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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 4×10^5 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

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(Genome analysis: comparison of the transport capabilities of several bacteria. M. Saier's transport classification page; http://www.biology.ucsd.edu/~msaier/transport/titlepage.html); members of this large superfamily are present in eubacteria and archaea (Aleshin *et al.*, 1999). They all have a very similar structure, and are possibly all involved in the export of small solutes. Most interestingly, in *Escherichia coli* there are five paralogues present which might also be related to amino acid export. One of the paralogues confers resistance to L-threonine upon overexpression of the corresponding gene (*rhtC*), and another one (*rhtB*) confers resistance to L-homoserine and L-homoserine lactone (Zakataeva *et al.*, 1999). Recently, another exporter of *E. coli* has been identified (*ydeD*), related to the export of cysteine or cysteine-related compounds, but not belonging to the LysE superfamily (Daßler *et al.*, 2000).

Since export mediated by carriers belonging to the LysE superfamily is obviously of relevance both to an understanding of basic microbial functions and to biotechnological applications, here we discuss expression control of *lysE* and the substrate specificity of the carrier.

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 overday 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 *lysGE′lacZ* fusion cassette was made by inserting *lacZ* with its ribosome-binding site obtained as an *Asp*718–*Xba*I fragment (3078 bp) from piWiT10 (Wilcken-Bergmann *et al.*, 1986) into the *Rsr*II site of pJC*lysGE* (Vrljić *et al.*, 1996). The resulting *lysGE′lacZ* cassette (the *Bam*HI cassette) with a *lysE′lacZ* transcriptional fusion at nt 62 of *lysE* was excised as a *Bam*HI fragment and ligated with *Bam*HI-digested pEM1dppc (Vašicová *et al.*, 1998) to give pEM1dppc*lysGE′lacZ*. Plasmid pEM1dppc-$lysG^{NarI}E'lacZ$ was made by deletion of the 739 bp *Nar*I fragment of *lysG* in pJC*lysGE′lacZ*, re-ligation, and further processing of the resulting *Bam*HI cassette as described above. By *Xho*I–*Stu*I digestion of pJC*lysGE′lacZ*, Klenow treatment, and blunt-end ligation, a *Bam*HI cassette was made to generate pEM1dppc$lysG^{StuI-XhoI}E'lacZ$ deleted of 567 nt within *lysG*. To construct pK18mobsacBΔ*lysA*, the *lysA* gene was obtained as a 1·3 kb *Pst*I fragment from pCT4-1, which was ligated with pUC18. The resulting vector was restricted with *Dra*III and *Eco*RV (deletion of the central 881 bp fragment) and re-ligated. The remaining 560 bp fragment of Δ*lysA* was obtained by restriction with *Sal*I and *Pst*I and ligated with *Sal*I–*Pst*I-restricted pK18mobsacB. Plasmid pK18mob$argF_{int}$ was made by amplifying a 551 nt internal fragment of *argF* by using primers to which *Eco*RI and *Pst*I restriction sites were attached. The resulting fragment was ligated with *Eco*RI–*Pst*I-restricted pUC18, excised by restriction with *Eco*RI and *Hin*dIII, and ligated with *Eco*RI–*Hin*dIII-treated pK18mob. To construct plasmid pEC7*lysE*, the primers 5′-CTCGAG-AGCGGATCCGCGCTGACTCAC-3′ and 5′-GGAGAGTA-CGGCCCATCCACCGTGACC-3′ were used to amplify *lysE* as a 1·0 kb fragment with attached *Bam*HI sites from pJC*lysGE*, before ligating it with pEC7 (Eikmanns *et al.*, 1991). Overexpression of *lysE* was verified by increased L-lysine export activity with strain 13032ΔGE.

RNA analysis. Total RNA was isolated from *C. glutamicum* clones harbouring the plasmids pET2P*lysE* or pET2P*lysG* as described by Börmann *et al.* (1992). For primer extension experiments, 30 μg RNA was hybridized to 0·5 pmol fluorescein-labelled primer (5′-GAAAATCTCGTCGAAGC-TCG-3′) complementary to vector sequences (Vašicová *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 (OD_{600}) was 8–10, corresponding to 2·4–3·0 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*-phthaldialdehyde 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

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
Escherichia coli		
DH5αMCR	Cloning strain	Grant *et al.* (1990)
S17-1	Mobilizing donor strain	Simon *et al.* (1983)
Corynebacterium glutamicum		
13032ΔEG	Wild-type deleted of the *Stu*I–*Mlu*I fragment of *lysEG*	Vrljić *et al.* (1996)
13032ΔEG::*lysGE′lacZ*	Deletion mutant with *lysGE′lacZ* cassette integrated at the d*ppc* site	This work
13032ΔEG::$lysG^{NarI}E'lacZ$	Deletion mutant with cassette deleted of 739 nt of *lysG*	This work
13032ΔEG::$lysG^{StuI-XhoI}E'lacZ$	Deletion mutant with cassette deleted of 567 nt of *lysG*	This work
13032Δ*lysA*	Wild-type with 560 bp of *lysA* deleted	This work
13032::*argF*	Wild-type with *argF* disrupted by pK18mob$argF_{int}$	This work
MH20-22B	Lysine producer, *lysC*(Fbr)	Schrumpf *et al.* (1992)
MH20-22B::*lysGE′lacZ*	Producer with *lysGE′lacZ* cassette integrated	This work
LE4	Isogenic to MH20-22B, but wild-type *lysC*	Schrumpf *et al.* (1992)
LE4::*lysGE′lacZ*	LE4 with *lysGE′lacZ* cassette integrated	This work
Plasmids		
pEM1dppc	Vector with d*ppc* integration site	Vašicová *et al.* (1998)
pEM1dppc*lysGE′lacZ*	pEM1dppc with *lysGE′lacZ* cassette	This work
pEM1dppc$lysG^{NarI}E'lacZ$	pEM1dppc with cassette deleted of the 739 nt *Nar*I fragment	This work
pEM1dppc$lysG^{StuI-XhoI}E'lacZ$	pEM1dppc with cassette deleted of the 567 nt *Stu*I–*Xho*I fragment	This work
pEC7	Shuttle vector, Cm^R	Eikmanns *et al.* (1991)
pEC7*lysE*	pEC7 carrying *lysE* as a 1·06 kb *Bam*HI fragment	This work
pK18mobsacB	Integration vector, Km^R $oriV_{E.c.}$oriT, *sacB*	Schäfer *et al.* (1994)
pJC*lysGE*	pJC1::2·4 kb *Bam*HI insert containing *lysG* and *lysE*	Vrljić *et al.* (1996)
pET2	Promoter probe vector	Vašicová *et al.* (1998)
pETP*lysE*	pET2 with 198 bp fragment of '*GE*'	This work
pETP*lysG*	As pETP*lysE*, but inverse orientation	This work
pVWEx2	Expression vector, Tet^R, $lacI^q$, *tac* promoter	Wendisch (1997)
pVWEx2′GE′	pVWEx2::455 bp intergenic fragment of *lysEG*	This work
pK18mobsacBΔ*lysA*	pK18mobsacB with *lysA* deleted of a 560 bp internal fragment	This work
pK18mob$argF_{int}$	pK18mob with 551 bp internal fragment of *argF*	This work
piWiT10	Source of *lacZ*	Wilcken-Bergmann *et al.* (1986)
pCT4-1	Shuttle vector with *lysAargS*	Cremer *et al.* (1991)

(5000 ***g***, 10 min, 4 °C) the supernatant was immediately used for the enzyme assay. The specific activity is given in U (mg protein)$^{-1}$.

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 13032ΔGE, thus providing the gene locus under study in one single copy. With this strain (13032ΔGE::*lysGE′lacZ*), a high β-galactosidase activity of 6·49 U mg^{-1} was obtained (Fig. 1). When the same fusion cassette was first deleted of most of its *lysG* sequences by excision of the 739 bp *Nar*I fragment and then integrated into the genome of 13032ΔGE, a drastically reduced β-galactosidase activity of only 0·33 U mg^{-1} was the result. This indicates that the presence of *lysG* is essential for increased expression of *lysE′lacZ*. Interestingly, with a separate *Stu*I–*Xho*I deletion construct in which an apparent helix–turn–helix motif within LysG is still present, we repeatedly quantified a weak, but sig-

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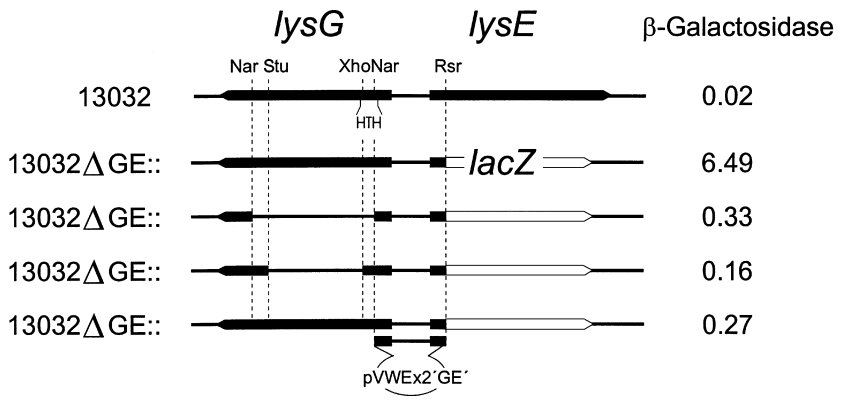

Fig. 1. Transcriptional *lysE′*lacZ fusions integrated in the chromosome to monitor the effects of *lysG* on *lysE* expression. The general arrangement of the locus is given at the top, together with the location of the helix–turn–helix (HTH) motif within LysG and the restriction enzyme sites used for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

nificantly reduced, *β*-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 *lysE* expression, plasmid pVWEx2′GE′ was constructed (Fig. 1). On its 455 bp insert, this multicopy plasmid contains the intergenic region between *lysG* and *lysE* which is assumed to contain the LysG binding site. With plasmid pVWEx2′GE′ in strain 13032ΔGE::*lysGE′lacZ*, a reduced *β*-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 *lysE* and *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 *lysG–lysE* region together with the 5′ ends of both genes was amplified and cloned into the promoter-probe vector pET2 (Vašicová *et al.*, 1998). As expected, this fragment conferred chloramphenicol resistance on *C. glutamicum* in both orientations through expression of the *cat* reporter gene. The position of the *lysE* and *lysG* transcript initiation sites was localized in several primer-extension experiments, using fluorescein-labelled primers which were hybridized to 30 μg RNA isolated from *C. glutamicum* clones harbouring plasmid pET2P*lysE* or plasmid pET2P*lysG*. Two fluorograms of these experiments, together with the sequencing reactions carried out in parallel, are shown in Fig. 2(a, b). This defines C_{1024} and G_{969} (on the opposite strand) in sequence X96471 as the transcript initiation sites for *lysE* and *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 transcript initiation site of *lysE* is in close proximity to the deduced translation initiation site, as inferred from sequence comparisons (Vrljić *et al.*, 1999). Apparently *lysE* belongs to those genes of *C. glutamicum* for which the transcript does not provide a ribosome-binding site (Morbach *et al.*, 2000). This seems to be a more general feature of the *Actinomyces* subdivision of Gram-positive bacteria to which *C. glutamicum* belongs (Strohl, 1992).

Intracellular basic amino acids induce *lysE* expression

As already mentioned, the specific *β*-galactosidase activities presented in Fig. 1 were quantified in strains grown on complex medium BHI. When we determined the activity in strain 13032ΔGE::*lysGE′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 *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 *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::*lysGE′lacZ* for 4 h, intracellular amino acid concentrations were determined by silica-oil centrifu-

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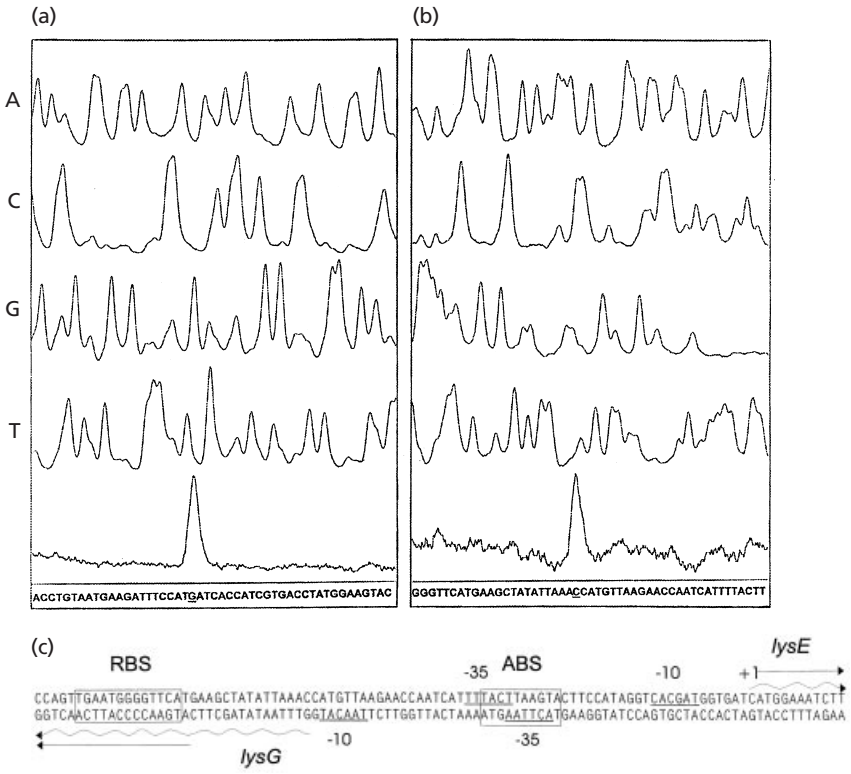

Fig. 2. Result of the primer-extension analyses and overview of the intergenic *lysGlysE* region (bp 936–1035 of sequence X96471). In (a), the primer-extension analysis of the *lysE* transcript is shown, and in (b) that of *lysG* is presented. The corresponding fluorograms of the sequence determinations 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.

Peptide added	Intracellular aa concentration (mM)			Specific β-galactosidase activity (U mg^{-1})
	Peptide-specific aa	Other aa		
		Ala	Phe	
None	–	3	<1	0·3
Lys–Ala	42 Lys	3	<1	5·5
Arg–Ala	98 Arg	4	<1	4·2
Cit–Phe	73 Cit	2	3	4·1
His–Ala	18 His	3	< 1	3·2
Thr–Ala	32 Thr	4	< 1	0·3
Ala–Phe	4 Phe	5	–	0·3

gation. The specific β-galactosidase activities were quantified in extracts made from the same culture. As shown in Table 2, a specific increase in the intracellular Lys, Arg, Cit, His and Thr concentrations was obtained, whereas with Phe no strong increase was present. The Ala concentration was comparable in all strains and was not substantially increased, indicating that it is rapidly metabolized. All the other peptide-specific amino acids quantified (for instance Thr or Phe in the cells supplied 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 *Nar*I 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

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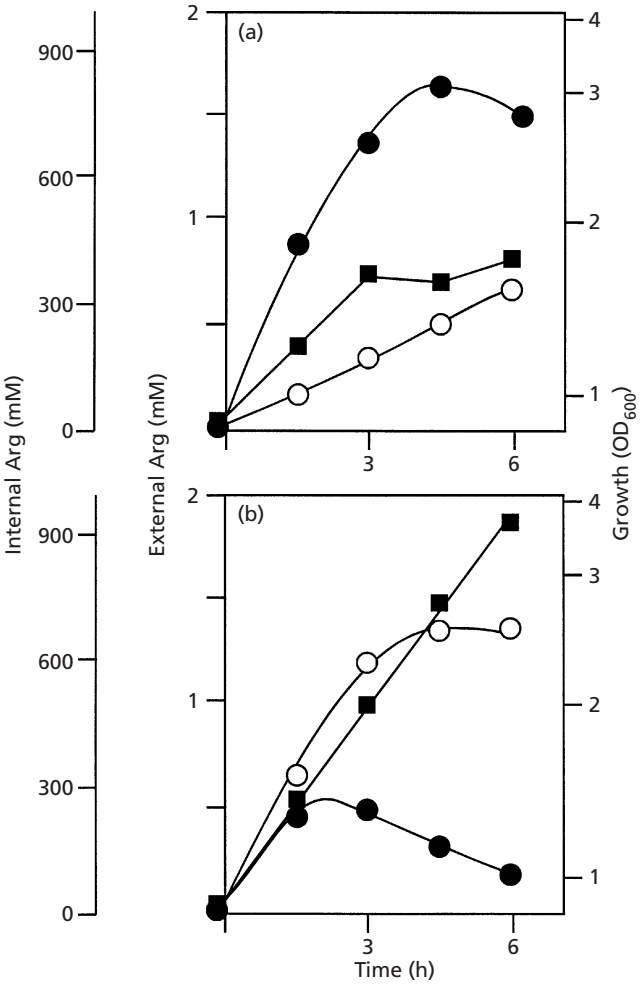

Fig. 3. Characteristics of strains 13032ΔEG (a) and 13032 (b) when incubated with the peptide 2 mM arginyl-alanine. Because of the deletion, intracellular L-arginine (●) accumulates, growth (■) is prevented, and excretion of extracellular L-arginine (○) is strongly reduced.

conditions to those given in Table 2 were 0·2–0·3 U mg^{-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 (Bröer *et al.*, 1993); the isogenic strain LE4, however, carries the feedback-sensitive wild-type aspartate kinase (Schrumpf *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 intial 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

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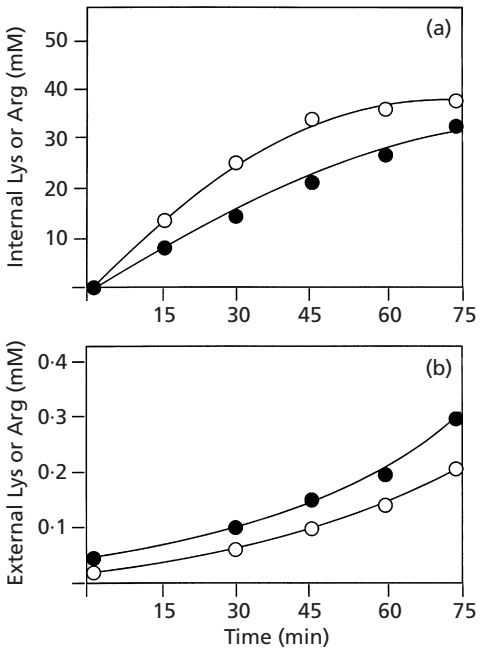

Fig. 4. Competition for L-lysine and L-arginine export in the wild-type. (a) Because of the addition of 2·72 mM lysyl-alanine and 1·28 mM arginyl-alanine, comparable intracellular concentrations of L-lysine (●) and L-arginine (○) are obtained. (b) The extracellular accumulation for both amino acids is almost identical. ●, L-Lysine; ○, L-arginine.

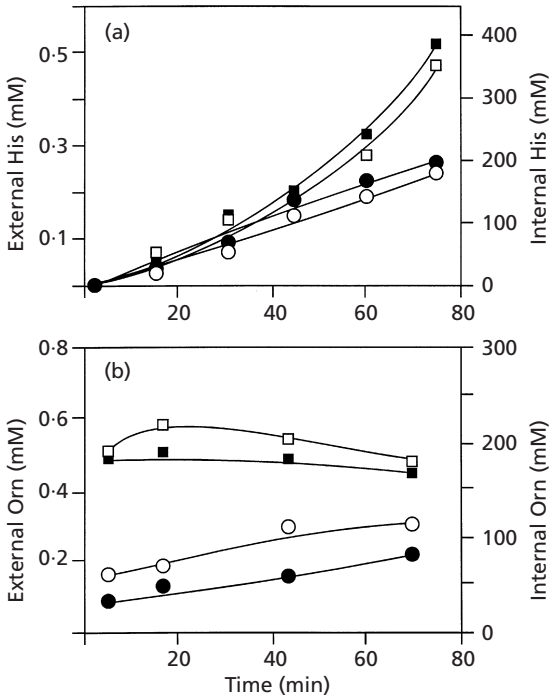

Fig. 5. (a) Intracellular (circles) and extracellular (squares) L-histidine concentrations in 13032 and its deletion mutant, 13032ΔEG, in the presence of 2 mM histidyl-alanine. Open symbols represent the deletion mutant, and solid symbols represent the wild-type. (b) Intracellular (squares) and extracellular (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.

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 2·72 mM Lys–Ala and 1·28 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). This clearly indicates that the LysE export system accepts and transports both amino acids at a similar rate, and might indicate a comparable affinity towards L-lysine and L-arginine. 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

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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 pEC7*lysE*, overexpressing *lysE*. In short-term fermentation experiments, the L-ornithine concentrations obtained with the *argF* mutant in the absence and the presence of overexpressed *lysE* were determined. As can be seen from Fig. 5(b), in both strains the intracellular L-ornithine concentration reached about 200 mM, whereas it was below 1 mM in 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 pVWEx2′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 palindrome (-TACTTAAGTA-) 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 (Vrljić *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), when 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).

The affinity of LysE towards L-lysine and L-arginine is comparable. The other basic amino acids assayed (L-histidine, L-citrulline, L-ornithine, DL-diaminopimelate) are not accepted as transport substrates by LysE. Basic amino acid exporters in other bacteria have not yet been identified. The uptake carrier of *Penicillium chrysogenum* accepts L-arginine, L-lysine and L-ornithine as

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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 μM 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 DL-diaminopimelate 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-cysteine 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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