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A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*

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The first gene of a family of prokaryotic proteases with a specificity for L,D-configured peptide bonds has been identified in *Escherichia coli*. The gene named *ldcA* encodes a cytoplasmic L,D-carboxypeptidase, which releases the terminal D-alanine from L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine containing turnover products of the cell wall polymer murein. This reaction turned out to be essential for survival, since disruption of the gene results in bacteriolysis during the stationary growth phase. Owing to a defect in muropeptide recycling the unusual murein precursor uridine 5′-pyrophosphoryl *N*-acetylmuramyl-tetrapeptide accumulates in the mutant. The dramatic decrease observed in overall cross-linkage of the murein is explained by the increased incorporation of tetrapeptide precursors. They can only function as acceptors and not as donors in the crucial cross-linking reaction. It is concluded that murein recycling is a promising target for novel antibacterial agents.

Keywords: antimicrobial targets/L,D-carboxypeptidase/murein precursor/murein recycling/stationary growth phase

Introduction

The cell envelope of most bacteria is stabilized by an exoskeleton made of the cross-linked heteropolymer murein (peptidoglycan). Its basic building block, a peptidyl-disaccharide, is polymerized by the formation of glycosidic and peptide bonds. Thereby, a bag-shaped structure, the murein sacculus, is synthesized which completely encloses the cell (reviewed by Höltje, 1998). In the case of *Escherichia coli* the subunits of murein consist of the peptide L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (L-Ala-D-Glu-*m*-A_2pm-D-Ala-D-Ala) or truncated forms of this peptide, which are linked via an amide bond to the lactyl group of the muramic acid of the disaccharide *N*-acetylglucosamine-β-1.4-*N*-acetylmuramic acid (GlcNAc-MurNAc; van Heijenoort, 1994). Importantly, the peptide side chains consist of D- and L- (or *meso*-) amino acids giving rise to the presence of unusual L,D-, as well as D,D-, peptide bonds in murein. Cross-linkage of the murein strands is achieved by a head-to-tail cross-linking of the peptides via a peptide bond between the carboxyl group of the terminal amino acid of one peptide moiety and the non-alpha amino group present in a peptide side chain protruding from a neighboring glycan strand. In *E.coli* two types of cross-links can be formed (Glauner *et al*., 1988). The majority is found between the carboxyl group of a terminal D-Ala and the ω-amino group at the D-center of the *meso*-diaminopimelic acid resulting in a cross-linking D,D-peptide bond. To a lesser extent also the carboxyl group at the L-center of a terminal *meso*-diaminopimelic acid present in a tripeptide stem peptide is linked to the ω-amino group at the D-center of the *meso*-diaminopimelic acid of another peptide side chain giving rise to the formation of an L,D-cross-linking peptide bond.

Not surprisingly, L,D- and D,D-peptide bonds are import ant targets for highly specific antibacterial agents since they are not found in eukaryotes. The most important group of antibiotics, the β-lactams, inhibit the enzymes specifically involved in the formation and cleavage of the D,D-peptide bonds (Waxman and Strominger, 1983). Since these enzymes bind penicillin covalently, they are referred to as penicillin-binding proteins (PBPs; Spratt, 1975). In addition to transpeptidases and endopeptidases, carboxypeptidases have also been described (Izaki *et al*., 1966). Being PBPs many of the D,D-enzymes from different sources have been characterized in some detail (Ghuysen, 1991).

By way of contrast, knowledge about enzymes with a specificity for L,D-peptide bonds is limited. In particular, no gene encoding an L,D-transpeptidase, an L,D-endopeptidase or an L,D-carboxypeptidase has been cloned to date and the physiological functions of these proteins are still under discussion (Templin and Höltje, 1998). The best characterized example of an enzyme of this group is an L,D-carboxypeptidase isolated from *E.coli* (Metz *et al*., 1986a,b; Ursinus *et al*., 1992). The enzyme cleaves the D-alanine in position 4 that is linked via an L,D-peptide bond to the L-center of the *meso*-diaminopimelic acid in position 3 of the peptide side chains. Such an activity was shown earlier to oscillate during the cell cycle of *E.coli* with highest activity during cell division (Beck and Park, 1976, 1977). The function(s) of L,D-carboxypeptidases remain(s) obscure, although an involvement in cell division has been proposed (Begg *et al*., 1990; Höltje, 1998). Here we report on the cloning, overexpression and deletion of a cytoplasmic L,D-carboxypeptidase which turned out to be essential at the onset of the stationary phase of growth by specifically interfering with murein recycling.

Results

Identification of the gene encoding the L,D-carboxypeptidase A

Classical protein purification methods yielded a highly enriched fraction of an L,D-carboxypeptidase, which could

be used for biochemical characterization of the enzyme (Ursinus *et al*., 1992). Nevertheless, the amount of protein was not high enough to obtain a partial peptide sequence that could have been used for identification of the gene encoding this enzyme. Since a simple and fast assay for L,D-carboxypeptidase using uridine 5′-pyrophosphoryl *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine (UDP-MurNAc-tetrapeptide) as a substrate, which can be used even with crude cell extracts, was available (Ursinus *et al*., 1992), we decided to screen an expression library for the corresponding gene. Therefore, the Kohara miniset library (Noda *et al*., 1991) was scored for phage-based overexpression of L,D-carboxypeptidase activity. Screening the 479 phages from the miniset library led to the identification of one clone with a reproducible increase in L,D-carboxypeptidase activity. This phage (λ 245) carries a 17.4 kb insert containing 13 potential reading frames. Only one of the predicted proteins, f304 (Blattner *et al*., 1997) had a calculated molecular mass close to the experimentally determined value of the L,D-carboxypeptidase (Ursinus *et al*., 1992). Therefore, this coding region was amplified from phage DNA by PCR and directly cloned into an expression vector. A transformant of MC1061 carrying this construct (pLD2) was induced to overproduce the cloned gene product by adding isopropyl-thio-β-D-galactoside (IPTG). A 4500-fold increase in L,D-carboxypeptidase activity was detected and it could be concluded that the gene for an L,D-carboxypeptidase active on UDP-MurNAc-tetrapeptide had been identified. This high degree of overproduction had no effect on growth rate or cell viability. Since this is the first example of the cloning of a gene coding for an enzyme possessing L,D-carboxypeptidase activity the gene was named *ldcA* (L,D-carboxypeptidase A).

Sequence analysis suggests a prokaryotic protease family

The reading frame for the LdcA consists of 912 base pairs, coding for a protein with a predicted molecular mass of 33 572 Da. No apparent N-terminal signal sequence could be detected by using the TopPred II algorithm (Claros and von Heijne, 1994); therefore, a cytosolic location was expected.

Interestingly, the *ldcA* gene and *emtA*, a recently identified gene coding for a lytic endo-transglycosylase (Kraft *et al*., 1998), are found side-by-side (Figure 1). Whereas the genes are transcribed in opposite directions, the promotors for both genes seem to overlap (M.F.Templin, unpublished). A coordinated expression might be significant, since some of the muropeptides released by EmtA are substrates for the L,D-carboxypeptidase.

Similarity searches with the predicted coding sequence were performed using the PSI-BLAST algorithm (Altschul *et al*., 1997) and revealed the presence of homologs in different bacterial species including *Synechocystis* sp., *Bacillus subtilis*, *Streptomyces coelicolor*, *Vibrio cholerae*, *Rickettsia prowazekii* and *Treponema pallidum* (e values $<10^{-63}$ in a PSI-BLAST search after the second iteration). No significantly similar proteins were detected in any eukaryote. When extending the searches to the available 'unfinished bacterial genomes' (last update 15 March 1999; preliminary sequence data were obtained from the Institute for Genomic Research website at http://www.tigr.org) further homologs of LdcA were detected in *Salmonella typhi*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Bordetella pertussis*, *Chlorobium tepidum*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Streptococcus mutans* and *Deinococcus radiodurans* (e values in the tblastn search $<10^{-5}$).

An alignment using the identified proteins shows three regions of high similarity (Figure 2). Therefore, the database searches suggest that these orthologs form a protease family present in a wide range of bacteria.

Subcellular localization of LdcA

Earlier experiments pointed to a periplasmic location of an L,D-carboxypeptidase that could be released from whole cells by Tris–EDTA treatment (Beck and Park, 1977). However, the sequence analysis discussed above favors a cytoplasmic location for LdcA. Therefore, cell fractionation experiments were performed. Following the method of Witholt *et al*. (1979), cells were fractionated into periplasmic extract, membrane fraction and cytosolic extract. Using UDP-MurNAc-tetrapeptide as a substrate, enzyme activity was exclusively found in the cytoplasm in wild-type *E.coli* MC1061 (Table I). Even upon high induction of expression of LdcA only traces of activity (>1%) were found in the membrane or in the periplasmic fraction (data not shown). The same result was obtained after Tris–EDTA treatment of the cells performed as described by Beck and Park (1977). This shows that the LdcA is a cytoplasmic enzyme.

Substrate specificity and inhibition of the LdcA

With the construction of an N-terminally His_6-tagged version of the enzyme it was possible to obtain sufficient amounts of highly enriched enzyme preparations (2.53×10^6 units/mg protein). This allowed us to determine the substrate specificity of the LdcA in some detail and to examine the sensitivity against different antibiotics. Cytoplasmic fractions containing 1.34 units of enzyme were incubated with a variety of murein derivatives carrying L-Ala-D-Glu-*meso*-A_2pm-D-Ala tetrapeptide side chains. As listed in Table II, LdcA does not interact with isolated high molecular mass murein sacculi or with cross-linked muropeptides. Thus, only monomeric muropeptides, free tetrapeptide and UDP-activated murein precursors are substrates for LdcA. This finding is in accordance with results obtained by others (Leguina *et al*., 1994), who showed that an L,D-carboxypeptidase is present in *E.coli* that acts on low molecular mass muropeptides, but not on the native polymer murein. The specificity determined is consistent with the fact that LdcA is a cytoplasmic enzyme that cannot come into contact with the murein sacculus in the periplasm.

L,D-carboxypeptidase activity had been shown to be inhibited competitively by β-lactam antibiotics containing a D-configured amino acid as a side chain (Hammes, 1978; Hammes and Seidel, 1978a; Ursinus *et al*., 1992). Metz *et al.* (1986b) reported an inhibition of the activity by penem antibiotics. The enriched enzyme fraction was used to test the senitivity of LdcA: little or no inhibition could be shown for Penicillin G and the penems Imipemem and CGP31608 (kindly supplied by Ciba-Geigy, Basel, Switzerland); β-lactams containing a D-configured amino

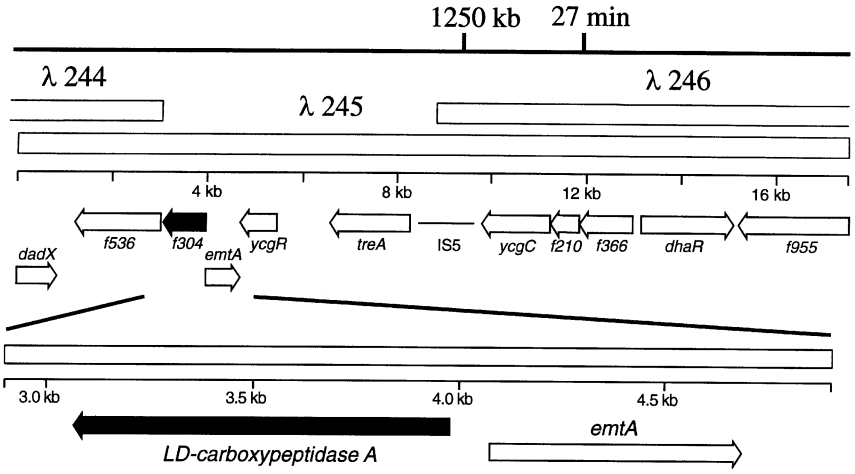

Fig. 1. Map of the region from 1241 to 1259 kb of the *E.coli* W3110 chromosome. The inserts encoded by the Kohara phages λ244, λ245 and λ246 are indicated. The unassigned open reading frame f304 (black arrow) codes for the L,D-carboxypeptidase A, *emtA* encodes the membrane-bound lytic endotransglycosylase A, an enzyme which releases muropeptides that are substrate for LdcA (Kraft *et al.*, 1998).

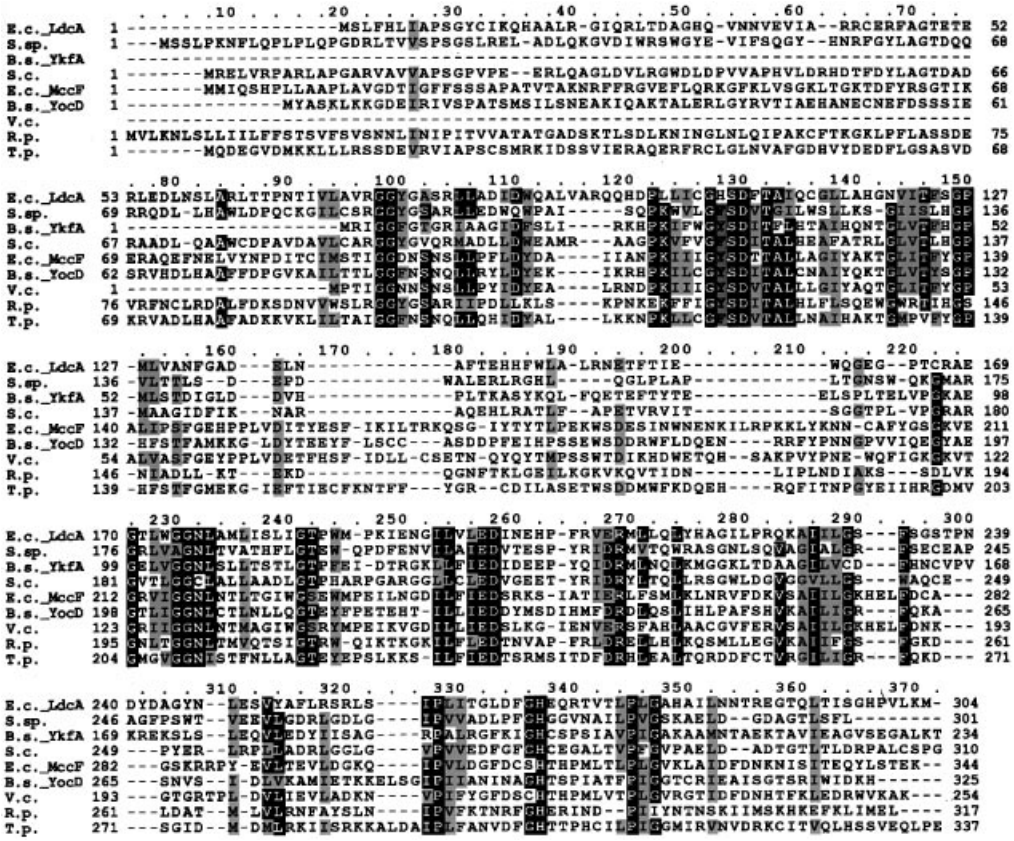

Fig. 2. Alignment of the LdcA with identified homologs from different bacterial species. Multiple sequence alignment was performed using the PIMA algorithm (Smith and Smith, 1992). E. c., *Escherichia coli*, *MccF* CAA40814; S. sp., *Synechocystis* sp., BAA18314; B. s., *Bacillus subtilis*, YkfA CAA05577, YocD AAB84435; S. c., *Streptomyces coelicolor*, CAA22737; V. c., *Vibrio cholerae*, AAB81977; R. p., *Rickettsia prowazekii*, CAA14859; T. p., *Treponema pallidum*, AAB38706.

acid as a side chain including Cefminox, Cephalosporin C and Norcardicin A (kindly supplied by Eli-Lilly, Indianapolis, IN) inhibited enzyme activity (Table II).

Construction and growth phenotype of a deletion mutant

To get an idea of the function of the *ldcA*, a plasmid-coded deletion was constructed and transferred to the chromosome of *E.coli* MC1061 by λ-phage-mediated transduction (Kulakauskas *et al.*, 1991). The obtained mutant named LD3 did not contain the coding sequence for the protein. No L,D-carboxypeptidase activity could be detected in soluble extracts when UDP-MurNAc-tetrapeptide was used as a substrate (data not shown), suggesting that the identified enzyme is the only activity of this type present in *E.coli*.

Despite the fact that viable clones could be obtained, growth during the stationary phase was severely affected.

Table I. L,D-carboxypeptidase activity in different cell fractions[a]

	Acid phosphatase[b]	G6P-DH[b]	L,D-carboxypeptidase[b]
A: Tris–EDTA–lysozyme treatment			
Cytoplasm	19.5%	91.5%	91.2%
Periplasm	80.5%	8.5%	8.8%
B: Tris–EDTA treatment			
Cytoplasm	55.3%	89.3%	93.1%
Periplasm	44.7%	10.7%	6.9%

[a]Cell fractionation was carried out following two different methods: A, according to Withold *et al*. (1979) and B, as described by Beck and Park (1977).
[b]Enzyme activities were determined using published methods for acid phosphatase (Atlung *et al*., 1989) as a marker for periplasmic proteins, for glucose-6-phosphate dehydrogenase (G6P-DH; Bergmeyer, 1974) as a marker for cytoplasmic enzymes and for LdcA as described in Materials and methods.

Table II. Substrate specificity of LdcA and inhibition of the enzyme by different antibiotics

A: Substrate specificity

Substrate	Relative activity (%)
UDP-MurNAc-tetrapeptide[a]	100
Disaccharide-tetrapeptide[b]	23.0
Anhydrodisaccharide-tetrapeptide[b]	25.7
MurNAc-tetrapeptide[b]	100
Bis-disaccharide-tetrapeptide[b]	n.d.[d]
Bis-anhydrodisaccharide-tetrapeptide[b]	n.d.[d]
Tetrapeptide[b]	132.8
Murein sacculi[c]	n.d.[d]

B: Inhibition by antibiotics

Antibiotic	Concentration (μg/ml)	Inhibition (%)
Imipenem	100	10.1
	250	13.9
Penicillin G	100	26.6
	250	23.7
CGP31608	100	35.3
	250	34.0
Cefminox (MT-141)	100	33.7
	250	50.5
Cephalosporin C	100	53.1
	250	63.1
Nocardicin A	100	81.7
	250	86.9

[a]L,D-carboxypeptidase activity with UDP-MurNAc-tetrapeptide was determined at a concentration of 0.7 mM with 1.3 U of the enzyme under standard conditions as described in Materials and methods. The value for UDP-MurNAc-tetrapeptide was set to 100%.
[b]L,D-carboxpeptidase activity with the muropeptides indicated was determined by separating substrate and product by RP-HPLC using the system to separate muropeptides described by Glauner (1988) except for terminating the elution when 50% of buffer B was reached, i.e. after 67.5 min.
[c]The reaction with isolated murein sacculi was determined by analyzing the muropeptide composition of 50 μg sacculi treated with 27 U L,D-carboxypeptidase under standard conditions by RP-HPLC as described by Glauner (1988).
[d]No activity detected.

After growth on Luria-Bertani (LB) agar for 72 h at 37°C, only a small percentage of the cells remained viable. Liquid cultures of wild-type bacteria and of the LdcA deletion mutant growing aerobically at 37°C had the same growth rate during the exponential phase (Figure 3). However, upon onset of the stationary phase, growth of the mutant culture seemed to slow down and the culture eventually started to lyse. Phase-contrast microscopy

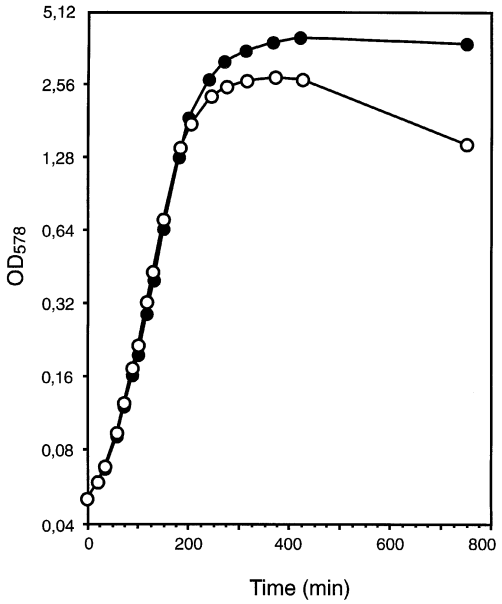

Fig. 3. Growth of the *ldcA* deletion mutant LD3 and its isogenic parent strain MC1061. Growth of *E.coli* LD3 (○) and MC1061 (●) in 50 ml LB medium with aeration at 37°C was followed by optical density readings. Inoculation was carried out with one single colony from a fresh plate.

(Figure 4) showed cells that had lost their shape and, in addition, many lysed cells were visible. The number of viable cells determined after 12 h of growth was reduced to 3.5×10^7 in the LdcA mutant culture compared with 1.8×10^9 in the control culture. Therefore, spontaneous autolysis occurred when the culture entered the stationary growth phase. This phenotype could be suppressed by a plasmid carrying the *ldcA* gene.

Changes in the murein composition of the mutant

Lysis of the LdcA deletion mutant in the stationary growth phase could be the result of some defects in the structure of the murein sacculus. Therefore, the composition of the murein of an exponentially growing culture (OD_{578} 0.5) and a culture just entering the stationary phase (OD_{578} 1.5) was analyzed. Isolated sacculi were digested completely using a muramidase and the degradation products (muropeptides) were separated and quantified by reversed-phase high-pressure liquid chromatography (RP-HPLC) (Glauner, 1988). The results shown in Table III reveal significant differences in the relative amounts of the

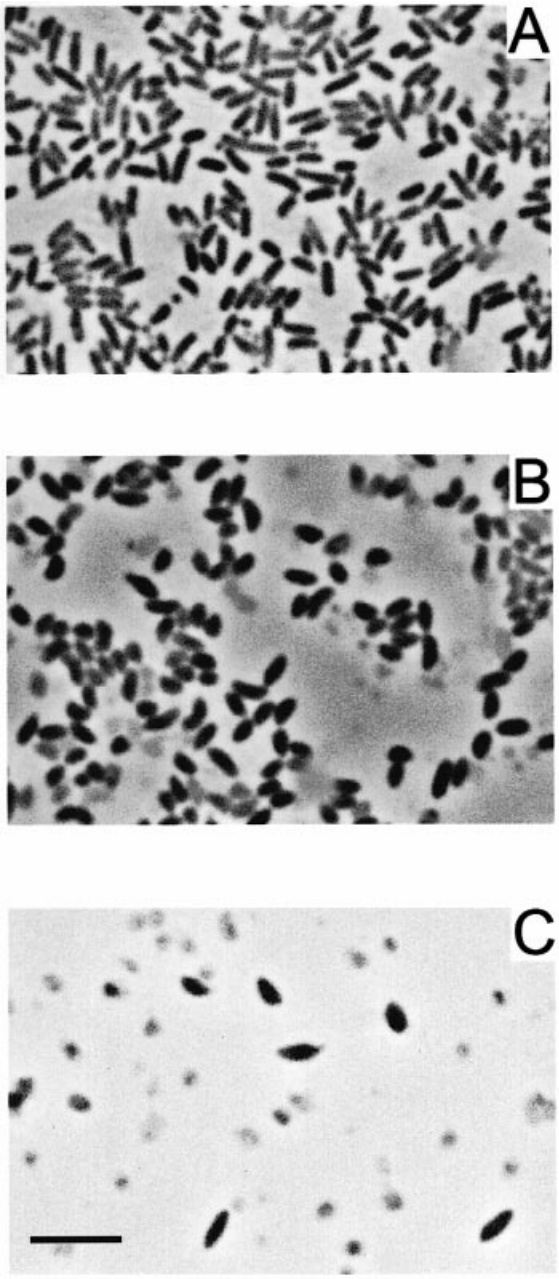

Fig. 4. Morphology of the *ldcA* deletion mutant LD3 and its isogenic parent strain MC1061. Phase-contrast micrographs were taken from cells grown aerobically at 37°C in LB medium at an OD_{578} of 1.5 (**A**, MC1061; **B**, LD3) and after growth overnight (**C**, LD3). The bar represents 5 μm.

various muropeptides between the *ldcA* mutant and the parental strain. Whereas these differences were obvious in the murein from cells harvested in the exponential phase of growth, they were more pronounced for a culture grown to an OD_{578} of 1.5. In particular the overall cross-linkage was reduced by 23% leading to an increase in the monomeric muropeptide disaccharide–tetrapeptide by 16% and disaccharide–tripeptide by 46%. Whereas the number of dimeric D,D-cross-bridges was reduced by 12%, an even more dramatic decrease was observed for the trimeric and tetrameric structures containing D,D-cross-links: the relative amounts decreased by 67 and 58%, respectively. Interestingly, the unusual L,D-cross-linkage between two *m*-A_2pm residues was increased. This finding is consistent with a number of reports showing a rise of L,D-cross-links when D,D-cross-links decrease for various reasons (Driehuis and Wouters, 1987; Kohlrausch and Höltje, 1991a). Therefore, formation of L,D-cross-bridges is considered to represent a salvage mechanism to keep the overall degree of cross-linkage at a high level even under conditions where normal D,D-cross-links, which depend on the presence of sufficient pentapeptides, can no longer be formed. It is important to note that the number of chain ends, which reflects the average length of the glycan strands, did not increase in the mutant indicating that the impending lysis event was not caused by autolytic processes described previously (Kitano and Tomasz, 1979; Kohlrausch and Höltje, 1991a). We conclude that autolysis of the *ldcA* mutant is the result of a dramatically reduced overall murein cross-linkage.

Determination of murein precursors and phenotypic suppression of ldcA mutants

Since LdcA is present in the cytoplasm it can interact only with cytoplasmic murein precursors and murein turnover products. The turnover products, however, are known to be recycled by their incorporation into the precursor molecules (Goodell, 1985). Therefore, the intracellular pools of the final UDP-activated murein precursors were determined in an LdcA mutant and compared with the pools present in wild-type cells. The precursors were radioactively labeled by growth of the cells in the presence of [^{3}H]diaminopimelic acid, extracted with hot water and separated by HPLC. As shown in Figure 5, in wild-type cells only UDP-MurNAc-pentapeptide could be found in the cytoplasm. By way of contrast in the deletion mutant HfrH-ldcA only a small amount of pentapeptide is detected but there is a prominent peak corresponding to UDP-MurNAc-tetrapeptide which has never been detected in *E.coli* before. In addition, free tetrapeptide is also found.

With a possible role in recycling, it was interesting to investigate the effects of mutations in other genes coding for proteins involved in muropeptide recycling. It was particularly interesting to study the effect of an *ampD* mutation, which is known to cause a severe block in muropeptide recycling, since cytoplasmic hydrolysis of muropeptides into the sugar and peptide part is abolished (Höltje *et al*., 1994; Jacobs *et al*., 1995). It was therefore expected that a mutation in *ampD* would counteract the toxic effect of a lack in LdcA. Indeed, a mutant carrying deletions in both *ampD* and *ldcA* showed no lysis during stationary growth. Analysis of precursor pools in this mutant showed a decrease in the amounts of free tetrapeptide and UDP-MurNAc-tetrapeptide, whereas MurNAc-pentapeptide was increased compared with the *ldcA* single mutant (Figure 5).

The observed phenotypic suppression by a mutation in *ampD* further supports the idea that LdcA takes part in the recycling pathway. But it should be noted that this type of phenotypic suppression might also occur in an *in vivo* situation when LdcA is inactivated by specific inhibitors. This would not only lead to restored viability, but in bacteria possessing a certain type of a chromosomally encoded β-lactamase (e.g. *Citrobacter freundii*; Lindberg *et al.*, 1987) this might even lead to a decreased

Table III. Muropeptide composition of the *ldcA* mutant and its isogenic parent strain[a]

	Relative amounts (%)					
	Exponential growth (OD_{578} 0.5)			Stationary growth (OD_{578} 1.5)		
	MC1061	LD3	Δ%	MC1061	LD3	Δ%
Muropeptides[b]						
Monomeric	55.35	60.43	+9	51.47	60.89	+18
Tri	7.35	9.86	+34	7.43	10.83	+46
Tetra	44.39	46.54	+5	38.75	44.81	+16
Penta	0.08	0.03	–63	0.05	0.03	–40
Dimeric	39.06	36.22	–7	38.54	36.01	–7
TetraTetra	27.71	23.24	–16	22.52	19.05	–15
TetraTri	2.80	2.77	–1	3.03	3.03	0
Trimeric	5.29	3.24	–39	9.68	2.98	–69
Tetrameric	0.30	0.12	–60	0.31	0.13	–58
Anhydro	4.19	3.83	–9	6.01	4.93	–18
LysArg-containing	1.81	2.33	+29	2.92	3.28	+12
Cross-linked	23.28	20.35	–13	25.96	20.08	–23
Ala-A_2pm	21.81	18.30	–16	23.03	16.81	–27
A_2pm-A_2pm	1.49	2.02	+36	3.07	3.34	+9

[a]Murein composition of isolated murein sacculi was determined as described previously (Glauner, 1988).
[b]Tri, disaccharide tripeptide; Tetra, disaccharide–tetrapeptide; Penta, disaccharide–pentapeptide; TetraTri/TetraTetra, bis-disaccharide-tetra-tri-/tetra-tetra-peptide; disaccharide, GlcNAc-β-1.4-MurNAc; tri-/tetra-/penta-peptide, -L-Ala-D-Glu-*m*-A_2pm-(D-Ala)-(D-Ala); A_2pm-A_2pm indicates a D,L-A_2pm-A_2pm cross-bridge; Ala-A_2pm indicates a D,D-Ala-A_2pm cross-bridge.

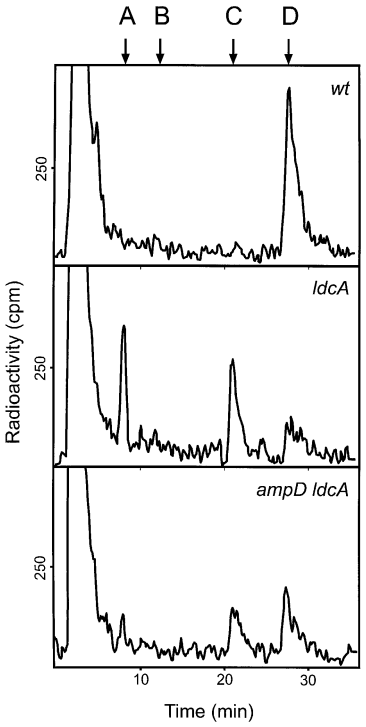

Fig. 5. Determination of the intracellular pools of soluble murein precursors. Murein precursors, radiolabeled with meso-2,6-diamino-[3,4,5-^{3}H]pimelic acid were extracted from *E.coli* HfrH (*wt*), HfrH *ldcA* (*ldcA*) and JRG582 *ldcA* (*ampDE ldcA*). Separation the precursors was performed by HPLC on Nucleosil ODS as described in Materials and methods. The identity of the indicated peaks was verified by authentic standards. A, tetrapeptide; B, UDP-NAcMur-tripeptide; C, UDP-NAcMur-tetrapeptide; D, UDP-NAcMur-pentapeptide.

sensitivity against penicillins or cephalosporins. A combination of the *ldcA* deletion with a mutation in the *N*-acetylglucosaminidase, encoded by *nagZ*, which also participates in muropeptide recycling did not change the intracellular pool of UDP-MurNAc-pentapeptide and did not suppress bacteriolysis in the stationary growth phase (data not shown).

Discussion

Recycling of the murein turnover products, which make up ~50% per generation of the total murein (Goodell, 1985), is an important energy-saving mechanism. Only a very small amount of turnover material, ~4–7% per generation, is lost to the medium (Goodell and Schwarz, 1985). Therefore, it is not surprising to find that *E.coli* has a number of systems which make recycling as efficient as possible. Several uptake systems have been described, which are involved in the transportation of the murein turnover products from the periplasm back into the cytoplasm. Muropeptides are imported via the AmpG transporter (Jacobs *et al*., 1994), whereas peptides that are released from the murein or from muropeptides by the action of periplasmic amidases are accepted by the Opp (Goodell and Higgins, 1987) and Mpp (Park *et al*., 1998) permeases.

The fate of the major turnover products, the disaccharide-anhydro tri- and tetrapeptides, has been of considerable interest in recent research. A close connection between the regulation of expression of certain chromosomally encoded β-lactamases and murein recycling could be demonstrated (Jacobs *et al*., 1997). The β-lactamase work showed that the amount of cell wall material released is used to sense the state of the murein sacculus. In a situation where the incorporation of new material is inhibited, e.g. treatment with β-lactam antibiotics, the amount of released turnover products increases dramatically. Certain bacterial species exploit this increase of released muropeptides to induce β-lactamase expression.

Actually, most of the known enzymes of the recycling pathway have been identified during studies of β-lactamase

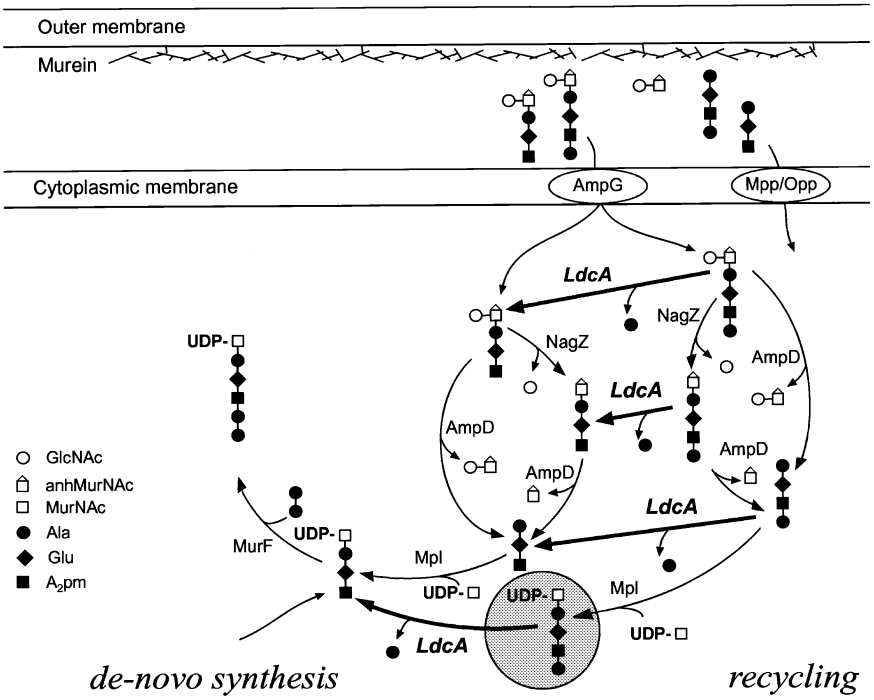

Fig. 6. Role of LdcA in murein recycling. Murein turnover products are taken up by the AmpG, Mpp and Opp systems and degraded to monosaccharides and oligopeptides by action of the amidase AmpD and the glucosaminidase NagZ. The muropeptide ligase Mpl adds the peptides to UDP-MurNAc. MurF, a dipeptide ligase adds D-alanyl-D-alanine to UDP-MurNAc-tripeptide which either originates from *de novo* synthesis of precursors or from the recycling process. L,D-carboxypeptidase guarantees that only tripeptides are present and thus exclusively UDP-MurNAc-tripeptides are formed. In a situation were *ldcA* is deleted, the toxic precursor UDP-MurNAc-tetrapeptide (encircled) accumulates.

induction. Not only has the uptake system AmpG been identified during a screen for genes involved in β-lactamase induction, but also the cytoplasmic amidase AmpD, which releases the peptide moiety from cytosolic muropeptides (Lindberg *et al*., 1987). Other enzymes known to be involved in recycling are the cytoplasmic β-*N*-acetylglucosaminidase NagZ (Park, 1996) and the muropeptide ligase Mpl, which has been shown to catalyze the addition of the released peptide to the nucleotide-activated MurNAc (Mengin-Lecreulx *et al*., 1996). Whereas mutations in these genes mainly affect the efficiency of the recycling process, the enzyme identified in this study is essential for the survival of bacteria that have entered stationary phase.

The reaction catalyzed by the LdcA, the conversion of cytosolic tetrapeptide-containing structures to tripeptide-containing structures, is obviously needed for recycling. The formation of the murein precursor UDP-MurNAc-tripeptide occurs by the sequential addition of amino acids to the activated sugar moiety (van Heijenoort, 1994). Alternatively, the muropeptide ligase Mpl uses peptides released during recycling directly (Park *et al.*, 1998). In the next step the general precursor for murein synthesis UDP-MurNAc-pentapeptide is made from UDP-MurNAc-tripeptide by the addition of the dipeptide D-alanyl-D-alanine. Since a single D-alanine cannot be added to a tetrapeptide, an activated tripeptide has to be present to facilitate the synthesis of the final precursor. In the absence of the L,D-carboxypeptidase the hydrolysis of tetrapeptide-conaining structures does not take place and the consequences are striking: the formation of UDP-MurNAc-tetrapeptide occurs, a dead-end intermediate that cannot be converted into a pentapeptide precursor (Figure 6). When incorporated into the murein sacculus, it affects the number of cross-links in the polymer since tetrapeptides can function only as acceptors and not as donors for transpeptidation. A decrease in the overall degree of cross-linkage in the sacculus is the consequence of this failure to synthesize a sufficient supply of pentapeptide precursors. This leads not only to a weakened cell wall, but also results finally in autolysis. Therefore this work was able to identify not only the gene coding for this new type of carboxypeptidase, but also its physiological role during recycling of the cell wall polymer murein. With an enzyme required for survival and which seems to be present in a wide range of bacterial species, a potential target for antimicrobial agents has been identified. Antibiotically active substances inhibiting L,D-carboxypeptidases have been known for quite a while (Hammes and Seidel, 1978b; Metz *et al*., 1986b). The best characterized example is an antibiotic called Nocardicin A. It has been shown that this monocyclic β-lactam is able to bind to LdcA. This property has been exploited and has led to the purification of the enzyme by affinity chromatography (Ursinus *et al*., 1992). Nocardicin A is a bi-headed structure: a β-lactam with a D-amino acid in its side chain. It interacts non-covalently via its D-amino acid residue with LdcA and, in addition, binds covalently with its β-lactam ring to the classical PBPs. It is expected that completely different structures, not related to β-lactams, may be picked up in a screen for specific inhibitors of LdcA and other L,D-enzymes.

Interestingly, the lethal effect of the absence of LdcA seems to be restricted to the stationary phase of growth. This suggests that the contribution of muropeptide recycling, compared with *de novo* murein precursor synthesis, is of greater importance when the cells enter this part of

their life cycle. Correspondingly, it has been shown that expression of the amidase AmiA, which is involved in periplasmic recycling, is under the control of the sigma factor RpoS (Troup *et al.*, 1994; Jahn *et al.*, 1996). First results indicate an increase in the expression of *ldcA* at this time, which is not dependent on RpoS (M.F.Templin, unpublished data). The phenotype of the *ldcA* mutant demonstrates that interference with murein recycling can result in autolysis. Thus a screen for compounds affecting recycling could lead to the identification of antimicrobial agents active against resting bacteria, which present a still unsolved problem in antibacterial chemotherapy.

Materials and methods

Bacterial strains, bacteriophages and plasmids

Escherichia coli MC1061 (Casabadan and Cohen, 1980) was used as a general cloning host and for the construction of a deletion mutant of *ldcA*. For the phage-based overexpression assay *E.coli* D456 (Edwards and Donachie, 1993) was used. The Kohara miniset λ library (Noda *et al*., 1991) was employed for the screen to identify the gene encoding the L,D-carboxypeptidase. Phage λ 245 (7C10) from this library was used as a DNA source for cloning the gene of interest and for PCR amplification of the flanking regions. The general cloning vector was pBluescriptII SK+ (Stratagene, La Jolla, CA); the kanamycin resistance determinant was taken from pUC4K (Pharmacia, Uppsala, Sweden). In order to construct an inducible expression system, the *ldcA* gene was cloned into plasmid pJFK118EH (Bishop and Weiner, 1993), an N-terminally His_6-tagged version of the enzyme was obtained after cloning of the coding region into pQE30 (Qiagen, Hilden, Germany). For construction of a double mutant in *ampDE* and *ldcA* JRG582 (Guest and Stephens, 1980) and its isogenic parent strain HfrH were used. To test the influence of a *nagZ* mutation in a *ldcA* background, the *ldcA* deletion was transduced into E593 (Yem and Wu, 1976) and its parent AB1157.

Growth conditions and phase-contrast microscopy

Bacteria were cultivated aerobically at 37°C in LB medium (Miller, 1972). Growth was monitored by determination of the optical density of the culture at 578 nm (OD_{578}) in an Eppendorf photometer (Eppendorf, Hamburg, Germany). When required, the medium was supplemented with either 50 μg/ml kanamycin and/or 50 μg/ml ampicillin. Radioactively labeled murein precursors were prepared from bacteria, which were grown for at least four generations in LB medium supplemented with 10 μCi/ml meso-2,6-diamino-[3,4,5-^{3}H]pimelic acid (A_2pm; ARC, St Louis, MO) (Kohlrausch *et al*., 1989).

Phase-contrast micrographs of bacterial cells were taken with a Zeiss Axiovert 135 TV microscope equipped with a Plan-Neofluar 100× lens (Zeiss, Oberkochen, Germany) and a Photometrics Series 200 image-grabbing system (Photometrics, Tucson, AZ). Pictures were edited using the IPLab Spectrum 3.0 software (Signal Analytics, Vienna, VA) and Adobe Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA).

Screening of a λ phage library of E.coli W3110

The λ-phage-mediated overexpression assay as described by Henderson *et al*. (1995) was used to screen a chromosomal library of *E.coli* W3110. After infection of 3 ml of *E.coli* D456 with high titer lysates of individual λ clones from the Kohara miniset λ library (Noda *et al*., 1991), the bacteria were incubated for 45 min at 37°C. Infected bacteria (2 ml) were cooled rapidly on ice, harvested by centrifugation (2 min, 15 000 *g*), resuspended in 60 μl buffer (0.1 M Tris–HCl pH 8.0, 10 mM $MgSO_4$, 1% Triton X-100) and frozen. For the L,D-carboxypeptidase assay 20 μl of the lysed cells were used in the standard enzyme assay.

*Assay for L,D-*carboxypeptidase activity

The general assay to determine L,D-carboxypeptidase activity employs RP-HPLC and has been described in detail previously (Ursinus *et al*., 1992). The substrate used in the assays was UDP-*N*-acetylmuramyl-L-Ala-D-Glu-*m*-A_2pm-D-Ala (UDP-MurNAc-tetrapeptide), which was prepared from UDP-MurNAc-pentapeptide, which contains an additional C-terminal D-Ala. UDP-MurNAc-pentapeptide was obtained from *Bacillus cereus* using a previously published method (Kohlrausch and Höltje, 1991b), converted to UDP-MurNAc-tetrapeptide by digestion with D,D-carboxypeptidase (PBP5) from *E.coli* and purified using RP-HPLC as described previously (Kohlrausch and Höltje, 1991b). Muramyl tetrapeptide was obtained after acid hydrolysis of UDP-MurNAc-tetrapeptide and purification by HPLC (Ursinus *et al*., 1992). Disaccharide–tetrapeptide (Tetra), anhydro-disaccharide–tetrapeptide (Tetra-anh), bis-disaccharide–tetrapeptide (Tetra-Tetra) and bis-anhydro-disaccharide–tetrapeptide (Tetra-Tetra-anh) were isolated from muramidase digests of murein sacculi and quantified using the method of Glauner (1988). Tetrapeptide was prepared from Tetra by digestion with purified human serum amidase (Harz *et al.*, 1990).

The substrate concentration in the assays was generally 0.7 mM; to save substrate the concentration of UDP-MurNAc-tetrapeptide in the library screen was adjusted to 0.14 mM. The reactions were performed in a final volume of 100 μl 0.1 M Tris–HCl pH 8.0 and incubated for 45 min at 37°C. The reaction was terminated by boiling the samples for 3 min. After a brief centrifugation (15 000 *g*, 2 min), 90 μl of the supernatant was frozen or used directly for HPLC analysis. The separation and quantification of substrate and reaction product (UDP-MurNAc-tripeptide) was performed by RP-HPLC on a 5 μm Hypersil ODS column (Bischoff, Leonberg, Germany) as described by Ursinus *et al*. (1992). However, slightly modified elution conditions were used. A linear gradient of 2–20% acetonitrile in 0.05% trifluoroacetic acid which was built up in 20 min was employed.

To determine L,D-carboxypeptidase activity on high molecular mass murein, LdcA was incubated with isolated murein sacculi (Harz *et al.*, 1990) for 2 h at 37°C and the reaction was stopped by boiling the samples for 3 min. Analysis of the murein was performed as described below.

DNA manipulations and PCR

Standard techniques were used to manipulate the plasmid DNA (Sambrook *et al*., 1989) and *E.coli* was transformed as described by Inoue *et al.* (1990). Restriction endonucleases were purchased from Boehringer Mannheim (Mannheim, Germany), oligonucleotides came from MWG-Biotech (Ebersberg, Germany). PCR (Saiki *et al*., 1988) was performed using 1.25 units of Pfu polymerase (Stratagene, La Jolla, CA) in a volume of 50 μl of the supplied buffer with 0.5 μM of each primer and 2 μl of Kohara λ phage 245 lysate as a template. After initial denaturation for 3 min at 92°C, touchdown PCR (Don *et al*., 1991) was performed with 0.5 min of annealing, 1 min of extension at 72°C and 0.5 min of denaturation at 92°C. The annealing temperatures were 61, 59, 57, 55 and 53°C for three cycles each and finally 51°C for another 15 cycles.

Construction of an inducible expression system

The gene encoding the L,D-carboxypeptidase was cloned into the plasmid pJFK118EH (Bishop and Weiner, 1993) to obtain an inducible expression system under the control of the *tac* promoter. The coding region was amplified from Kohara phage λ245 DNA by touchdown PCR by using the primers 5′-TTGGAATTCCAACGGCACACCTAAGCGGC-3′ and 5′-AGAGTCGACTTTACTTAGCCCTGAAGCG-3′, introducing an *Eco*RI restriction site and a *Sal*I restriction site at the ends. The amplified product was purified and digested with *Eco*RI and *Sal*I and ligated into pJFK118EH cut with these enzymes and dephosphorylated with shrimp alkaline phosphatase (Boehringer Mannheim). The resulting construct, pLD2, was transformed into *E.coli* MC1061. For induction of overproduction of LdcA 0.4 mM IPTG was added to a growing culture.

Construction of a His_6–LdcA fusion protein

To allow a simple purification of the L,D-carboxypeptidase, a fusion protein possessing a His_6 tag at the N-terminus of the LdcA was constructed. Using touchdown PCR with Kohara phage λ245 lysate as a template and 5′-CGTGTCGACCGGTTAATGGAATTATCGGCG-3′ and 5′-CTCGAGCTCTCGACGACCAAAAAAGGCGAT-3′ as primers the coding sequence of *ldcA* was amplified, cut (*Sal*I, *Sac*I) and cloned into pQE30 (digested with *Sal*I, *Sac*I). Transformation of LD3 with this construct (pHLD5) complemented the growth defect of the mutant, indicating that the fusion protein is functional. To purify the fusion protein, 100 ml MC1061 freshly transformed with pHLD5 was grown to an OD_{578} of 0.8 and harvested by centrifugation. After washing with 50 ml ice-cold buffer (100 mM Tris–HCl pH 8.0), the cells were resuspended in 5 ml buffer and disrupted by one passage through a precooled French pressure cell at 15 000 lb/in^2 and centrifuged (30 min, 300 000 *g*). L,D-Carboxypeptidase activity in the supernatant was found to be 2.5×10^6 units/mg protein. To determine the substrate specificity and the sensitivity against different antibiotics, 1.3 units from this preparation were used.

Construction of a ldcA deletion mutant

The gene exchange method of Kulakauskas *et al*. (1991) was used to construct a deletion of the coding region of the *ldcA* gene. First, the flanking regions of the gene were amplified (750 base pairs upstream with 5′-GAAGTCGACGCGCGACTGATTTACATCG-3′ and 5′-CAGGATGACCGAATTCCATGGCAATTCCTTGCTGAC-3′; 800 base pairs downstream with 5′-GAAGCGGCCGCAATGGATATAATCCGGCG-3′ and 5′-GAATTGCCATGGAATTCGGTCATCCTGTTCTTAAAATG-3′), fused by PCR digested with *Sal*I and *Not*I and cloned into pBluescriptII SK+ (cut with *Not*I and *Sal*I). The introduction of a selectable marker [the *Eco*RI fragment containing the *npt* gene from pUC4K (Pharmacia, Uppsala, Sweden)] into the *Eco*RI site of this construct resulted in a plasmid-borne deletion. This mutation was transferred to the chromosome of MC1061 by λ-mediated transduction using Kohara phage λ245 as an intermediate as described (Kulakauskas *et al*., 1991). The obtained transductants were checked by PCR for the presence of the mutated copy of the *ldcA* gene. One of the clones LD3, showing no L,D-carboxypeptidase activity on UDP-MurNAc-tetrapeptide, was chosen for further experiments. To examine the effect of the absence of LdcA in a *ampDE* mutant background, the described *ldcA* allele was transferred to HfrH and JRG by P1 transduction (Miller, 1972). The *ldcA* deletion was also transduced into E593, a strain which has been shown to lack a cytoplasmic *N*-acetylglucosaminidase active on isolated muropeptides (Yem and Wu, 1976) and into its parent strain AB1157.

Cell fractionation

To determine the subcellular localization of LdcA, periplasmic proteins were isolated by preparing sphaeroplasts with Tris-EDTA and lysozyme under osmotic protection (Witholt *et al*., 1979). The cytoplasmic fraction was obtained after disruption of the sphaeroplasts with glass beads and ultracentifugation (20 min, 300 000 *g*) to sediment the membranes. As an alternative method, periplasmic proteins were released by EDTA treatment (Levy and Leive, 1970; Beck and Park, 1977). Fractionation efficiency was determined by measuring acid phosphatase activity as a periplasmic marker enzyme (Atlung *et al*., 1989) and glucose-6-phosphate dehydrogenase as a specific cytoplasmic enzyme (Bergmeyer, 1974).

Murein structure analysis

Murein sacculi were isolated as described previously (Glauner, 1988). After digestion with α-amylase and pronase, the murein was hydrolyzed with cellosyl (kindly provided by Hoechst-Marion-Roussel AG, Frankfurt, Germany). The resulting muropeptides were reduced with sodium borohydride and fractionated by RP-HPLC as described by Glauner (1988).

Determination of soluble murein precursors

To analyze the precursor pools in different bacterial strains, the cells were grown in LB medium containing 10 μCi/ml *meso*-2,6-diamino-[3,4,5-^{3}H] pimelic acid (20 Ci/mmol; ARC) for at least four generations. At an optical density of OD_{578} 1.0 aliquots (1.8 ml) were withdrawn for analysis of the precursors. The cells were chilled rapidly on ice, centrifuged (3 min, 15 000 *g*), washed once with 1 ml ice-cold medium and resuspended in 250 μl ice-cold water. In order to extract the soluble murein precursors the sample was boiled for 10 min. After centrifugation (3 min, 15 000 *g*) the radiolabeled precursors from 100 μl of the supernatant were determined by RP-HPLC on 5 μm Hypersil-ODS (4.6 mm × 125 mm). Chromatography was performed at room temperature at a flow rate of 1 ml/min using 0.05% (w/v) trifluoracetic acid (buffer A) and 60% (w/v) acetonitrile in buffer A. Samples were injected in 1% buffer B and elution started with a gradient from 2 to 3% buffer B for 5 min which was followed by isogratic elution with 3% buffer B for 30 min. The column was washed with 100% buffer B for 5 min and then equilibrated.

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