated as *otsA* and *otsB*, respectively, because they putatively encode polypeptides with significant similarity to the enzymes trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase of the OtsA-OtsB pathway (52% and 28% identity with *M. tuberculosis* OtsA and OtsB, respectively). The two genes are separated by an additional ORF (NCgl2536) with the same orientation as *otsA* and *otsB* but unknown function (Fig. 2a). One of the ORFs upstream of *otsA* (NCgl2533) encodes a transmembrane threonine exporter (Simic *et al.*, 2001). An oppositely oriented ORF downstream of *otsB* is predicted to encode a LacI-family-type transcription regulator which might be involved in the regulation of the *otsA* and *otsB* genes.

The *C. glutamicum* ORFs NCgl2045 and NCgl2037 were selected because of their 48% and 44% identity with the *M. tuberculosis* TreY and TreZ enzymes, respectively, which are involved in trehalose synthesis from glycogen. Their chromosomal organization in *C. glutamicum* (Fig. 2b) differs significantly from that of similar genes in other organisms, where both genes are clustered together, often even overlapping each other (Maruta *et al.*, 1996a, b, c; Cole *et al.*, 1998). Although localized in the same region of the *C. glutamicum* chromosome, the *treY* and *treZ* genes of this organism are separated by a stretch of more than 8 kb which contains seven ORFs. In *Sulfolobus acidocaldarius*, *M. tuberculosis* and *Arthrobacter* sp. Q36 the *treY* and *treZ* genes constitute an operon with a third gene designated as *treX*, which is thought to have a glycogen-debranching function in the trehalose biosynthesis process

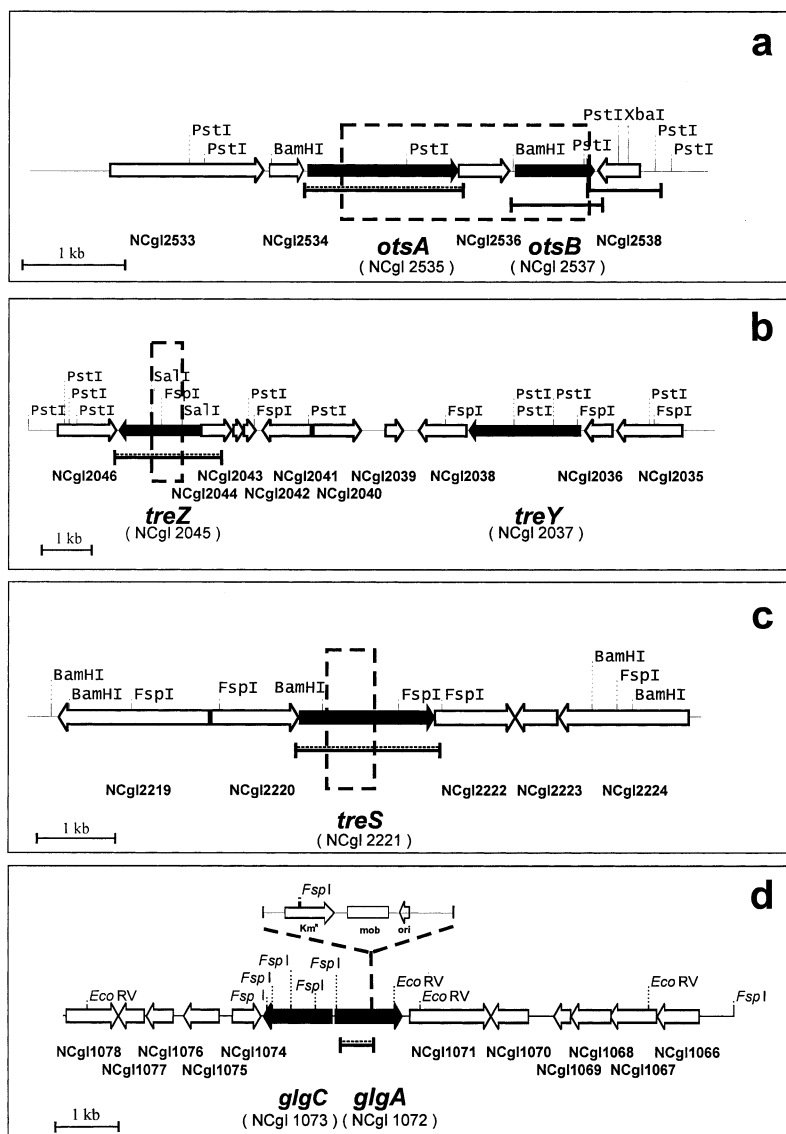


Fig. 2. Organization of the trehalose (a, b, c) and glycogen (d) biosynthesis genes on the *C. glutamicum* chromosome. The genes directly involved in biosynthesis of trehalose [*otsA/otsB* (a), *treY/treZ* (b) and *treS* (c)] and of glycogen [*glgA/glgC* (d)] are drawn in black. The deletions made during chromosomal gene inactivation are marked with dashed boxes (a, b, c). The scheme of integrational disruption of *glgA* is shown in (d). The fragments amplified by PCR and used for cloning and expression, or for inactivation of biosynthesis genes, are shown as black lines below the genes. For details of cloning and gene inactivation see the text.

(Maruta *et al.*, 1996c, 2000; Cole *et al.*, 1998). A possible *treX* homologue, NCgl2026, was identified in the *C. glutamicum* genome 10 kb upstream of *treY* gene (data not shown). The fact that *treY*, *treZ* and NCgl2026 all have the same orientation on the *C. glutamicum* genome and are separated from each other merely by several kilobases may indicate that this distribution is the result of intragenomic rearrangements of originally clustered genes.

Also, an ORF (NCgl2221) was identified in the *C. glutamicum* genome which is significantly related (up to 64% identity) to the trehalose synthase genes of other bacteria. This gene was designated *treS*. The start of the ORF located immediately downstream of *treS* (NCgl2222; Fig. 2c) overlaps the 3'-end of the *treS* ORF by 4 bp. ORFs with high similarity to NCgl2222 are found also directly downstream of *treS* in *Streptomyces coelicolor* and *M. tuberculosis*. In other bacteria like *Ralstonia solanacearum*, *Pseudomonas aeruginosa* and *Chlorobium tepidum*, the *treS* and NCgl2222 homologues are fused in one ORF.

Although nothing is known about the properties and physiological role of these putative NCgl2222-similar proteins, the genome data suggest a close functional connection with trehalose synthase.

To check the possibility of glycogen serving as a substrate for trehalose biosynthesis via the TreY-TreZ pathway, the *C. glutamicum* genome was scoured for putative genes for enzymes that may be involved in glycogen synthesis (Preiss & Greenberg, 1965). Two ORFs, NCgl1073 and NCgl1072, were found whose translation products are highly similar to the (putative) enzymes ADP-glucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA). The deduced *C. glutamicum* GlgC and GlgA amino acid sequences are related to the corresponding *M. tuberculosis* homologues at 61% and 59% identity, respectively. The two ORFs are situated next to each other but are oriented divergently, with their start codons separated by 51 bp (Fig. 2d). An additional ORF (NCgl0389) with significant similarity to (putative) glycogen synthase enzymes was found. However,

due to the genetic surroundings of NCgl1072 this gene and not NCgl0389 was preferred for investigation of its role in glycogen synthesis.

In summary, exploration of the *C. glutamicum* genome data indicated the presence of all three pathways for trehalose biosynthesis observed in bacteria, thus suggesting a similar gene outfit for this purpose as in the related *M. tuberculosis*. In addition, the genome data suggested the presence of the pathway for glycogen synthesis in *C. glutamicum*.

Accumulation of free trehalose by *C. glutamicum*

As described by Vallino & Stephanopoulos (1993) and Wittmann & Heinzle (2001), lysine-overproducing mutants of *C. glutamicum* accumulate up to 6 g trehalose l⁻¹ in the culture broth under conditions close to those used for industrial lysine production. Attempts to connect this trehalose accumulation with changes in the osmolarity of the growth medium, using the type strain of *C. glutamicum* and NaCl addition to increase the osmolarity, were not successful (data not shown). On the other hand, when sucrose was used instead of NaCl for adjustment of the medium's osmolarity, a significant long-term increase of the extracellular trehalose was observed.

The growth and trehalose accumulation by the type strain of *C. glutamicum* in minimal BMC medium with two different sugar concentrations, i.e. 0.5% (w/v) sucrose (Fig. 3a) and 10% (w/v) sucrose (Fig. 3b), was followed. In the case of the low-sugar medium *C. glutamicum* stopped its growth at an OD₆₀₀ of about 12, due to substrate limitation. In this case the trehalose accumulated in the culture broth did not exceed 0.1 g l⁻¹. In contrast, when grown with an excess of sucrose the bacteria reached a final OD₆₀₀ of more than 16. Under these conditions, the type strain accumulated up to 0.9 g trehalose l⁻¹ during the late exponential and the stationary phase. Monitoring of the intracellular trehalose level showed that in the case of high sucrose supply, intracellular levels of about 20 µg trehalose per mg dry cell weight were reached, which is about four times the maximum intracellular trehalose level detected in the case of low-sucrose supplementation. Under low- as well as high-sucrose conditions, the intracellular trehalose concentration dropped to extremely low values in stationary-phase cells (Fig. 3).

The correlation of extracellular trehalose accumulation with sugar excess in the medium, in concert with the knowledge of the presence of putative genes for trehalose production from glycogen in the *C. glutamicum* genome, prompted us to check for the presence of glycogen in the cells as a possible substrate for trehalose production. Indeed, it was shown that *C. glutamicum* is able to produce glycogen when supplied with a surplus of sucrose. Under conditions of excess sucrose, glycogen accumulation was found to correlate with trehalose accumulation (Fig. 3).

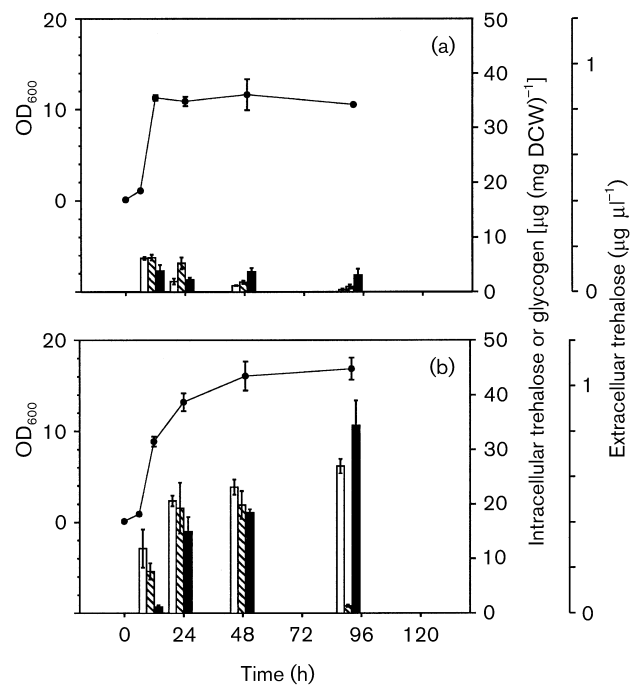


Fig. 3. Dependence of trehalose and glycogen accumulation by *C. glutamicum* on the sucrose concentration in the medium. The type strain of *C. glutamicum* was grown in minimal BMC broth supplemented with 0.5% sucrose (a) or 10% sucrose (b). Growth curves were recorded by monitoring the OD₆₀₀ of the cultures (●). Intracellular (hatched bars) and extracellular (black bars) trehalose, and intracellular glycogen (white bars), were measured after 12, 24, 48 and 96 h of growth. The results shown are from at least three replicate experiments. DCW, dry cell weight.

Inactivation of the *C. glutamicum* trehalose biosynthesis pathways

In order to determine the role of the different pathways proposed from the genome data analysis in *C. glutamicum* trehalose biosynthesis *in vivo*, three mutants were constructed by chromosomal inactivation of at least one gene of each pathway (Fig. 1; see Methods). For inactivation of the OtsA-OtsB pathway a 2.4 kb chromosomal fragment was removed, resulting in the in-frame fusion of truncated *otsA* and *otsB* genes. In this mutant, designated *C. glutamicum* Δ *otsAB*, more than 70% of the *otsA* gene, the entire ORF NCgl2536, and more than 95% of *otsB* were deleted (Fig. 2a). Inactivation of the TreY-TreZ pathway was achieved by in-frame deletion of a 645 bp fragment of the *treZ* gene (Fig. 2b). Preceding efforts to inactivate the first gene of the pathway (*treY*) were unsuccessful, perhaps due to polar effects of such deletions on the NCgl2038 ORF. The third proposed pathway for trehalose synthesis in *C. glutamicum*, i. e. the TreS pathway, which uses maltose as a precursor (Fig. 2c), was inactivated by the in-frame deletion of a 459 bp internal fragment of *treS*, resulting in a truncated gene which no longer encoded a functional

trehalose synthase (data not shown). Thus, three *C. glutamicum* DSM 20300 single mutants were obtained and named \DeltaotsAB , $\Delta treZ$ and $\Delta treS$, according to the pathway targeted for inactivation in each case.

Based on the single mutants just described, all possible combinations of double mutants ($\DeltaotsAB/\Delta treZ$, $\DeltaotsAB/\Delta treS$, $\Delta treZ/\Delta treS$), as well as a triple mutant inactivated in all three trehalose synthesis pathways ($\DeltaotsAB/\Delta treZ/\Delta treS$), were constructed. During the construction of the $\DeltaotsAB/\Delta treZ$ and the $\DeltaotsAB/\Delta treZ/\Delta treS$ mutants we faced difficulties in obtaining the second-step (vector excision) recombinants carrying the desired deletion. Instead of obtaining nearly equal numbers of the desired deletion variants and clones resulting from reversion of the vector integration event (Schäfer *et al.*, 1994), only the latter type of second-step recombinants were obtained. The problem was overcome after addition of 2% (w/v) trehalose to the medium used for the *sacB*-based selection of clones carrying the second recombination event. This interesting observation was a first indication that these two mutant strains had severe difficulties in growing without trehalose in the medium.

During incubation of the $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ mutant strains in liquid minimal medium without trehalose with moderate shaking (about 150 r.p.m.) aggregates of cells were observed which rapidly sedimented at the bottom of the culture tubes. Although the increase of culture agitation to 210 r.p.m. resulted in the improvement of growth, the strains carrying mutations in both the OtsA-OtsB and the TreY-TreZ pathways were significantly impaired in their growth in minimal media compared to

the other trehalose synthesis mutants and the type strain (Fig. 4a).

Experiments to measure the intra- and extracellular accumulation of trehalose by the *C. glutamicum* type strain and the mutants were made using cultures grown in 5 ml 10% (w/v) sucrose-containing BMC medium. Intracellular trehalose was determined via HPLC analysis, while extracellular trehalose was measured enzymically and confirmed via HPLC analysis (see Methods). A more than 50% decrease of the intracellular trehalose concentration was observed in the mutants carrying either the \DeltaotsAB or the $\Delta treZ$ mutation, and the complete absence of intracellular trehalose was noted in the strains simultaneously carrying both mutations (Fig. 4b). Also, in comparison with the wild-type strain, the \DeltaotsAB , $\Delta treZ$, $\DeltaotsAB/\Delta treS$ and $\Delta treZ/\Delta treS$ mutants showed a significant (about 20–50%) decrease in the levels of extracellular trehalose accumulation (Fig. 4c). In the double mutant $\DeltaotsAB/\Delta treZ$ and the triple mutant $\DeltaotsAB/\Delta treZ/\Delta treS$ no significant amount of extracellular trehalose was detected (Fig. 4c). In contrast, the mutant inactivated only in the TreS pathway showed only a slight decrease in the intracellular and almost no change in the extracellular trehalose levels compared to the type strain.

Growth of the mutants $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ on different substrates known to be utilized by *C. glutamicum* was investigated by cultivation at 30 °C at 150 r.p.m. in 5 ml BMC medium supplemented with different carbon sources at 1% (w/v) (Table 2). It is noteworthy in this context that *C. glutamicum* DSM 20300 is unable to grow on trehalose as the sole source of carbon

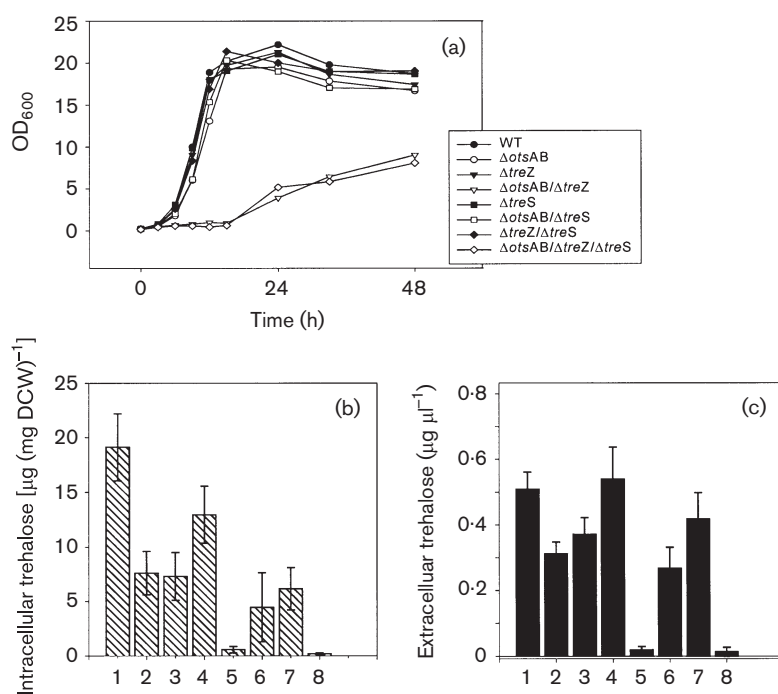


Fig. 4. Phenotypic characterization of mutants with inactivated trehalose biosynthesis pathways. Growth curves in BMC medium containing 1% sucrose in comparison with the wild-type are shown in (a). In addition, all mutants and the type strain were grown in 10% sucrose BMC medium. The intracellular trehalose concentration was determined after 48 h (b), and extracellular trehalose was measured after 72 h (c). The bars in (b) and (c) correspond to the following strains: wild-type (1), \DeltaotsAB (2), $\Delta treZ$ (3), $\Delta treS$ (4), $\DeltaotsAB/\Delta treZ$ (5), $\DeltaotsAB/\Delta treS$ (6), $\Delta treZ/\Delta treS$ (7), $\DeltaotsAB/\Delta treZ/\Delta treS$ (8). All data shown are mean values from at least three replicate experiments. DCW, dry cell weight.

Table 2. Comparison of the growth of the double mutant $\DeltaotsAB/\Delta treZ$ and the triple mutant $\DeltaotsAB/\Delta treZ/\Delta treS$ with that of the type strain

The strains were grown at 30 °C, 150 r.p.m., in tubes containing 5 ml BMC broth supplemented with different substrates as specified, at a final concentration of 1 % (w/v) (unless noted otherwise). \pm , Weak growth.

Carbon source	WT	$\DeltaotsAB/\Delta treZ$	$\DeltaotsAB/\Delta treZ/\Delta treS$
Glucose	+++	\pm	\pm
Fructose	+++	+	+
Sucrose (1 %)	+++	\pm	\pm
Sucrose (10 %)	+++	\pm	\pm
Maltose	+++	+++	\pm
Trehalose (2 %)	–	–	–
Sucrose + trehalose (2 %)	+++	++	++
<i>myo</i> -Inositol	+++	+	+
Pyruvate	+++	++	++
Acetate	++	+(+)	+(+)

and energy. On most of the sugar substrates tested the wild-type strain reached a maximum OD₆₀₀ of above 15, while the mutant strains displayed significantly impaired growth. In contrast, growth of the mutants on acetate or pyruvate was not as severely affected as growth on sugar substrates. Trehalose addition to sucrose cultures largely relieved the growth defect of the mutants. This phenomenon of complementation of the mutants by trehalose addition was investigated in more detail by recording growth curves (see below, Fig. 5).

Complementation of $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ by addition of trehalose

The mutants $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ were significantly impaired in their ability to grow in minimal BMC medium (Fig. 4a), but their growth rates did not differ significantly from that of the type strain when grown on complex LB medium (not shown), indicating that LB

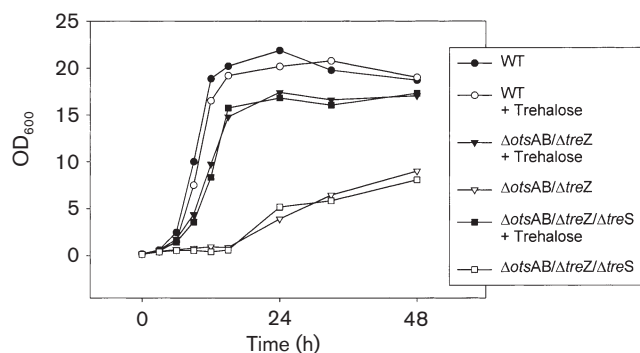


Fig. 5. Growth characteristics of trehalose-non-producing mutants in 1% sucrose BMC in the absence or presence of 2% trehalose. Addition of trehalose led to the nearly complete restoration of growth of the mutants $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$. The data shown are mean values from at least three replicate experiments.

contained a component(s) needed for normal growth of the mutants which is absent in minimal medium. Addition of the osmoprotectants L-proline or betaine (at 20 mM) did not improve the mutants' growth (data not shown), while the addition of 2 % (w/v) trehalose to BMC medium resulted in nearly the same growth rate and final culture density as the wild-type control (Fig. 5). Thus the simultaneous inactivation of both the OtsA-OtsB and the TreY-TreZ pathways leads to trehalose auxotrophy of *C. glutamicum*.

Growth of $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ on maltose

The fact that the double mutant $\DeltaotsAB/\Delta treZ$ and the triple mutant $\DeltaotsAB/\Delta treZ/\Delta treS$ displayed similar growth behaviour in minimal medium with most of the substrates tested (Table 2) indicates that the presence of an intact *treS* gene had no significant effect on growth under these conditions. Taking into account that trehalose synthase (TreS) catalyses trehalose production from maltose we investigated the growth phenotype of both mutants on BMC minimal media supplemented with 1 % (w/v) maltose as the sole carbon source (Table 2; Fig. 6a). While growth of the triple mutant $\DeltaotsAB/\Delta treZ/\Delta treS$ was significantly impaired in this medium, the $\DeltaotsAB/\Delta treZ$ strain with an intact *treS* gene displayed a similar growth rate to the wild-type.

In addition, the intra- and extracellular accumulation of trehalose by both mutants and the type strain grown at a high maltose concentration was checked (Fig. 6b, c). Under these conditions, the intracellular trehalose level in the mutant $\DeltaotsAB/\Delta treZ$ was similar to the type strain, while the triple mutant $\DeltaotsAB/\Delta treZ/\Delta treS$ was devoid of intracellular trehalose (Fig. 6b). This result, in concert with the differences observed between the $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ mutants grown on maltose in comparison to growth on the other substrates (Table 2), suggests that the TreS pathway is functional and able to

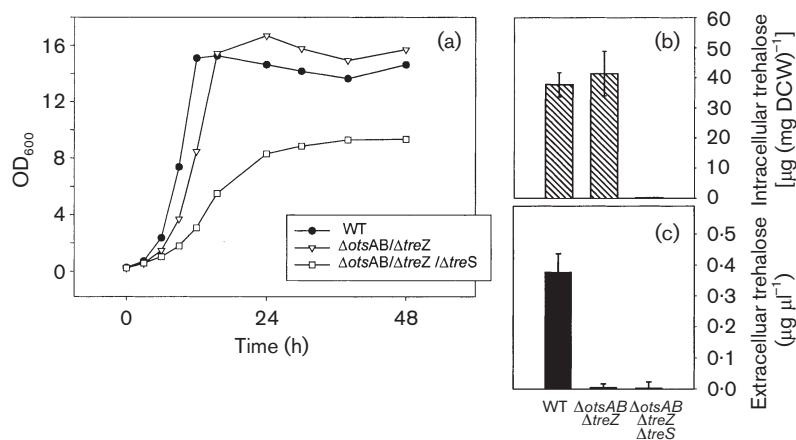


Fig. 6. Phenotypic differences between Δ otsAB/ Δ treZ, Δ otsAB/ Δ treZ/ Δ treS and the wild-type grown in BMC minimal medium supplemented with maltose as the carbon source. Bacterial growth was determined on minimal medium with 1% maltose (a); the accumulation of intracellular (b) and extracellular (c) trehalose was measured after growth for 48 h and 72 h, respectively, in BMC medium with 10% maltose. The values for extracellular trehalose were corrected by subtraction of the assay background as described in Methods. All data shown are mean values from at least three replicate experiments. DCW, dry cell weight.

supply sufficient amounts of trehalose for *C. glutamicum* growth only in the presence of maltose. In contrast to the wild-type, both mutants did not accumulate extracellular trehalose (Fig. 6c). A possible explanation for the lack of extracellular trehalose accumulation by the Δ otsAB/ Δ treZ mutant on maltose could be that the rate of trehalose production via the TreS pathway is low and supplies sufficient trehalose to meet the requirements of the cell but not a significant surplus, whereas the rate of trehalose synthesis in cells with active OtsA-OtsB and TreY-TreZ pathways may be significantly higher and may lead to the accumulation of the surplus trehalose in the culture broth.

Plasmid complementation of Δ otsAB mutations

C. glutamicum Δ otsAB/ Δ treZ strains carrying expression plasmids with the *otsA* gene (pWLQ2::*otsA*) and both *ots* genes (pWLQ2::*otsAB*) were constructed and checked for their ability to grow in 1% (w/v) sucrose-containing BMC medium in the absence of trehalose (Fig. 7). The plasmid carrying both *otsA* and *otsB* efficiently complemented the

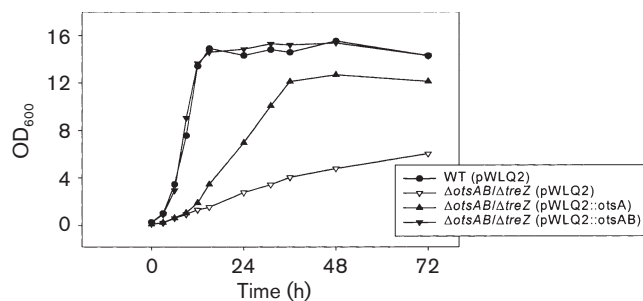


Fig. 7. Complementation of the *C. glutamicum* Δ otsAB/ Δ treZ mutant using plasmid copies of the *otsA* and *otsB* genes. Plasmids carrying only the *otsA* gene or both *otsA* and *otsB* were transformed into the Δ otsAB/ Δ treZ mutant. The transformants were grown in 1% sucrose BMC broth containing 20 μ g kanamycin ml⁻¹ and 1 mM IPTG for induction of gene expression. The data shown are mean values from at least three replicate experiments.

mutant's growth deficiency under these conditions. This observation excludes the possibility that the mutant's growth phenotype is a result of polar effects that could have been caused by the deletion introduced into the chromosome, and also shows that ORF NCgl2536, the ORF located between *otsA* and *otsB* on the chromosome (see Fig. 2) which was not supplied on the plasmid, is not essential for trehalose production and normal growth in minimal medium. Transformation of the Δ otsAB/ Δ treZ double mutant with pWLQ2::*otsA* led to a significant improvement of growth in 1% (w/v) sucrose BMC broth, but did not result in the complete complementation of the mutant's growth deficiency (Fig. 7). An explanation for this could be the *in vivo* substitution of the function of trehalose phosphate phosphatase (OtsB) by a different, perhaps non-specific, phosphatase, or the assumption that the presence of trehalose 6-phosphate instead of trehalose in the *C. glutamicum* cell is sufficient for a partial restoration of bacterial growth.

Lipid composition of the trehalose-non-producing mutant *C. glutamicum* Δ otsAB/ Δ treZ

The importance of trehalose for *C. glutamicum* growth could be connected with its structural role in the cell. Trehalose is found in *C. glutamicum* cells not only in its free form but also as trehalose mono- (TMCM) and di- (TDCM) corynomycolates, which are the dominant components in the non-covalently bound corynomycolate-containing lipid fraction of the outer cell wall permeability barrier (Puech *et al.*, 2000, 2001). Our results show that the inability of *C. glutamicum* to synthesize trehalose has a significant influence on the composition of its cell wall lipid fraction.

The Δ otsAB/ Δ treZ mutant was grown in 30 ml 1% (w/v) sucrose-containing BMC broth with or without the addition of 2% (w/v) trehalose. The cells were harvested after 10 h and equal amounts of wet cells were used for cell wall lipid isolation. The lipids were separated using silica-gel TLC plates developed with a chloroform/methanol/water solvent system and compared with the lipids isolated from the type

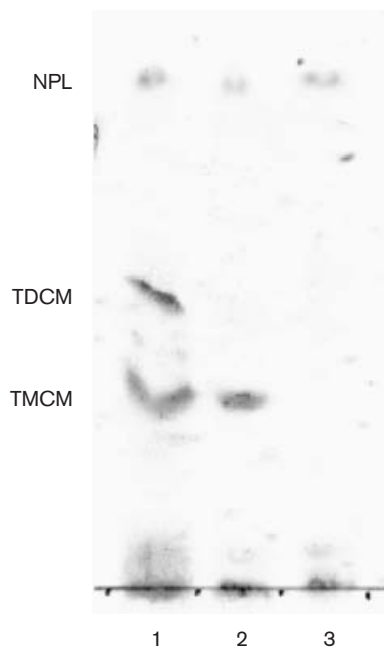


Fig. 8. TLC analysis of cellular lipid extracts. Equal amounts (100 μ g each) of cell lipid extracts from the type strain grown in 1% sucrose BMC medium (lane 1), and the mutant Δ otsAB/ Δ treZ grown in 1% sucrose BMC medium with addition of 2% trehalose (lane 2) and without trehalose (lane 3), were separated on TLC silica-gel plates and developed with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (30:8:1, by vol.) in a tightly sealed chamber at 4°C. The spots were visualized by spraying with 0.2% anthrone dissolved in conc. H_2SO_4 followed by heating at 100°C. NPL, non-polar lipids; TDCM, trehalose dicorynomycolate; TMCM, trehalose monocorynomycolate.

strain grown under the same conditions (Fig. 8). The spots detected after anthrone staining were identified based on the *C. glutamicum* glycolipid profile described by Puech *et al.* (2000). When grown in the absence of trehalose, the mutant strain lacked both major trehalose-containing glycolipids in its cell wall lipid fraction. The missing trehalose-corynomycolates were not substituted by other, trehalose-less corynomycolates (such as glucose monocorynomycolate, GMCM, which was observed to be accumulated in a *csp1*-inactivated *C. glutamicum* mutant; Puech *et al.*, 2000). In the presence of trehalose in the culture broth, the Δ otsAB/ Δ treZ mutant is able to produce trehalose corynomycolates. However, in contrast to the wild-type strain, the trehalose-supplemented mutant contained TMCM as the predominant glycolipid while TDCM was missing. Based on the proposed trehalose corynomycolate biosynthetic pathway (Shimakata & Minatogawa, 2000), it may be possible that a high concentration of trehalose present in the medium results in a shift of the equilibrium in the TDCM synthesis reaction in favour of TMCM. Analysis of the lipids from the type strain DSM 20300 grown in medium with 2% trehalose supports this hypothesis (authors' unpublished data).

Construction and characterization of a *glgA* mutant

C. glutamicum is able to accumulate glycogen in the presence of excess sucrose in the culture medium (Fig. 3). In accordance with this observation, a cluster of ORFs was found in the *C. glutamicum* genome (NCgl1073–NCgl1072) whose predicted translation products display high similarity with enzymes or predicted enzymes of glycogen biosynthesis from some bacteria (data not shown). We decided to disrupt the ORF NCgl1072, which encodes a putative glucosyl transferase suspected to represent glycogen synthase (*glgA*), with two goals in mind: (i) to investigate whether the gene cluster containing this gene is indeed involved in glycogen production by *C. glutamicum*, and (ii) to find out if glycogen synthesis plays a role in trehalose production.

A mutant designated as *glgA*::Km was obtained after site-specific integration of pCLiK6::*glgA'* into the chromosome of *C. glutamicum*, resulting in disruption of the NCgl1072 ORF (see Fig. 2d). The mutant was unable to accumulate glycogen under conditions of excess sucrose (see legend to Fig. 9). Two additional mutants were made by disruption of the NCgl1072 ORF in the chromosome of the Δ otsAB and Δ otsAB/ Δ treS mutants. The mutants were designated as Δ otsAB/*glgA*::Km and Δ otsAB/ Δ treS/*glgA*::Km, respectively. The phenotypic comparison of the *C. glutamicum* Δ otsAB/ Δ treZ and Δ otsAB/ Δ treZ/ Δ treS mutants with the two isogenic mutants lacking glycogen synthase (GlgA) instead of TreZ did not reveal differences between the four mutant strains with respect to their ability to grow in minimal media without trehalose (Fig. 9) and their inability to produce and accumulate trehalose (see legend to Fig. 9). The fact that the *glgA*::Km and Δ treZ mutants showed identical phenotypes in the Δ otsAB as well as the Δ otsAB/ Δ treS background strongly supports the idea that TreZ and GlgA are involved in one and the same pathway for trehalose biosynthesis. Also, these results provide evidence for the importance of trehalose synthesis from glycogen in *C. glutamicum*.

DISCUSSION

Genetic dissection of trehalose and glycogen biosynthesis pathways in *C. glutamicum*, and their operation under various growth conditions

Some of the *C. glutamicum* mutants with a single *tre* biosynthetic pathway knocked out by chromosomal mutagenesis showed a decrease in trehalose synthesis but none of them displayed a total lack of trehalose production, suggesting that synthesis of this disaccharide in *C. glutamicum* is not accomplished by a single pathway, but is based on two or more, presumably coordinately regulated pathways. The subsequent construction of double mutants, in which only one of the three proposed pathways for trehalose synthesis was still active, showed that either the OtsA-OtsB pathway or the TreY-TreZ

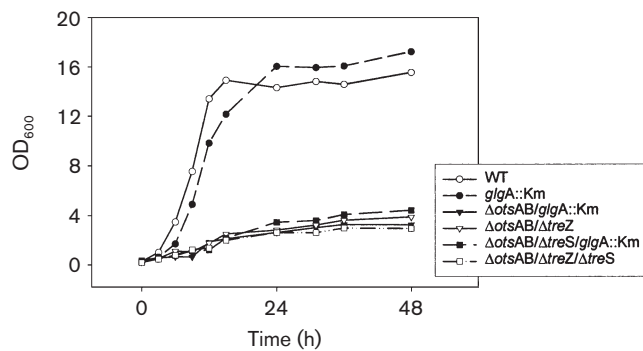


Fig. 9. Phenotypic comparison of the *glgA::Km* and Δ *treZ* mutants. The single recombination mutant *glgA::Km* and the mutants Δ *otsAB/glgA::Km* and Δ *otsAB/ΔtreS/glgA::Km* were compared with Δ *otsAB/ΔtreZ*, Δ *otsAB/ΔtreZ/ΔtreS*, and the wild-type. Growth was monitored in 1% sucrose BMC broth supplemented with 20 μ g kanamycin ml^{-1} to maintain the marker insertion in *glgA::Km* mutants. The data shown are mean values from at least three replicate experiments. While the wild-type produced about 68.1 ± 14.3 μ g glycogen [mg dry cell weight (DCW)]⁻¹ after 24 h growth, no glycogen was found in the *glgA::Km* mutant. The level of intracellular trehalose after 24 h growth was 18.6 ± 3.6 μ g (mg DCW)⁻¹ and 10.5 ± 1.9 μ g (mg DCW)⁻¹, respectively, for the wild-type and the *glgA::Km* mutant, while the mutants Δ *otsAB/glgA::Km*, Δ *otsAB/ΔtreS/glgA::Km*, Δ *otsAB/ΔtreZ* and Δ *otsAB/ΔtreZ/ΔtreS* did not reveal significant amounts of intracellular trehalose [all assay values below 0.4 μ g (mg DCW)⁻¹]. Extracellular trehalose accumulation, which was measured after 72 h growth in 10% sucrose BMC broth supplemented with 20 μ g kanamycin ml^{-1} where appropriate, was 0.43 ± 0.12 μ g ml^{-1} and 0.52 ± 0.09 μ g ml^{-1} , respectively, for the wild-type and the *glgA::Km* mutant, while the mutants Δ *otsAB/glgA::Km*, Δ *otsAB/ΔtreS/glgA::Km*, Δ *otsAB/ΔtreZ* and Δ *otsAB/ΔtreZ/ΔtreS* did not reveal significant amounts of extracellular trehalose (all assay values below 0.03 μ g ml^{-1}).

pathway alone was sufficient to ensure trehalose synthesis at a level meeting the requirements of the bacteria. Even trehalose excretion to the outside of the cells was not dramatically decreased as long as the mutated bacteria possessed one of these two biosynthesis pathways. On the other hand, the inactivation of both the OtsA-OtsB and TreY-TreZ pathways, or in addition also of the TreS pathway, led to the inability of the corresponding mutants to synthesize trehalose and to grow efficiently under most conditions tested. Thus the pathway-inactivation experiments indicate the dominant role of the two pathways involving OtsA-OtsB and TreY-TreZ for the *in vivo* trehalose synthesis in *C. glutamicum*.

It is not known if the OtsA-OtsB and TreY-TreZ pathways are used simultaneously in wild-type cells and, if so, if the quantitative contribution of both pathways to trehalose production is similar. From the energetic point of view, the OtsA-OtsB pathway is more efficient than the TreY-TreZ

pathway. The synthesis of 1 mol trehalose via the OtsA-OtsB pathway is achieved from 1 mol glucose 6-phosphate and 1 mol UDP-glucose, while 1 mol trehalose produced via the TreY-TreZ pathway consumes 2 mol ADP-glucose (for glycogen synthesis). If one assumes that trehalose is produced mainly for synthesis of the cell wall lipids TDCM and TCM, and that trehalose phosphate and not free trehalose is needed as a precursor for this purpose (also see below; Shikimakata & Minatogawa, 2000), the energy balance is even more in favour of the OtsA-OtsB pathway, because phosphorylated trehalose is an intermediate of the OtsA-OtsB but not of the TreY-TreZ pathway. Therefore it seems reasonable to speculate that only under energy- and substrate-excess conditions could the TreY-TreZ pathway be preferred over the OtsA-OtsB pathway. On the other hand, our results show that glycogen, which can serve as a substrate for the TreY-TreZ pathway, is present in *C. glutamicum* cells also under conditions of low sugar supply, although not in the same amounts as under sugar-excess conditions. Also, we observed that the TreY-TreZ pathway alone is sufficient to support *C. glutamicum* growth not only under sugar excess (Fig. 4a) but also under low-sugar conditions (0.5%, w/v, sucrose; data not shown). Further experiments are needed to determine the individual contribution of each of the OtsA-OtsB and TreY-TreZ pathways to trehalose biosynthesis in wild-type *C. glutamicum* cells under different growth conditions.

Our data suggest that the TreS pathway plays only a supporting role in trehalose synthesis. Analysis of the growth and trehalose accumulation characteristics of the Δ *otsAB/ΔtreZ* and Δ *otsAB/ΔtreZ/ΔtreS* mutants (Fig. 6) demonstrated that this pathway is involved in trehalose synthesis during growth on maltose-containing medium. It is interesting to note that while the wild-type strain and the Δ *otsAB/ΔtreZ* mutant revealed similar levels of intracellular trehalose, the Δ *otsAB/ΔtreZ* mutant accumulated much less extracellular trehalose than the wild-type (Fig. 6b, c), whose extracellular trehalose level after growth on maltose was about the same as on sucrose (Fig. 4c). At present it is not known if the wild-type strain, which contains all three functional trehalose biosynthesis pathways, preferentially utilizes the TreS pathway during growth on maltose. However, the difference in extracellular trehalose accumulation between the wild-type strain and the mutant retaining the TreS pathway as the only trehalose biosynthesis pathway after growth on maltose suggests that in the wild-type both other pathways have a dominant role for trehalose synthesis also when the bacteria are grown on an excess of maltose.

Our results show that *C. glutamicum* accumulates glycogen when grown under conditions of sugar excess. A glycogen synthesis pathway using ADP-glucose as precursor, similar to that in other bacteria (Preiss & Greenberg, 1965), was predicted from the genome data. Using chromosomal insertion mutagenesis, we showed that the ORF NCgl1072 (together with its neighbour NCgl1073) is involved in

glycogen synthesis in *C. glutamicum*. We were able to connect glycogen synthesis with trehalose synthesis, showing that *otsAB* mutants simultaneously impaired in glycogen synthesis ($\DeltaotsAB/glgA::Km$ and $\DeltaotsAB/\Delta treS/glgA::Km$) displayed an identical growth and trehalose synthesis phenotype as the *otsAB* mutants with an inactivated TreY-TreZ pathway ($\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$) (see Fig. 9). The growth deficiency of the mutant blocked simultaneously in glycogen synthesis and in the OtsA-OtsB pathway was observed under most growth conditions, including low (1%) sucrose (Fig. 9), which confirms the important role of trehalose synthesis from glycogen not only under sugar-excess growth conditions.

Impact of trehalose biosynthesis on the growth physiology and cell wall lipid composition of *C. glutamicum*

The trehalose dependence of growth of the $\DeltaotsAB/\Delta treZ$ and the $\DeltaotsAB/\Delta treZ/\Delta treS$ mutants on the majority of the substrates tested indicates the importance of this disaccharide for these bacteria. This is in accordance with the fact that *C. glutamicum*, just like the related mycobacteria (De Smet *et al.*, 2000), has established three independent pathways for trehalose biosynthesis. One of the possible roles of trehalose in *C. glutamicum* cells is to act as a compatible solute in osmotic shock conditions, as found in other bacteria (Argüelles *et al.*, 2000). This hypothesis is supported by the observation of the accumulation of free trehalose in *C. glutamicum* and *Brevibacterium lactofermentum* cells under hyperosmotic conditions (Skjerdal *et al.*, 1995). Our own initial experiments to analyse if NaCl-induced changes in the osmolarity of the medium elicited the intracellular and extracellular accumulation of free trehalose were not successful (unpublished results). However, a significant increase of the free trehalose level was obtained at a high sugar concentration in the growth medium, a finding that correlates with the observation that significantly higher amounts of trehalose were accumulated by the type strain when hyperosmotic stress was induced by sucrose rather than NaCl or glutamate (Skjerdal *et al.*, 1996). In order to further specify the role of trehalose we used the mutants $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$, which are defective in its synthesis. Both mutants were unable to grow efficiently in minimal medium in the absence of trehalose on most of the carbon sources tested. This inability to grow normally was not linked to hyperosmotic stress imposed on the cells. On the contrary, the mutants were unable to grow on minimal medium containing 1% (w/v) sucrose (less than 50 mosmol kg⁻¹), which is far below the concentrations that elicit an osmotic response in *C. glutamicum* cells (Skjerdal *et al.*, 1996). Only the addition of trehalose, but not of other compatible solutes, restored the growth of the mutants. All these results argue against a major role for trehalose as a compatible solute in *C. glutamicum*. The intracellular and extracellular trehalose accumulation was shown to be connected with an excess of carbon source in the medium and was observed in the late

exponential and stationary phases. All these prerequisites for trehalose synthesis are reminiscent of conditions known to favour the accumulation of carbon and energy storage compounds such as glycogen in other bacteria. A role for trehalose as a reserve compound in *C. glutamicum* is unlikely since the intracellular trehalose level is extremely low in stationary-phase cells. The possibility that trehalose accumulation is only a direct result of the glycogen increase in the corynebacterial cells does not accord with the fact that mutants impaired in their ability to synthesize trehalose from glycogen ($\Delta treZ$, $\Delta treZ/\Delta treS$) still accumulate significant amounts of trehalose both intracellularly and extracellularly (Fig. 4).

The *C. glutamicum* mutants $\DeltaotsAB/\Delta treZ$ and $\DeltaotsAB/\Delta treZ/\Delta treS$ are unable to grow properly under a variety of conditions, and only the addition of trehalose restored growth. These mutants' tendency to form large cell aggregates suggests an altered cell surface or a defect in a late stage of cell division. In both mycobacteria and corynebacteria it was shown that trehalose in the form of corynomycolic esters is involved in a second permeability barrier outside the cytoplasmic membrane (Puech *et al.*, 2001; Sathyamoorthy & Takayama, 1987). Our data (Fig. 8) show that one striking consequence of the inability to synthesize trehalose is the absence of trehalose-containing TMCM and TDCM, which are thought to be important constituents of the outer lipid bilayer in *C. glutamicum*. The growth problems of the trehalose-deficient mutants may be connected with their inability to constitute such a cell wall lipid layer. It has been shown for *Corynebacterium matruchotii* that trehalose is not only essential at the final stage of corynomycolate ester metabolism but also, as trehalose phosphate, plays a key role in the entire process of corynomycolic acid synthesis (Shimakata & Minatogawa, 2000), i.e. trehalose 6-phosphate was suggested to serve as an acceptor for the freshly synthesized corynomycolic acid. The resulting TMCM is then a common precursor for the synthesis of all esterified corynomycolates of the cell wall, of TDCM, and of free corynomycolic acid (Shimakata & Minatogawa, 2000; Puech *et al.*, 2000). Thus, the inability to synthesize trehalose or trehalose 6-phosphate by some of the *C. glutamicum* mutants constructed here could lead not only to the absence of both trehalose-containing glycolipids but also of all other corynomycolate esters. The mechanism just described, where trehalose is used as a carrier for the corynomycolic acid and then is (partially) liberated outside the cells, may provide an explanation for the presence of extracellular trehalose.

It is interesting to note that on some substrates such as acetate and to some extent pyruvate the trehalose-deficient *C. glutamicum* mutants were able to grow quite normally, reaching similar final culture densities as the wild-type strain, which stands in contrast to the severely impaired growth on sugar substrates. This phenomenon may be explained by differences in the effects an altered cell wall lipid bilayer could have on the uptake of different substrates.

Interestingly, in the case of acetate it has been reported that a 50% decrease in cell wall-linked corynomycolates facilitated acetate uptake (Puech *et al.*, 2000).

Importantly, the results of the genetic and physiological dissection of trehalose biosynthesis in *C. glutamicum* reported here may be of general relevance for the whole phylogenetic group of mycolic-acid-containing coryneform bacteria, which contains a number of different genera, including medically and biotechnologically important species (see Liebl, 2001). Additional transcriptional and enzyme activity studies are required to reveal the regulation of the trehalose synthesis pathways. Regulation studies are expected to reveal more information about the physiological role of the free extracellular and intracellular trehalose accumulated in *C. glutamicum* during growth under sugar-excess conditions.

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