Expression control and specificity of the basic amino acid exporter LysE of Corynebacterium glutamicum

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LysE of Corynebacterium glutamicum belongs to a large new superfamily of translocators whose members are probably all involved in the export of small solutes. Here, the transcript initiation site of lysE, and its divergently transcribed regulator gene, lysG, are identified. Single-copy transcriptional fusions of lysE with lacZ, and titration experiments, show that LysG is the positive regulator of lysE expression enabling its up to 20-fold induction. This induction requires the presence of a coinducer, which is either intracellular L-lysine, or L-arginine. A competition experiment showed that LysE exports these two basic amino acids at comparable rates of about 0.75 nmol min⁻¹ (mg dry wt)⁻¹. Although L-histidine and L-citrulline also act as coinducers of lysE expression, these two amino acids are not exported by LysE. As is evident from the analysis of a lysEG deletion mutant, the physiological role of the lysEG system is to prevent bacteriostasis due to elevated L-lysine or L-arginine concentrations that arise during growth in the presence of peptides or in mutants possessing a deregulated biosynthesis pathway. C. glutamicum has additional export activities other than those of LysE for exporting L-histidine, L-citrulline and L-ornithine.

Keywords: carrier, transcriptional regulator, LTTR, basic amino acid export, peptide hydrolysis

INTRODUCTION

We are engaged in studying the physiology of Corynebacterium glutamicum, which is used industrially for the production of amino acids (Leuchtenberger, 1996). Together with the genera Mycobacteria and Nocardia, this bacterium belongs to the CMN group of the Gram-positive eubacteria. About 4 x 10⁷ tons per year of L-lysine are produced using C. glutamicum. Lysine synthesis has therefore been studied thoroughly, and its regulation is sufficiently well understood that it can be overcome in order to achieve production (Cremer et al., 1991; Eggeling et al., 1996). Surprisingly, the extracellular accumulation of L-lysine is determined not only by the properties of its biosynthetic pathway, but also by the properties of its efflux from the cell (an active process driven by the membrane potential) (Bröer & Krämer, 1991). We were able to isolate mutants with modified efflux (Vrljić et al., 1995) and thus we succeeded in cloning lysE, the gene encoding the L-lysine export carrier (Vrljić et al., 1996).

Studies on the physiological function of lysE showed that, in the absence of the carrier, L-lysine can reach an intracellular concentration of more than 1100 mM, which prevents cell growth. Therefore, in addition to the synthesis of this amino acid, its export apparently also represents a possible means of regulating its intracellular concentration. Such a mechanism, not previously known in bacteria, is necessary in the wild-type of C. glutamicum if lysine-containing peptides are present in the environment (Erdmann et al., 1993). Thus, LysE serves as a valve for exporting excess L-lysine that might be harmful to the cell. Nothing is yet known about the specificity of LysE and its expression control.

In addition to its novel function, the carrier LysE also represents a novel type of structure within the membrane proteins (Vrljić et al., 1999). It is a rather short polypeptide of 233 amino acyl residues which might span the membrane five times. The carrier is therefore a prototype of a new group of translocators, termed the LysE superfamily.
resistance based on the absence of vector-encoded sacB

measurement of Plasmid pEM1dppc with the chromosomal deletions, double selection was required, which annealing in the chromosome and the integrated vector. For analysis or PCR, respectively, with appropriate primers transformants and transconjugants were analysed by plasmid transformed via electroporation (Liebl et al. 1998) to give pEM1dppclysGElacZ. Plasmid pEM1dppc-lysGsr-F1lacZ was made by deletion of the 739 bp NarI fragment of lysG in pJClaysElacZ, re-ligation, and further processing of the resulting BamHI cassette as described above. By Xhol–Stul digestion of pJClysElacZ, Klenow treatment, and blunt-end ligation, a BamHI cassette was made to generate pEM1dppclysGsr-F1lacZ deleted of 567 nt within lysG. To construct pK18mobbsacBΔlysA, the lysA gene was obtained as a 1-kb PsI fragment from pCT4-1, which was ligated with pUC18. The resulting vector was restricted with DralIII and EcoRV (deletion of the central 881 bp fragment) and re-ligated. The remaining 560 bp fragment of ΔlysA was obtained by restriction with SalI and PsiI and ligated with SalI–PsiI-restricted pK18mobbsacB. Plasmid pK18mobargF2 was made by amplifying a 551 nt internal fragment of argF by using primers to which EcoRI and PsiI restriction sites were attached. The resulting fragment was ligated with EcoRI–PsiI-restricted pUC18, excised by restriction with EcoRI and HindIII, and ligated with EcoR1–HindIII-treated pK18mob. To construct plasmid pEC7lysE, the primers 5’-CTCGAG-AGCCGATCCTCGGATACCTAG-3’ and 5’-GGAGATAGCGCCCATCCACCTGAC-3’ were used to amplify lysE as a 1-kb fragment with attached BamHI sites from plJClaysE, before ligating it with pEC7 (Eikmanns et al., 1991). Overexpression of lysE was verified by increased l-lysine export activity with strain 13032ΔGEl.

METHODS

Bacteria, plasmids and growth conditions. The strains and plasmids used in this work are listed in Table 1. Luria–Bertani (LB) medium was used as a complex medium for E. coli, and also for C. glutamicum in the case of DNA isolation. For the measurement of β-galactosidase activities, C. glutamicum was cultivated on either brain–heart infusion (BHI) medium (Difco) or salt medium CGXII (Keilhauer et al., 1993). To inoculate the latter medium, 1 ml of a 5 ml overnight BHI preculture was used first to inoculate an overnight preculture of 50 ml CGXII, which, after being washed with 50 ml cold CGXII, was finally used to inoculate the main culture. When appropriate, ampicillin (50 μg ml⁻¹), kanamycin (25 or 50 μg ml⁻¹), chloramphenicol (15 μg ml⁻¹) or nalidixic acid (50 μg ml⁻¹) was added. E. coli was grown at 37 °C and C. glutamicum at 30 °C.

Strain and plasmid constructions. The plasmid constructions were made in E. coli DH5αMCR. C. glutamicum was transformed via electroporation (Liebl et al., 1989) or, in the case of vector integration into the chromosome, by intergeneric conjugation with E. coli S17-1 (Schäfer et al., 1990). All transformants and transconjugants were analysed by plasmid analysis or PCR, respectively, with appropriate primers annealing in the chromosome and the integrated vector. For chromosomal deletions, double selection was required, which was done according to Schäfer et al. (1994) using kanamycin resistance for the selection of vector integration, with subsequent selection for loss of vector sequences by sucrose resistance based on the absence of vector-encoded sacB. Clones were checked at each step by kanamycin and sucrose resistance/sensitivity and verified by PCR analyses.

Plasmid pEM1dppc with the lysGElacZ fusion cassette was made by inserting lacZ with its ribosome-binding site obtained as an Asp718–XbaI fragment (3078 bp) from pWit10 (Willeken-Bergmann et al., 1986) into the Rsfl II site of pJChysGE (Vrljic et al., 1996). The resulting lysGElacZ cassette (the BamHI cassette) with a lysElacZ transcriptional fusion at nt 62 of lysE was excised as a BamHI fragment and ligated with BamHI-digested pEM1dppc (Vasićová et al., 1998) to give pEM1dppclysGElacZ. Plasmid pEM1dppc-lysGsrF1lacZ was made by deletion of the 739 bp NarI fragment of lysG in pJClaysElacZ, re-ligation, and further processing of the resulting BamHI cassette as described above. By Xhol–Stul digestion of pJClysElacZ, Klenow treatment, and blunt-end ligation, a BamHI cassette was made to generate pEM1dppclysGsr-F1lacZ deleted of 567 nt within lysG. To construct pK18mobbsacBΔlysA, the lysA gene was obtained as a 1-kb PsI fragment from pCT4-1, which was ligated with pUC18. The resulting vector was restricted with DralIII and EcoRV (deletion of the central 881 bp fragment) and re-ligated. The remaining 560 bp fragment of ΔlysA was obtained by restriction with SalI and PsiI and ligated with SalI–PsiI-restricted pK18mobbsacB. Plasmid pK18mobargF2 was made by amplifying a 551 nt internal fragment of argF by using primers to which EcoRI and PsiI restriction sites were attached. The resulting fragment was ligated with EcoRI–PsiI-restricted pUC18, excised by restriction with EcoRI and HindIII, and ligated with EcoR1–HindIII-treated pK18mob.

RNA analysis. Total RNA was isolated from C. glutamicum clones harboring the plasmids pET22lysE or pET22lysG as described by Börmann et al. (1992). For primer extension experiments, 30 μg RNA was hybridized to 0.5 pmol fluorescein-labelled primer (5’-GAAATTCCTCGTGAC-3’) complementary to vector sequences (Vasićová et al., 1998). Denaturation, annealing and reverse transcription were as described by Peters-Wendisch et al. (1998), and the products were analysed by using an automated laser fluorescence DNA sequencer with sequencing reactions carried out in parallel.

Assay of amino acid export. For the determination of export rates in short-term experiments, pre-grown cells were washed twice with ice-cold CGXII and used to inoculate new CGXII. After growth overnight, the cells were harvested by centrifugation (5000 g, 10 min), washed again (twice with ice-cold CGXII), then amino acid excretion was initiated by resuspending the cells in pre-warmed CGXII containing 2 mM of the appropriate dipeptide (see Results). The resulting cell density (OD600) was 8–10, corresponding to 2.4–3 mg dry weight ml⁻¹. The cells were stirred and incubated at 30 °C. Samples for silica oil centrifugation (Klingenberg & Pfaff, 1977) were taken after 1.5 min and then every 15 min for a period of 1.25 h. The procedures for deriving cellular and extracellular fractions and for the quantification of the amino acids as their o-phthalaldehyde derivatives via HPLC were as described by Bröer & Krämer (1991).

Determination of the specific β-galactosidase activity. For the determination of the specific β-galactosidase activity, pre-grown cells (BHI, Difco) were transferred into BHI medium and cultivated for 4 h at 30 °C. After the cells had been harvested by centrifugation (5000 g, 10 min), they were washed with ice-cold 0.1 M potassium phosphate buffer, pH 7.0, centrifuged (5000 g, 10 min), resuspended in 1 ml β-galactosidase reaction buffer (5 mM Tris, pH 7.5; 5% glycerol; 10 mM KCl) and disrupted by sonication in the same buffer. After pelleting of the cellular debris by centrifugation

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Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>Escherichia coli</td>
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<tr>
<td>DH1sMCR</td>
<td>Cloning strain</td>
<td>Grant et al. (1990)</td>
</tr>
<tr>
<td>S17-1</td>
<td>Mobilizing donor strain</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>Corynebacterium glutamicum</td>
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<tr>
<td>13032AEG</td>
<td>Wild-type deleted of the Stul–MluI fragment of lysEG</td>
<td>Vrljić et al. (1996)</td>
</tr>
<tr>
<td>13032AEG::lysGE'lacZ</td>
<td>Deletion mutant of lysGE'lacZ cassette integrated at the dppc site</td>
<td>This work</td>
</tr>
<tr>
<td>13032AEG::lysG'stat::XhoE'lacZ</td>
<td>Deletion mutant with cassette deleted of 739 nt of lysG</td>
<td>This work</td>
</tr>
<tr>
<td>13032AEG::lysG'stat::XhoE'lacZ</td>
<td>Deletion mutant with cassette deleted of 567 nt of lysG</td>
<td>This work</td>
</tr>
<tr>
<td>13032::argF</td>
<td>Wild-type with argF disrupted by pK18mobargF&lt;sub&gt;int&lt;/sub&gt;</td>
<td>Wang et al. (1996)</td>
</tr>
<tr>
<td>MH20-22B</td>
<td>Lysine producer, lysC(Fbr)</td>
<td>Schrumpf et al. (1992)</td>
</tr>
<tr>
<td>MH20-22B::lysGE'lacZ</td>
<td>Producer with lysGE'lacZ cassette integrated</td>
<td>This work</td>
</tr>
<tr>
<td>LE4</td>
<td>Isogenic to MH20-22B, but wild-type lysC</td>
<td>Schrumpf et al. (1992)</td>
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<tr>
<td>LE4::lysGE'lacZ</td>
<td>LE4 with lysGE'lacZ cassette integrated</td>
<td>This work</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pEM1dppc</td>
<td>Vector with dppc integration site</td>
<td>Vasićová et al. (1998)</td>
</tr>
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<td>pEM1dppclysGE'lacZ</td>
<td>pEM1dppc with lysGE'lacZ cassette</td>
<td>This work</td>
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<tr>
<td>pEM1dppclysG'stat::XhoE'lacZ</td>
<td>pEM1dppc with cassette deleted of the 739 nt NarI fragment</td>
<td>This work</td>
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<tr>
<td>pEM1dppclysG'stat::XhoE'lacZ</td>
<td>pEM1dppc with cassette deleted of the 567 nt Stul–Xhol fragment</td>
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<tr>
<td>pEC7</td>
<td>Shuttle vector, Cm&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Eikmanns et al. (1991)</td>
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<td>pEC7/lysE</td>
<td>pEC7 carrying lysE as a 106 kb BamHI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pK18mob sacB</td>
<td>Integration vector, Kmr&lt;sup&gt;B&lt;/sup&gt; ori&lt;sub&gt;V&lt;/sub&gt;.ori&lt;sub&gt;T&lt;/sub&gt;, sacB</td>
<td>Schäfer et al. (1994)</td>
</tr>
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<td>pJClysGE</td>
<td>pJC1::2.4 kb BamHI insert containing lysG and lysE</td>
<td>Vrljić et al. (1996)</td>
</tr>
<tr>
<td>pET2</td>
<td>Promoter probe vector</td>
<td>Vasićová et al. (1998)</td>
</tr>
<tr>
<td>pETlyphsE</td>
<td>pET2 with 198 bp fragment of ‘GE’</td>
<td>This work</td>
</tr>
<tr>
<td>pETlyphsG</td>
<td>As pETlyphsE, but inverse orientation</td>
<td>This work</td>
</tr>
<tr>
<td>pVWE Ex2</td>
<td>Expression vector, Tet&lt;sup&gt;C&lt;/sup&gt;, lacI&lt;sup&gt;B&lt;/sup&gt;, tac promoter</td>
<td>Wendisch (1997)</td>
</tr>
<tr>
<td>pVWE Ex2'GE'</td>
<td>pVWE Ex2:: 455 bp intergenic fragment of lysEG</td>
<td>This work</td>
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<tr>
<td>pK18mob sacB lysA</td>
<td>pK18mob sacB with lysA deleted of a 560 bp internal fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pK18mob argF&lt;sub&gt;int&lt;/sub&gt;</td>
<td>pK18mob with 551 bp internal fragment of argF</td>
<td>This work</td>
</tr>
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<td>pWitT10</td>
<td>Source of lacZ</td>
<td>Wilcken-Bergmann et al. (1986)</td>
</tr>
<tr>
<td>pCT4-1</td>
<td>Shuttle vector with lysA&lt;sub&gt;argS&lt;/sub&gt;</td>
<td>Cremer et al. (1991)</td>
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</table>

(5000 g, 10 min, 4 °C) the supernatant was immediately used for the enzyme assay. The specific activity is given in U (mg protein)<sup>−1</sup>.

RESULTS

LysG is a positive regulator of lysE transcription

The gene lysG is located upstream of the carrier gene lysE (Fig. 1). The features of lysG, which is divergently transcribed from lysE and whose deduced polypeptide exhibits similarities to LTTR-type regulators of Gram-negative bacteria (Schell, 1993), suggest that it is involved in the control of lysE transcription. To quantify the lysG effects on the expression of lysE, the fusion cassette lysGE'lacZ was made, carrying the putative regulator, as well as the intergenic region, and the carrier gene fused with lacZ. This fusion cassette was integrated downstream of the ppc gene (Eikmanns et al., 1989) into the chromosome of the wild-type derivative 13032AEG, thus providing the gene locus under study in one single copy. With this strain (13032AEG::lysGE'lacZ), a high β-galactosidase activity of 6.49 U mg<sup>−1</sup> was obtained (Fig. 1). When the same fusion cassette was first deleted of most of its lysG sequences by excision of the 739 bp NarI fragment and then integrated into the genome of 13032AEG, a drastically reduced β-galactosidase activity of only 0.33 U mg<sup>−1</sup> was the result. This indicates that the presence of lysG is essential for increased expression of lysE'lacZ. Interestingly, with a separate Stul–Xhol deletion construct in which an apparent helix–turn–helix motif within LysG is still present, we repeatedly quantified a weak, but sig-
significantly reduced, \( \beta \)-galactosidase activity. This could indicate the formation of a truncated peptide still interacting with target sequences.

To obtain further evidence that LysG is required for \( \text{lysE} \) expression, plasmid pVWEx2'GE' was constructed (Fig. 1). On its 455 bp insert, this multicopy plasmid contains the intergenic region between \( \text{lysG} \) and \( \text{lysE} \) which is assumed to contain the LysG binding site. With plasmid pVWEx2'GE' in strain 13032ΔGE::\( \text{lysGE} \), a reduced \( \beta \)-galactosidase activity of 0.27 U mg\(^{-1}\) was the result. Thus the intergenic fragment titrates LysG away. This experiment identifies the intergenic region as its binding site. Determinations of the intracellular amino acid concentrations ensured that in these experiments, in which cells had been grown on complex medium BHI, l-lysine was present in all strains in high concentrations of about 60 mM.

Transcript initiation sites of \( \text{lysE} \) and \( \text{lysG} \)

To define the transcript initiation sites of the regulatory gene and its target gene, a fragment of 198 bp carrying the 70 bp intergenic \( \text{lysG}-\text{lysE} \) region together with the 5' ends of both genes was amplified and cloned into the promoter-probe vector pET2 (Vasićová et al., 1998). As expected, this fragment conferred chloramphenicol resistance on \( \text{C. glutamicum} \) in both orientations through expression of the \( \text{cat} \) reporter gene. The position of the \( \text{lysE} \) and \( \text{lysG} \) transcript initiation sites was localized in several primer-extension experiments, using fluorescein-labelled primers which were hybridized to 30 μg RNA isolated from \( \text{C. glutamicum} \) clones harbouring plasmid pET2P\( \text{lysE} \) or plasmid pET2P\( \text{lysG} \). Two fluorograms of these experiments, together with the sequencing reactions carried out in parallel, are shown in Fig. 2(a, b). This defines \( \text{C}_{1994} \) and \( \text{G}_{969} \) (on the opposite strand) in sequence X96471 as the transcript initiation sites for \( \text{lysE} \) and \( \text{lysG} \), respectively. The two promoters overlap and share a palindromic sequence of 10 bp, labelled ‘ABS’ (for activating binding site) in Fig. 2(c), which could be an activation binding site of LysG. The

\[
\text{lysG} \quad \text{lysE} \quad \beta\text{-Galactosidase}
\]

\[
\begin{array}{ccc}
13032 & \\
13032ΔGE::{\text{lacZ}} & 6.49 \\
13032ΔGE::{\text{lacZ}} & 0.33 \\
13032ΔGE::{\text{lacZ}} & 0.16 \\
13032ΔGE::{\text{lacZ}} & 0.27 \\
\end{array}
\]

\[
\text{Fig. 1. Transcriptional } \text{lysE}^{-}\text{lacZ} \text{ fusions integrated in the chromosome to monitor the effects of } \text{lysG} \text{ on } \text{lysE} \text{ expression. The general arrangement of the locus is given at the top, together with the location of the helix-turn-helix (HTH) motif within } \text{lysG} \text{ and the restriction enzyme sites used for the constructions. The fusions were integrated into the wild-type deleted of its original } \text{lysGE} \text{ locus (13032ΔGE) at the } \text{ppc} \text{ downstream site useful for this purpose (Vasićová et al., 1998). Plasmid pVWEx2'GE' is a multicopy plasmid carrying the intergenic } \text{lysG}^{-}\text{lysE} \text{ region. The } \beta\text{-galactosidase activities are given in U (mg protein)}^{-1} \text{ and were determined after growth of the cells for 5 h on complex medium.}
\]

Intracellular basic amino acids induce \( \text{lysE} \) expression

As already mentioned, the specific \( \beta\)-galactosidase activities presented in Fig. 1 were quantified in strains grown on complex medium BHI. When we determined the activity in strain 13032ΔGE::\( \text{lysGE}\text{'lacZ} \) grown on mineral salts medium CGXII (Keilhauer et al., 1993), a drastically reduced specific activity of only 0.3 U mg\(^{-1}\) was obtained. The marked difference was present at all time points assayed during the growth curve (not shown). Only when grown into the late stationary phase on CGXII, after overnight incubation, did the specific activity increase to 2.8 U mg\(^{-1}\). Therefore, in growing cells, the activity is only about 1/20th of that of cells grown on complex medium (Fig. 1). This indicates, on the one hand, a tight regulation of \( \text{lysE} \) expression and, on the other hand, the involvement of an inducer, possibly an amino acid, present in cells grown on BHI. This is in agreement with the notion that almost all LTTR-type transcriptional activators require an inducer in order to become active (Schell, 1993).

To assay for a specific intracellular increase in amino acids, we used the addition of peptides, some of which are known to be taken up and hydrolysed by \( \text{C. glutamicum} \) (Erdmann et al., 1993). The dipeptides used are given in Table 2. They contained the basic amino acids Lys, Arg, Cit (l-citrulline) and His. The Thr- and Phe-containing peptides served as controls. These peptides were added at a concentration of 0.5 mM to salt medium CGXII, and, after growth of strain 13032ΔGE::\( \text{lysGE}\text{'lacZ} \) for 4 h, intracellular amino acid concentrations were determined by silica-oil centrifuga-
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Fig. 2. Result of the primer-extension analyses and overview of the intergenic lysGlysE region (bp 936–1035 of sequence X96471). In (a), the primer-extension analysis of the lysE transcript is shown, and in (b) that of lysG is presented. The corresponding fluorograms of the sequence determinations are shown, and the bottom curve shows the product of the primer extension. The residues identified on the coding strand of the respective transcriptional start sites are given underneath the fluorograms and are underlined. In the overview (c), the start of the transcript is indicated by a wavy line, and the polypeptides are indicated by the straight lines. The consensus promoter sequences are underlined. Boxed and labelled ‘RBS’ is a dyad element which is the putative recognition binding site of LysG. Boxed and marked ‘ABS’ is a palindrome which is a putative activating binding site involved in the induction of lysE expression.

Table 2. Specific amino acid (aa) increase, as obtained by peptide addition, and the resulting β-galactosidase activities

Peptide was added at a concentration of 0.5 mM, and intracellular determinations, as well as specific enzyme determinations, were performed after growth of the cells for 4 h on mineral salts medium. After 5 h, comparable values were determined.

<table>
<thead>
<tr>
<th>Peptide added</th>
<th>Intracellular aa concentration (mM)</th>
<th>Specific β-galactosidase activity (U mg⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>Peptide-specific aa</td>
<td>Other aa</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Lys–Ala</td>
<td>42 Lys</td>
<td></td>
</tr>
<tr>
<td>Arg–Ala</td>
<td>98 Arg</td>
<td></td>
</tr>
<tr>
<td>Cit–Phe</td>
<td>73 Cit</td>
<td></td>
</tr>
<tr>
<td>His–Ala</td>
<td>18 His</td>
<td></td>
</tr>
<tr>
<td>Thr–Ala</td>
<td>32 Thr</td>
<td></td>
</tr>
<tr>
<td>Ala–Phe</td>
<td>4 Phe</td>
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</tbody>
</table>

...with Lys–Ala) were at concentrations below 1 mM (not shown). A high β-galactosidase activity, elevated 11–18-fold relative to the controls, was present when the concentration of Lys, Arg, Cit or His was increased. This is evidence that these basic amino acids might serve as inducers of lysE expression. With the strain carrying the NarI deletion construct of lysGE'lacZ' in its chromosome (Fig. 1) being used as a control, the specific β-galactosidase activities in cells grown under identical
conditions to those given in Table 2 were 0.2–0.3 μg ml⁻¹. This clearly indicates that LysG is required, together with one of the four basic amino acids, for lysE expression, and that no additional regulator, other than LysG, is present in the cell able to interact with Lys, Arg, His or Cit and capable of binding upstream of the fusion.

To address the question of the intracellular level of L-lysine required for induction, we used different Lys–Ala concentrations. The highest specific β-galactosidase activity (7.2 U mg⁻¹) was present upon the use of 0.75 mM peptide containing the extremely high intracellular L-lysine concentration of 225 mM (not shown). Note that at 42 mM L-lysine, the activity was already 5.5 U mg⁻¹ (Table 2). However, attempts to correlate expression levels with the intracellular L-lysine concentration failed, since the intracellular concentration is not freely selectable in the course of an experiment (Erdman et al., 1993). We therefore used an entirely different approach to assay lysine-dependent lysE expression. For this purpose we used a pair of strains of C. glutamicum differing only in their aspartate kinase activity, which is the key enzyme of cellular L-lysine synthesis (Cremer et al., 1991). In strain MH20-22B, the aspartate kinase is not feedback-inhibited by L-lysine, and the strain therefore has an elevated intracellular L-lysine concentration (Broer et al., 1993); the isogenic strain LE4, however, carries the feedback-sensitive wild-type aspartate kinase (Schrampf et al., 1992). The lysGE/lac-fusion cassette was introduced into these two strains, which were grown on minimal medium CGXII for the determination of enzyme activities and internal amino acid concentrations. The resulting β-galactosidase activity in strain MH20-22B was 6.1 U mg⁻¹ at a cytosolic L-lysine concentration of 35 mM, whereas the activity in strain LE4 was 0.4 U mg⁻¹ at a cytosolic L-lysine concentration of 0.5 mM. In the cassette-less strains, assayed as controls, almost identical L-lysine concentrations were determined. This shows that intracellularly synthesized L-lysine also induces lysE expression and that a concentration of 35 mM L-lysine is already sufficient to obtain a 20-fold induction.

**LysE exports L-arginine and L-lysine**

lysE was isolated as the gene encoding the L-lysine export carrier (Vrljić et al., 1996). In view of the finding that lysE is induced by several basic amino acids, we studied the specificity of LysE in terms of its catalytic activity, and first focused on L-arginine as a possible exported cytoplasmic solute. In a growth experiment the behaviour of wild-type strain 13032 and strain 13032ΔEG was studied. When 2 mM of the peptide Arg–Ala was added to the mutant, after an initial doubling, growth was arrested, which was not the case with the wild-type (Fig. 3). Determination of the intracellular L-arginine concentration revealed its very high accumulation (up to 823 mM) relative to the wild-type (245 mM). These characteristics are in accord with the known growth-inhibiting effects of high intracellular L-lysine concentrations (Vrljić et al., 1996). It illustrates that LysE also exports L-arginine, but does not exclude induction of a second exporter during the growth experiment. However, when the export activity was quantified over a 1 h period directly after inoculation, the efflux rate for L-arginine in the deletion mutant was below 0.01 nmol min⁻¹ (mg protein)⁻¹ (not shown). This is of the order of the efflux mediated by passive diffusion (Vrljić et al., 1996) and thus shows that, under these conditions, LysE is the only relevant system for L-arginine and L-lysine export present in C. glutamicum.

A more direct approach for proving the specificity of LysE towards L-arginine and L-lysine was to apply a competition experiment, i.e. measure the efflux when these two amino acids were present at comparable concentrations in the cell. To achieve this metabolic situation, we carried out a set of short-term experiments.
with the *C. glutamicum* wild-type by using different mixtures of Lys–Ala and Arg–Ala, in which the l-lysine and l-arginine content of the peptide mixture was varied systematically (not shown). At concentrations of 272 mM Lys–Ala and 128 mM Arg–Ala, after a 1 h incubation period we obtained an equal intracellular concentration of the two basic amino acids. Under these conditions, the intracellular l-lysine and l-arginine concentrations were increased to 34 and 38 mM (Fig. 4a), respectively, and the kinetics of the intracellular and extracellular amino acid concentrations was monitored. The time-courses of the extracellular accumulation of l-lysine and l-arginine were almost identical (Fig. 4b). The export rates calculated from these data were 0.83 nmol min⁻¹ (mg dry wt)⁻¹ for l-lysine, and 0.70 nmol min⁻¹ (mg dry wt)⁻¹ for l-arginine.

**L-Histidine and l-citrulline are exported by transport systems different from LysE**

We were also interested in studying whether His and Cit are also exported by LysE. Similarly to above, we used short-term experiments to compare the *C. glutamicum* wild-type with the deletion mutant 13032ΔEG. We found that the addition of 2 mM His–Ala resulted in comparable export of l-histidine by both strains (Fig. 5a). Without the addition of His–Ala, no amino acid excretion occurred (not shown). This shows, on the one hand, that l-histidine can in fact be exported by *C. glutamicum* if present at high intracellular concentrations, but, on the other hand, that LysE is not involved in the export of this amino acid. Although it could be argued that in the wild-type LysE exports His, and that in the deletion mutant a second exporter takes over the role of LysE, the simplest explanation of this experiment is that LysE does not accept l-histidine as a substrate. This is a remarkable result in view of the fact that we have shown that l-histidine might act as an inducer of LysG (Table 2). However, a similar situation exists for the LAO uptake system of *E. coli* (Wissenbach *et al.*, 1995). Here it was shown that the system is induced by l-lysine, l-arginine and l-ornithine, but that it is only able to take up l-lysine and l-ornithine. The calculated export rate for l-histidine of *C. glutamicum* is 1.4 nmol min⁻¹ (mg dry wt)⁻¹. Furthermore, we tested a Cit–Phe dipeptide in this kind of assay. Under similar experimental conditions, an intracellular accumulation of about 150 mM l-citrulline was obtained. Compared to the *C. glutamicum* wild-type, however, the *lysE* deletion mutant did not exhibit reduced l-citrulline export.

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**Fig. 4.** Competition for l-lysine and l-arginine export in the wild-type. (a) Because of the addition of 272 mM lysyl-alanine and 128 mM argyl-alanine, comparable intracellular concentrations of l-lysine (●) and l-arginine (○) are obtained. (b) The extracellular accumulation for both amino acids is almost identical. ●, l-Lysine; ○, l-arginine.

**Fig. 5.** (a) Intracellular (circles) and extracellular (squares) l-histidine concentrations in 13032 and its deletion mutant, 13032ΔEG, in the presence of 2 mM histidy-alanine. Open symbols represent the deletion mutant, and solid symbols represent the wild-type. (b) Intracellular (squares) and extracellular (circles) l-ornithine concentrations in 13032 with disrupted argF, along with those in the same strain with overexpressed *lysE*. The open symbols represent the strain with overexpressed *lysE*, and the solid symbols represent the control.
export, and efflux in the range observed for l-histidine occurred (not shown).

Other structurally related basic amino acids which could be transported by LysE are DL-diaminopimelate and L-ornithine. However, specific peptides were not available (as in the case of DL-diaminopimelate), or they were found not to be hydrolysed (as in the case of Orn–Orn and Orn–Asp). Therefore, we disrupted argF, the gene encoding the ornithine carbamoyltransferase, in the chromosome of the wild-type (see Methods). This resulted in a mutant requiring L-arginine for growth and which accumulated 60 mM L-ornithine in the medium within 24 h (not shown). This argF mutant was transformed with pEC7lysE, overexpressing lysE. In short-term fermentation experiments, the wild-type (not shown). The ornithine export rate was 0.6 nmol min⁻¹ (mg dry wt)⁻¹ and was not increased by overexpressing lysE. We therefore conclude that LysE is not responsible for L-ornithine export, but that the L-ornithine export of C. glutamicum is catalysed by a separate exporter. The presence of an L-ornithine exporter is in accord with known producer strains of C. glutamicum for this amino acid (Choi et al., 1995).

In a similar approach using chromosomal gene inactivation, we derived a strain suitable for assaying for the capacity of LysE to accept intracellular DL-diaminopimelate as a substrate. A C. glutamicum lysA mutant preventing the decarboxylation of DL-diaminopimelate to L-lysine was constructed. This mutant accumulated up to 1600 mM DL-diaminopimelate in the cytoplasm (results not shown). However, in the medium, only concentrations of about 3 mM accumulated within 2 d, with the wild-type and with the lysE-overexpressing strain as well. This shows that LysE does not accept the basic amino acid DL-diaminopimelate as a substrate. Furthermore, it strengthens the belief that the basic amino acids have an extremely low permeability via the lipid bilayer of C. glutamicum.

**DISCUSSION**

The functional data obtained with the lacZ fusions in which most of lysG was deleted, and those obtained with plasmid pVWEEx2'GE' titrating LysG away such that it was unable to bind to the lysE lacZ promoter are in full accord with the requirement of the LysG protein for the induction of lysE expression. This establishes LysG as a positive regulator of lysE transcription. Sequence identities of LysG, its gene arrangement with respect to its target gene, and additional features of the LysG interacting sites (see below) identify LysG of C. glutamicum as an LTTR-type regulator (Schell, 1993).

The identification of the transcript initiation sites of lysG and lysE locate the perfect palindromes (AAC-TAC-\*TTAAGTA-) directly in the middle of the intergenic regions where both promoter regions overlap (Fig. 2). This palindrome probably represents the activating binding site characteristic of LTTR-regulated genes. A second dyad element present is the recognition binding site (RBS), which was selected by analogy to other LTTR systems (McFall et al., 1998). Both elements share the inverted repeats -TAAGT- and -TACTT- within or directly adjacent to them; these could represent DNA contact sites for LysG protein. The transcription initiation sites identified allow us to locate the translation initiation site of the LysE polypeptide more precisely. It shifts the initiating methionine that we previously inferred from the sequence further downstream to the following methionine, which is only three aminoacyl residues away at nt 1025–1027 (Vrljic et al., 1996).

As inducers of lysE for mediating transcriptional activation by LysG, L-lysine and L-arginine, as well as L-histidine and L-citrulline, were identified. One of the very few LTTR regulators for which an attempt has been made to quantify the intracellular inducer concentration is NhaR. This regulator controls the synthesis of the Na⁺/H⁺ antiporter of E. coli. It is known that it is fully induced at an extracellular Na⁺ concentration of 50 mM (Dover et al., 1997), while the intracellular Na⁺ concentration is around 60 mM (Harel-Bronstein et al., 1995). With the C. glutamicum wild-type derivative carrying the lysE-lacZ fusion, a specific β-galactosidase activity of 5·5 U at a concentration of 42 mM (Table 2) was obtained and an activity of 6·5 U was obtained at an intracellular l-lysine concentration of about 60 mM (Fig. 1). This fits very well with the values obtained with C. glutamicum MH20-22B (5·8 U at 35 mM). Even higher values of 7·2 U were achieved (not shown), but these values were not given consideration since growth was already retarded at the extraordinarily high internal concentration of 225 mM l-lysine. In summary, these data indicate that a comparatively moderate l-lysine concentration of around 30–40 mM is sufficient for almost full induction of lysE. The range of induction obtained is about 20-fold (see Table 2). Such strong control might be required to prevent expression of the export carrier under conditions in which l-lysine is synthesized from glucose, otherwise the viability of the cell would be endangered. In any case, l-lysine and l-arginine, and also l-histidine and l-citrulline, act as inducers (though the latter two amino acids are not exported by LysE). This differential specificity of LysG and LysE is not unexpected, since both proteins, of course, have entirely different structures. A wide range of different inducer structures is known for the LTTR NahR of salicylate-degrading pseudomonads (Cebolla et al., 1997).
substrates (Hillenga et al., 1996). E. coli has at least five importers for basic amino acids, which have different specificities. Three of them exhibit a high substrate affinity. Similarly, the lysine importer of C. glutamicum encoded by lysI (Seep-Feldhaus et al., 1991) has a $K_m$ of 10 $\mu$M for lysine (Bröer & Krämer, 1991). Obviously, a high substrate affinity is reasonable for carriers transporting amino acids into the cell, as it allows the cell to cope with low substrate concentrations in the environment. However, it would be harmful for amino acid exporters, because of the inevitable loss of these metabolites when synthesized from carbohydrates. Accordingly, the $K_m$ of LysE of C. glutamicum for L-lysine is three orders of magnitudes higher (20 mM) than that of the importer (Bröer & Krämer, 1991).

However, it should be borne in mind that in particular metabolic situations, for instance when intracellular L-arginine is low and L-lysine high, co-export of L-arginine by the lysine exporter, which would be active under these conditions, would be disadvantageous for the cell. This scenario possibly explains why argS and lysA in C. glutamicum form an operon (Marcel et al., 1990). The gene argS encodes the arginyl-tRNA synthetase and lysA encodes the D-lysinopimelate decarboxylase, which is the only specific gene of L-lysine synthesis. Both L-arginine and L-lysine control expression of this operon (Oguiza et al., 1993); this might serve to counteract intracellular amino acid imbalances possibly arising from the action of the lysine exporter.

In 1979 and 1982, Payne and coworkers had already observed the formation of amino acids derived from peptides with E. coli and Streptococcus faecalis, respectively (for a review, see Payne & Smith, 1994). Thus, export of amino acids is a general phenomenon and there is now firm evidence that in E. coli and, in particular, in C. glutamicum, distinct transporters catalysing amino acid export are present (Krämer, 1994). For instance, in C. glutamicum export of L-isoleucine and L-threonine (Palmieri et al., 1996) is also a carrier-mediated process, and, as indicated in the present work, further transport systems specific for the export of at least L-citrulline, L-histidine and L-ornithine are present in C. glutamicum. In E. coli, it has been shown that overexpression of rhtB results in increased extracellular accumulation of L-homoserine, and that rhtC overexpression confers resistance to L-homoserine lactone (Zakataeva et al., 1999). It may be assumed, however, that the physiological function of rhtB, as well as the functions of its four paralogues present in E. coli, might be to serve cell-to-cell communication. Consequently, the observed amino acid export in E. coli might be due to limited specificity. Similarly, L-cystine export in E. coli is probably due to limited specificity of the exporter YdeD (Daßler et al., 2000). In contrast, the function of LysE of C. glutamicum is obviously to control the intracellular L-lysine and L-arginine pools. This is in line with the fact that C. glutamicum is unable to use these amino acids for catabolic purposes. In combination with LysG, the whole system is designed to sense and export intracellular L-lysine and L-arginine. The cell is therefore able to respond to imbalances in cytosolic amino acid pools. These might occur both physiologically under particular environmental conditions (during growth in the presence of peptides) as well as during amino acid production.

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