Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*

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*Corynebacterium glutamicum* is used for the large-scale production of L-glutamate, but the efflux of this amino acid is poorly understood. This study shows that addition of ethambutol (EMB) to growing cultures of *C. glutamicum* causes L-glutamate efflux at rates of up to 15 nmol min⁻¹ (mg dry wt)⁻¹, whereas in the absence of EMB, no efflux occurs. EMB is used for the treatment of *Mycobacterium tuberculosis*, and at a molecular level it targets a series of arabinosyltransferases (EmbCAB). The single arabinosyltransferase-encoding *emb* gene of *C. glutamicum* was placed under the control of a Tet repressor (TetR). Experiments with this strain, as well as with an *emb*-overexpressing strain, coupled with biochemical analyses showed that: (i) *emb* expression was correlated with L-glutamate efflux, (ii) *emb* overexpression increased EMB resistance, (iii) EMB caused less arabinan deposition in cell wall arabinogalactan, and (iv) EMB caused a reduced content of cell-wall-bound mycolic acids. Thus EMB addition resulted in a marked disordering of the cell envelope, which was also discernible by examining cellular morphology. In order to further characterize the cellular response to EMB addition, genome-wide expression profiling was performed using DNA microarrays. This identified 76 differentially expressed genes, with 18 of them upregulated more than eightfold. Among these were the cell-wall-related genes *ftsE* and *mepA* (encoding a secreted metalloprotease); however, genes of central metabolism were largely absent. Given that an altered lipid composition of the plasma membrane of *C. glutamicum* can result in L-glutamate efflux, we speculate that major structural alterations of the cell envelope are transmitted to the membrane, which in turn activates an export system, perhaps via increased membrane tension.

**INTRODUCTION**

*Corynebacterium glutamicum* and *Mycobacterium tuberculosis* share characteristic cell walls common to all *Corynebacterineae* (Minnikin, 1982; Stackebrandt et al., 1997). They contain an unusual lipid-rich outer layer, unique among Gram-positive bacteria, containing mycolic acids, either as organic soluble lipids, such as trehalose mono- and dimycolates (TMM and TDM) or glucose monomycolates (GMM), or bound to arabinose residues of the cell wall polymer arabinogalactan (Besra et al., 1995). The major physical properties of the cell wall and its biosynthesis are key to our understanding of cell wall permeability and resistance to front-line *M. tuberculosis* agents (Brennan & Nikaido, 1995). This unique lipid-rich layer represents a barrier for the access of antibiotics, and its synthesis is a target of several effective antibiotics, such as isoniazid and ethambutol (EMB).

Our interest in *C. glutamicum* stems from the use of this bacterium for amino acid production and amino acid export. Currently, more than 1·8 × 10⁶ t of amino acids are produced annually with this bacterium, and it is clear that specific mechanisms must exist for amino acids to pass through the cell wall and its lipid layers. We succeeded in identifying specific exporters for L-lysine (Vrljic et al., 1996), L-threonine (Simic et al., 2001) and L-isoleucine (Kennerknecht et al., 2002), each representing a new functional and structural type of transporter (Vrljic et al., 1999; Eggeling & Sahm, 2003). The L-lysine exporter and its subfamilies are widely distributed in a large number of...
bacteria. The native physiological function of the L-lysine export system of C. glutamicum is the sensing of intracellular concentrations of either L-lysine or L-arginine by the regulator LysG, with subsequent induction of lyeE, the exporter gene, at an elevated intracellular concentration of about 20–30 mM to prevent toxic accumulation of these basic amino acids (Bellmann et al., 2001). L-GLutamate has been manufactured for almost 50 years using C. glutamicum, and, with an annual production of 1·2·10⁶ t, this amino acid represents by far the greatest fraction produced from this bacterium. Nevertheless, the efflux of L-glutamate remains the least understood. The reason for this is that specific treatments are necessary for L-glutamate excretion, e.g. addition of detergents, biotin limitation or a temperature upshift (Uy et al., 2003). Consequently, a number of diverse hypotheses exist to explain L-glutamate excretion by C. glutamicum (Hoischen & Krämer, 1990; Eggeling et al., 2001; Kimura, 2003). In addition, no exporter for L-glutamate export has been identified to date.

We report here that EMB treatment results in L-glutamate efflux of C. glutamicum, and we also report on genome-wide consequences of gene expression caused by the addition of EMB, which is discussed in the context of the mode of action of EMB in M. tuberculosis.

METHODS

Plasmids and strains. The strains and plasmids used are listed in Table 1. To achieve overexpression of emb, plasmid pEKEx2emb was constructed. For this purpose, emb was amplified using the primers 5′-AAGGAGATATAAGTGTAGTTGAGTCGAAGA-3′ and 5′-CCGGAATTCCGAGTACTGATCAAGGCTT-ATT-3′, with a ribosome-binding site attached to the primer complementary to the start of emb and an EcoRI site attached to the primer complementary to the end of emb using KOD Hot Start DNA Polymerase (Novagen). The PCR product was cleaved with EcoRI, and ligated into EcoRI- and EcoCRI-cleaved pEKEx2.

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>E. coli</td>
<td>Wild-type</td>
<td>Culture collection</td>
</tr>
<tr>
<td>DH5αMCR</td>
<td>Wild-type with chromosomal emb promoter exchanged by Ptet</td>
<td>This work</td>
</tr>
<tr>
<td>C. glutamicum ATCC 13032</td>
<td>C. glutamicum expression vector with Ptac</td>
<td>Schäfer et al. (1994)</td>
</tr>
<tr>
<td>13032::Ptet-emb</td>
<td>Shuttle vector, Kanª oriVc, oriVcB</td>
<td>Eikmanns et al. (1991)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td>Cremer et al. (1990)</td>
</tr>
<tr>
<td>pK19mobsacB</td>
<td>Exchange vector, Kanª oriVc, oriT sacB</td>
<td>This work</td>
</tr>
<tr>
<td>pEKEx2</td>
<td>C. glutamicum expression vector with Ptac</td>
<td>Schäffer, Res. Centre Jülich</td>
</tr>
<tr>
<td>pIC1</td>
<td>Shuttle vector, Kanª oriVc, oriVcE, emb</td>
<td>This work</td>
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<tr>
<td>pEKEx2emb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK19mobsacB-Ptet</td>
<td>Exchange vector with tetA promoter flanked by clpP1P2 sequences</td>
<td>S. Schaffer, Res. Centre Jülich</td>
</tr>
<tr>
<td>pIC1-Pgap-tetR</td>
<td>pIC1 tetR modified by an integration of Pgap upstream of tetR</td>
<td>S. Schaffer, Res. Centre Jülich</td>
</tr>
<tr>
<td>pK19mobsacB-Ptet-emb</td>
<td>Exchange vector with tetA promoter flanked by upstream and downstream regions of emb promoter</td>
<td>This work</td>
</tr>
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</table>

To enable chromosomal exchange of the emb promoter region with the controllable tet promoter, the vector pK19mobsacB-Ptet-emb was made, containing the tet promoter flanked by upstream and downstream sequences of the emb promoter. The upstream fragment was amplified using the primer pair Ptet_up_XmaI (5′-TCCCCCCC-GGGGGGAACGCTGTGCAGACGACAC-3′; with an XmaI site attached to the 5′ end) and Ptet_up_NsiI (5′-AACCTCGACAC-CATGCTATTGGTTGAGAACTCATTGT-3′; with an NsiI site attached to the 5′ end). The downstream fragment was amplified using the primer pair Ptet_down_Xhol (5′-CCGCTCG-AGGGATCAACTGACTGATCAAGGCTT-3′; with an Xhol site attached to the 5′ end) and Ptet_down_NsiI (5′-GCTCTAGACGGGAAACATCACGTCAGAGGTAC-3′; with an Xhol site attached). Both fragments were subsequently ligated with pK19mobsacB::Ptet, which was digested using the restriction sites endowed to the fragments to eventually yield pK19mobsacB-Ptet-emb.

Genomic mutations. To replace Pemba with Ptet, the plasmid pK19mobsacB-Ptet-emb was introduced into C. glutamicum by electroporation. Recombinants with the integrated vector used 15 μg kanamycin ml⁻¹. Positive selection for the loss of vector in a second round of homologous recombination was by growth in the presence of 10 % sucrose. Finally, clones were analysed for successful exchange using PCR and two different sets of primer pairs. The resulting strain was transformed with pJC1-Pgap-tetR (in the presence of 200 ng anhydrotetracycline ml⁻¹) to provide the Tet repressor, and to yield 13032::Ptet-emb(pJC1-Pgap-tetR), in which emb expression is under control of TetR.

Cultivation conditions. Pre-cultures of C. glutamicum were grown on complex medium CGXII, and growth experiments were done using salt medium CGXII containing 4 % (w/v) glucose and 30 mg protocatechuic acid l⁻¹ in 50 ml Erlemeyer baffled flasks with shaking at 120 r.p.m. (Keilhauer et al., 1993). EMB was added after sterilization as an aqueous solution. Where appropriate, C. glutamicum was cultured with kanamycin at a concentration of 25 μg ml⁻¹. After transformation, a lower concentration of kanamycin (15 μg ml⁻¹) was used. Growth was followed as optical density (OD600) of appropriately diluted culture samples.

To cultivate 13032::Ptet-emb(pJC1-Pgap-tetR), a pre-culture was grown in BHI (brain heart infusion; Difco) containing 200 ng anhydrotetracycline ml⁻¹. After 24 h, aliquots were harvested, washed with 0·9 % NaCl, resuspended in CGXII, and used for the
inoculation of a CGXII culture to an OD<sub>600</sub> of 1.0. After 24 h, this culture, without anhydrotetracycline, was used to inoculate a second CGXII culture without washing. After 24 h, this procedure was repeated. Only in the third CGXII culture was the anhydrotetracycline-dependent growth clearly detected.

Strain 13032(pEKE2x2mb), and the control strain containing the empty plasmid, were pre-cultivated in CGIII without induction. After washing with 0.9% NaCl, the CGXII culture (containing 0.1 mM IPTG) was inoculated to give a starting OD<sub>600</sub> of 0.5.

**Electron microscopy.** Cells were grown on the salt medium CGXII, either with or without 100 mg EMB l<sup>−1</sup>. At an OD<sub>600</sub> of about 10, 1 ml cells were pelleted, and resuspended in 0.1 M sodium phosphate buffer, pH 7.2, containing 3% glutaraldehyde. The cells were embedded in 3% agarose, followed by fixation with 1% OsO<sub>4</sub>, water removal, and embedding in epoxy resin. Ultrathin sections were placed on a copper grid, stained with uranyl acetate and lead citrate, and inspected with a Philips EM 400 transmission electron microscope.

**Biochemical analysis.** To quantify arabinose and galactose in cell wall mycolinolactan, cultures with and without EMB (50 μg ml<sup>−1</sup>) were grown for 4 h, then 1 μCi ml<sup>−1</sup> (37 kBq ml<sup>−1</sup>) [<sup>14</sup>C]glucose (230 mCi mmol<sup>−1</sup>; 9-25 GBq mmol<sup>−1</sup>; AP Biotech) was added, and after 4 h cultivation, the cells were harvested. They were immediately disrupted by sonication, followed by preparation of the insoluble cell wall mycolyl-arabinogalactan-peptidoglycan complex as described by Westphal & Jann (1965). Briefly, the disrupted cell extract was harvested by centrifugation and treated with 2% SDS in PBS (0.1 M K<sub>2</sub>HPO<sub>4</sub>, 0.01 M NaCl, pH 7.4) at 95 °C for 1 h. After centrifugation, the pellet was washed with water, acetone/water (80:20, v/v) and acetone, and allowed to dry. An aliquot was resuspended in 400 μl M trifluoroacetic acid and incubated for 3 h at 120 °C. After drying under nitrogen, the hydrolysed residue was resuspended in water (1 ml) and chloroform (1 ml), sedimented by centrifugation, and the upper aqueous phase was recovered, dried, and finally resuspended in 100 μl water. An aliquot (50 000 c.p.m.) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F254; Merck), developed in pyridine/ethyl acetate/glacial acetic acid/water (3:1:3:1, by vol.). Autoradiograms were produced by 2–3 days’ exposure to Kodak X-Omat AR film to reveal <sup>14</sup>C-labelled sugars, and they were compared with known standards.

For lipid analysis, cells grown on CGXII plus 5 μCi (185 kBq) [<sup>14</sup>C]acetate ml<sup>−1</sup> (62 mCi mmol<sup>−1</sup>; 2-3 GBq mmol<sup>−1</sup>; Amersham) were used, which were harvested, washed and freeze-dried. Free lipids were extracted as described by Gande et al. (2004), and the de-lipidated extracts were used to release and isolate the bound lipids. Aliquots from each strain were subjected to TLC using silica gel plates (5735 silica gel 60F254; Merck), either developed in CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O (60:16:2, by vol.) for the free lipids, or in petroleum ether/acetone (95:5, v/v) for the bound lipids. Autoradiograms were produced by 2–3 days’ exposure to Kodak X-Omat AR film to reveal <sup>14</sup>C-labelled lipids, <sup>14</sup>C-labelled fatty acid methyl esters (FAMES) and <sup>14</sup>C-labelled mycolic acid methyl esters (MAMES), which were compared with known standards (Puech et al., 2000, 2001; Gibson et al., 2003).

**Preparation of total RNA.** Total RNA was prepared as described previously (Wendisch et al., 2001) from control and EMB cultures (25 ml) treated at OD<sub>600</sub> 1.0, which were harvested in the exponential and early-stationary growth phases. The quality of RNA was analysed by UV spectrophotometry and denaturing formaldehyde agarose gel electrophoresis. The prepared RNA was stored at −70 °C until use.

**DNA microarray analyses.** The generation of whole-genome DNA microarrays, synthesis of fluorescently labelled cDNA from total RNA, microarray hybridization, washing and data analysis were performed as described previously (Ishige et al., 2003; Polen & Wendisch, 2004). Genes that exhibited significantly changed mRNA levels (P<0.05 in a Student’s t-test) by a factor of at least three were determined in two series of DNA microarray experiments. Series one consisted of six comparisons of the wild-type cultivated in CGXII with or without 500 μg EMB ml<sup>−1</sup> harvested in the exponential growth phase after 5–6 h at fixed OD<sub>600</sub> whereas series two consisted of 14 comparisons of the wild-type cultivated either with or without EMB, but harvested at fixed times (0, 2, 4, 6, 8, 10, 15 and 25 h).

Genes that were differentially expressed in at least one of these experiments were tabulated. If one of the genes identified belonged to a putative operon, the operon genes were also added to a table consisting of a total of 107 genes. For those 76 genes that exhibited detectable signals in more than 3/4 of the experiments, a hierarchical cluster analysis was made as described (Eisen et al., 1998; Polen & Wendisch, 2004).

**RESULTS**

**EMB inhibits growth**

*C. glutamicum* is a fast-growing bacterium which reaches an OD<sub>600</sub> of 53 on salt medium CGXII supplemented with 220 mM glucose. This is equivalent to approximately 14 g dry wt l<sup>−1</sup> of cells within 30 h, at a maximal growth rate of 0.33 h<sup>−1</sup> (Fig. 1a). At a concentration of 10 mg l<sup>−1</sup>, EMB reduced growth rate and yield. Interestingly, the growth of *C. glutamicum* over a wide range of EMB concentrations (up to 500 mg l<sup>−1</sup>) was almost identical. We also observed a decreased foaming of EMB-treated cultures, and we detected proteins in supernatants analysed at the end of cultivations that were absent in untreated cultures. These were, in addition to the mycolyltransferases CmytA and CmytC (Brand et al., 2003; De Sousa-D’Auria et al., 2003), a putative secreted protein (NCg11761), a carbohydrate hydrolase (NCg1255) and an enolase (NCg0935), with the latter present in a conserved cluster of hypothetical lipo-exported proteins. In addition, proteins such as phosphoglycerate kinase (NCg1525), catalase (NCg0251), methylisocitrate synthase (NCg2450) and chorismate synthase (NCg1561) were clearly visible, which are indicative of partial cell lysis.

**EMB results in L-glutamate efflux**

Despite the lower cell densities obtained in cultures that received EMB, the carbon source glucose was totally consumed at the end of the cultivation (Fig. 1a). We therefore analysed for possible products formed. Glutamate accumulated in concentrations of up to 39 mM at 10 mg EMB l<sup>−1</sup>, and 56 mM at 30 mg EMB l<sup>−1</sup> (Fig. 1b), whereas only traces of L-glutamate were present without the addition of EMB. No further accumulation of L-glutamate occurred even if 500 mg l<sup>−1</sup> EMB was used, and no other amino acids accumulated due to EMB addition (not shown). As derived from the detailed production curves, the maximal excretion rate of 15–20 nmol glutamate min<sup>−1</sup> (mg dry wt)<sup>−1</sup> was observed at 32–35 h (Fig. 1b) when cells were in transition.
from exponential to stationary growth, and when a concentration of more than 50 mM glucose was present.

**Resistance is related to emb expression**

*Mycobacterium avium* and *M. tuberculosis* have three *emb* genes, and at least one of these (*embB*) is suggested to be the target of EMB (Escuyer et al., 2001). However, *Corynebacterium* species have only one *emb* gene. This is in accordance with the notion that the genome of *Corynebacterium* species is considered to represent the archetype of *Corynebacterineae*, and has a low frequency of structural alterations and gene duplications (Nakamura et al., 2003). The *emb* gene of *C. glutamicum* was cloned into pEKEx2, and the *emb*-overexpressing recombinant was assayed for EMB sensitivity. As shown in Fig. 2, this rendered the strain no longer susceptible to EMB at the concentration of 15 mg l⁻¹, and no glutamate efflux occurred (data not shown). The MIC (Etest) of EMB for the wild-type was 0·75 µg ml⁻¹, and for the overexpressing strain it was 1·5 µg ml⁻¹. Direct proof of overexpression was obtained by real-time PCR using *emb* cDNA as standards, resulting in a 5·2 ± 0·8-fold increased *emb* transcript in the overexpressing strain. This illustrates that overexpression of the single *emb* gene of *C. glutamicum*, which is more related to the mycobacterial *embC* than to *embA* or *embB*, was sufficient to confer EMB resistance.

**emb expression is related to L-glutamate efflux**

To reduce *emb* expression in *C. glutamicum*, the chromosomal copy was placed under the control of the tetracycline repressor (TetR) of Tn10 (Wissmann et al., 1991). Firstly, the non-replicative exchange vector pK19mobSacBPtet-emb was constructed, and used to exchange Pemb for PtetA. Plasmid pJC1-Pgap-tetR was introduced to provide TetR. The transformation was made in the presence of 200 ng anhydrotetracycline ml⁻¹ to prevent binding of the repressor TetR to PtetA in front of *emb*. The resulting strain 13032::Ptet-emb(pJC1-Pgap-tetR) was cultivated with and without anhydrotetracycline, as described in Methods. As seen in Fig. 4, the absence of anhydrotetracycline resulted in reduced growth. This was accompanied by L-glutamate efflux, which accumulated to 24 ± 7 mM within 48 h. In the presence of anhydrotetracycline, when Ptet is enabled to drive *emb* expression, the L-glutamate concentration was below 1 mM. This shows that regardless of any possible additional effects caused by EMB (Ramaswamy et al., 2000), the effect of EMB on L-glutamate efflux is related to *emb* expression.

**EMB causes altered cell morphology**

Light microscopy showed that *C. glutamicum* cells appeared to be altered when grown in the presence of EMB. In transmission electron microscopy at low magnification (Fig. 4), cells exposed to EMB were more rounded in shape. Moreover, where cells were separating from each other they were connected by a wider electron-translucent region than in the controls. Both morphological changes are indications...
of cell wall alterations. This was even more apparent at higher magnification, where the clear delimitation of cell wall layers was absent upon EMB addition. The pronounced cell wall layers (Puech et al., 2001) of the control are the wide transparent outer layer (WTOL), which is thought to represent the mycolic acid layer, and the small outer opaque layer (SOOL), which is probably dominated by polysaccharides (Brennan & Nikaido 1995).

**EMB results in altered arabinogalactan and mycolic acid content**

To assay for cell wall alterations at the biochemical level, the mycolyl-arabinogalactan complex of *C. glutamicum* was isolated, and its sugar content was analysed (Fig. 5a). In the absence of EMB, roughly equal radiometric intensities were observed for arabinose and galactose. However, in the presence of 50 μg EMB ml⁻¹, a strongly reduced arabinose content was observed. This is in accord with the action of EMB, shown to reduce *emb*-encoded arabinosyltransferase activity in *M. avium*, which transfers arabinose from β-D-Araf-1-monophosphorylpolyprenol to arabinogalactan (Belanger et al., 1996). Other possible alterations of free and bound glycolipids were less pronounced. However, slightly increased amounts of TMM, TDM and GMM (Fig. 5b) were apparent, which was verified in a separate cultivation and extraction experiment (data not shown), as well as a slightly reduced amount of bound mycolic acids.

**Fig. 3.** *emb* expression is required for optimal growth. Growth of strain 13032::Ptet-emb(pJC1-Pgap-tetR) with TetR-dependent *emb* expression is reduced when anhydrotetracycline is absent (▲), whereas it is normal in the presence of the inducer (●).

**Fig. 4.** Morphological changes due to growth of *C. glutamicum* in the presence of EMB. Left, electron micrographs of thin-sectioned cells grown on salt medium without EMB addition. Right, cells received 100 μg EMB ml⁻¹ (see text for details). SOOL, small opaque outer layer; WTOL, wide transparent outer layer. Bars: upper micrographs, 2 μm; lower micrographs, 0-1 μm.
These results are consistent with an EMB-mediated inhibition of arabinogalactan biosynthesis.

Global gene expression response to EMB addition

The physiological and biochemical consequences of EMB addition suggested that major alterations of gene expression occur. To assay for differentially expressed genes, two cultures were grown in parallel on CGXII, with one culture receiving 500 mg EMB l$^{-1}$. Both cultures were grown to an OD$_{600}$ of 4.5, and RNA was prepared and processed; this was repeated twice. In addition, a colour swap, exchanging Cy5-dUTP versus Cy3-dUTP, was performed to obtain statistically significant data. Probes were hybridized to DNA microarrays representing 95.5% of the ORFs of \textit{C. glutamicum}. A total of 77 ORFs showed significantly altered mRNA levels by a factor of two or more ($P<0.05$), with 20 of them exhibiting increased mRNA steady-state levels due to EMB addition. When the threshold level was set to a threefold alteration, 24 ORFs were differently expressed, with eight of them increased. The most strongly upregulated gene (7.9-fold) in this experiment was NCgl2411 (\textit{mepA}), which encodes a putative secreted metalloprotease. In addition to this quantification at equal OD$_{600}$ early after EMB addition, a separate experiment was done where RNA of cultures (with or without EMB) was extracted after identical cultivation times of 2, 4, 6, 8, 10, 15 and 25 h. This procedure yielded 31 additional genes (threefold change) compared with the first analysis, and in most cases this was due to a reduced expression at later time points.

Comparative analysis of the effect of EMB on RNA levels

The hierarchical cluster analysis of the global gene expression patterns in response to EMB addition is shown in Fig. 6. The experiments are shown in columns, and the ORFs in rows. As might be expected, the clustering of the experiments is according to the time points where samples were taken, including those experiments where cells were grown to a constant OD$_{600}$ (which took 5–6.5 h). The closest clustering was at around 4–10 h, indicating that at
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this point in time global gene expression levels remained comparatively unaltered.

The genes strongly upregulated from the beginning of EMB addition are present in cluster 1. There were also weakly upregulated genes present, including NCgl2313, NCgl2314, NCgl2319 and NCgl2315, which encode proteins predicted to be involved in the degradation of protocatechuate; however, a response of these genes has been observed under phosphate limitation (Ishige et al., 2003), indicating a rather unspecific effect of EMB. For 18 genes, the mRNA levels were strongly increased (up to 21-fold). The characterized genes were phoR and phoS, encoding the two-component system for phosphate sensing and regulation (Ishige et al., 2003), yggB, encoding a mechanosensitive channel (Nottebrock et al., 2003) which is also present in M. tuberculosis (Rv3104c), and mepA, encoding a secreted metalloprotease whose overexpression in Escherichia coli leads to lysis upon an osmotic downshift (Möker et al., 2004). Furthermore, two putative operons were present (NCgl0057, NCgl0059 and NCgl2033, NCgl2034).

Clusters 3 and 4 contained downregulated genes. The strongest response (0-3-fold decrease) was present for NCgl0511 (a permease in an operon with NCgl0509–13), and NCgl1131 and NCgl1466, which both encode putative secreted hydrolases. The known genes of the clusters depicted in Fig. 6 all encode membrane proteins. Furthermore, ORFs NCgl1915–17, proposed to encode components of an oligopeptide uptake system, were also reduced in their expression. Interestingly, in a comparative analysis of global expression changes in E. coli, a high number of membrane proteins exhibited altered expression as a consequence of ampicillin addition (Shaw et al., 2003). Thus, envelope stress might preferentially affect the expression of membrane proteins, which could also be the case for EMB. The cytochrome supercomplex genes, and that of the F_{0}F_{1}-type ATP synthase, are presumably downregulated due to poor growth of the cultures, as described for other organisms (Morita, 1997).

**DISCUSSION**

In the present work, the following effects were demonstrated for C. glutamicum. (i) EMB reduces growth partially, (ii) EMB causes decreased arabinan deposition in arabinogalactan, (iii) EMB reduces mycolic acid content, and (iv) emb overexpression increases EMB resistance. These findings suggest that similar mechanisms of EMB toxicity exist in both C. glutamicum and M. tuberculosis. Although it is clear that the Emb proteins of Mycobacterium species are intimately involved in the processes of cell wall arabinan deposition (Belanger et al., 1996; Zhang et al., 2003), the exact function of the Emb proteins is less clear. The possibilities discussed are that these proteins could be involved in transport of activated arabinose, recognition of the arabinose-containing polymer, and transfer of arabinose to the growing polymer. Unexpectedly, the presumed target of EMB, emb, is not among the genes upregulated. Instead, emb expression shows a weak decrease (Fig. 7), suggesting that the cell does not counteract the effect of EMB by mechanisms providing more mRNA for this gene. Also, there is no obvious regulator present in the C. glutamicum genome, as is the case with embR in M. avium (Belanger et al., 1996).

The putatively secreted acid phosphatase in cluster 1 (NCgl0576) is 21-fold upregulated. Interestingly, upstream in the genome a specialized RNA polymerase sigma subunit (NCgl0575) is present, which was occasionally upregulated in our EMB experiments. This putative operon is similar in sequence and genomic organization in all Corynebacterinae. In M. tuberculosis, this sigma subunit (SigD) is increased by mild cold shock (Manganelli et al., 1999), indicating a stress response of these genes, and confirming, together with their syntenic organization, an ancestral and important function. Since SigD belongs to the so-called ECF sigma factors, and a common feature of this subfamily is regulation of extracytoplasmic functions, its increased expression could be directly related to cell envelope restructuring. There are other genes that are clearly cell-wall-related, like the highly expressed NCgl2411, encoding a putative secreted metalloprotease (mepA). A homologue of this gene is present in M. tuberculosis and E. coli, and in the latter organism is assumed to be involved in stationary-phase survival. Overexpression of the mepA gene of C. glutamicum in E. coli leads to elongated cells that are hypersensitive to an osmotic downshift (Möker et al., 2004), supporting the idea that the protein is involved in peptidoglycan restructuring. This could be similar to the case of NCgl0768, encoding the cell-division ATP-binding protein PtsE, which is involved in transport of septation components across the membrane. Furthermore, there is one gene upregulated, NCgl0394, which encodes a permease belonging to a family of proteins predicted to transport lipids.
targeted to the outer membrane across the inner membrane in an ATP-dependent manner. Some of these genes could link changes to the topology of the cell wall when its arabinogalactan is altered to influence events in the plasma membrane.

Of course we inspected the upregulated genes in particular for carriers which could be candidates for the claimed L-glutamate exporter (Hoischen & Krämer, 1990). Cluster 1 includes the gene for the mechanosensitive ion channel YggB, which has been shown to enable betaine efflux, but this is not known to be involved in glutamate export (Nottebrock et al., 2003). In principle, each permease could be the carrier in demand, requiring inactivation of carrier genes for efflux assays.

With respect to the consequences of EMB addition on the mRNA levels of cytosolic enzymes, it is striking that no genes for enzymes of central metabolism are affected. Only a weak decrease of the odhA and lpd mRNA levels is apparent (Fig. 7); the products of these genes are likely to be constituents of the 2-oxoglutarate dehydrogenase complex competing with glutamate dehydrogenase for the common substrate (Kimura 2003; Uy et al., 2003). This, and the fact that sugar conversion to L-glutamate occurs although growth is retarded (Fig. 1a), means that transcription of genes of central metabolism is rather robust despite a strongly disordered cell envelope. This agrees with similar observations made for M. tuberculosis in response to the addition of antibiotics (Waddell et al., 2004).

There are a number of genes known whose altered expression facilitates L-glutamate efflux. This holds for overexpression of cma, acp, plsC and fadD15 (Nampoothiri et al., 2002), genes of phospholipid biosynthesis, reduced expression of dtsR1 encoding the β-subunit of acetyl-CoA carboxylase (Kimura et al., 1997; Gande et al., 2004), and reduced expression of afr, the alanine racemase gene (Eggelting et al., 2001), as well as ltsA encoding an amidotransferase (Hirasawa et al., 2000). Differential expression of these genes might alter lipid or peptidoglycan composition of the cell envelope. The emb gene represents the first description that an alteration of the arabinogalactan within the cell envelope results in L-glutamate efflux. Nevertheless, it appears that there is no single molecular event directly causing efflux. Instead, the common feature is merely a strongly disordered cell envelope (Eggelting & Sahm, 2001). Since, in addition, a carrier must be present for L-glutamate export (Hoischen & Krämer 1990), we speculate that as a consequence of the disordered cell envelope a carrier becomes activated due to an increased membrane tension or an altered lipid environment (Tillman & Cascio, 2003). Alternatively, the carrier gene could be expressed as a consequence of the obviously disordered cell envelope. In the latter case, comparative genome-wide expression profiles using not only EMB addition, but also the other conditions eliciting L-glutamate efflux, might help to further track the export carrier, and to unravel L-glutamate efflux and production by C. glutamicum.

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