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Mapping of catalytically important domains in *Escherichia coli* leader peptidase

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Leader peptidase (Lep) is a central component of the secretory machinery of *Escherichia coli*, where it serves to remove signal peptides from secretory proteins. It spans the inner membrane twice with a large C-terminal domain protruding into the periplasmic space. To investigate the importance of the different structural domains for the catalytic activity, we have studied the effects of a large panel of Lep mutants on the processing of signal peptides, both *in vivo* and *in vitro*. Our data suggest that the first transmembrane and cytoplasmic regions are not directly involved in catalysis, but that the second transmembrane region and the region immediately following it may be in contact with the signal peptide and/or located spatially close to the active site of Lep.
Key words: Escherichia coli/leader peptidase/membrane proteins/structure-function relationship

Introduction

In both prokaryotic and eukaryotic cells, proteins destined for secretion are initially made with an N-terminal signal peptide that serves to route the attached polypeptide into the secretory pathway. The signal peptide is subsequently removed by a membrane-bound protease that recognizes a fairly well-conserved pattern of amino acids at the C-terminal end of the presequence (von Heijne, 1986b). In *Escherichia coli*, most signal peptides are cleaved by the enzyme leader peptidase (Lep; also called signal peptidase I), a 323 residue long protein of the inner membrane (Wolfe *et al.*, 1983) which is insensitive to all known protease inhibitors (Zwizinski *et al.*, 1981). Lep has been extensively used as a model for studying the sequence determinants of membrane protein assembly (Wickner, 1988), but the relationships between the structure and the catalytic activity of this important enzyme have remained largely unexplored.

Lep has a somewhat unusual membrane topology (Figure 1) (Moore and Miura, 1987; von Heijne, 1989), with two hydrophobic transmembrane domains H1 and H2, a highly charged cytoplasmic domain P1 between H1 and H2, and a large periplasmic domain P2. H1 and H2 are both required for the correct membrane insertion of Lep, and the overall balance of charged residues between the periplasmic and cytoplasmic parts of the molecule serves as an additional topological determinant (Dalbey and Wickner, 1987; Laws and Dalbey, 1989; von Heijne, 1989; von Heijne *et al.*, 1988).

As a first step towards understanding how these domains contribute to the catalytic activity of Lep, we have developed *in vivo* and *in vitro* assays for monitoring, respectively, the processing of pro-OmpA to mature OmpA and of M13 procoat protein to its mature form, and have determined the kinetics of these signal peptide cleavage reactions as catalyzed by a large number of Lep mutants. Our data suggest that H1 and P1 are not directly involved in catalysis, but that H2 and the region immediately following H2 exert a strong influence on the catalytic activity of Lep and may be in contact with the signal peptide.

Results

In vivo assay for leader peptidase activity

In order to provide a sensitive measure of leader peptidase activity, we have developed a new *in vivo* assay that takes advantage of strain IT41 which has a temperature-sensitive mutation in the chromosomal *lep* gene (Inada *et al.*, 1989). At the non-permissive temperature (42°C), pro-OmpA is processed slowly to OmpA, with a characteristic reaction time t^* ($= 1/k_{app}$) of ~100 s (Figure 2). In contrast, when IT41 is transformed with plasmid pRD8 carrying the wild-type *lep* gene under control of the *ara* promoter (Dalbey and Wickner, 1985; Johnston *et al.*, 1985), the characteristic time for pro-OmpA processing drops >10-fold to $t^* \approx 7$ s, even under non-induced conditions where only very low amounts of Lep are produced. Below, we report t^* values for both uninduced and induced IT41 cells transformed with plasmids expressing different Lep mutants as $t^* = t^*_{unind}/t^*_{ind}$ (in duplicate experiments, the t^* times vary by not more than 10-20%).

The first transmembrane domain is required for assembly but not for catalytic activity

Lep has two transmembrane domains H1 and H2, both of which are needed for its membrane assembly. Deletion of H1 (residues 5-22, mutant Δ5-22) prevents H2 from acting as a signal for the translocation of the P2-domain (von Heijne *et al.*, 1988); accordingly, this mutant is completely inactive *in vivo*, even when induced to high levels ($t^* = 133/118$ s), Table I. The characteristic reaction-time for this mutant is even longer than for non-transformed IT41 cells; this may be related to the observation that expression of the Δ5-22 mutant impairs cell growth (not shown).

Since, in contrast to normal cleaved and uncleaved signal peptides (von Heijne, 1986a,c), H2 is bracketed by an N-terminal negatively charged Glu-residue and a C-terminal positive charged Arg (Figure 1), we tried to restore translocation of P2 in Δ5-22 by changing either Glu61 to Val or Arg77 to Leu (numbering refers to the wild-type sequence). To determine whether these mutants assemble

normally into the membrane, we employed a protease accessibility assay. Cells expressing the mutants were pulse-labeled with [^{35}S]Met for 1 min, treated with lysozyme and EDTA in a high-sucrose buffer to permeabilize the outer membrane, and then incubated with trypsin. Both mutant (Δ5-22,V61) and mutant (Δ5-22,L77) were accessible to trypsin (Figure 3), indicating that the P2 domain was translocated to its normal location on the periplasmic side of the inner membrane in both cases. AraB, a cytoplasmic marker protein, was not digested, showing that there was little if any cell lysis. To our surprise, the (Δ5-22,V61)-mutant was found to be almost indistinguishable from wild-type Lep in terms of catalytic activity (t^* = 16/7 s), demonstrating that H1 has at best a very small effect on catalysis. In contrast, mutant (Δ5-22,L77) is inactive (not shown, cf. below).

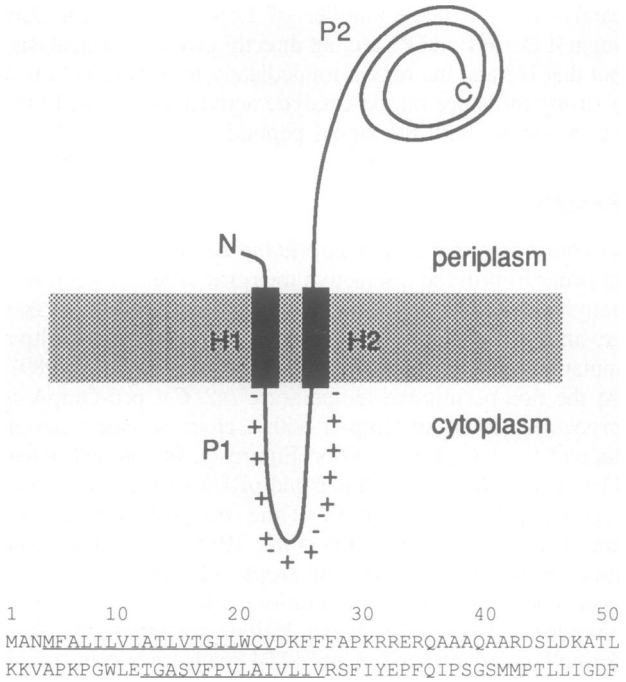

Fig. 1. Domain structure of leader peptidase, and the amino acid sequence of residues 1−100 (regions H1 and H2 are underlined).

The second transmembrane domain has a strong influence on the catalytic activity

To test the importance of H2, we first replaced this domain (residues 62−76) by an unrelated hydrophobic sequence composed of eight (Ala-Leu) repeats. In the protease accessibility assay, >95% of this $(AL)_8$-mutant was digested by trypsin even at the earliest chase point (Figure 4). Thus, the $(AL)_8$-mutant assembles only slightly more slowly than wild-type Lep. Despite this, it has very low enzymatic activity (t^* = 44/30 s).

This result prompted more detailed studies of H2. A short N-terminal deletion, Δ62-65, has no effect on the catalytic activity (t^* = 8/7 s), whereas a longer deletion, Δ62-68, while still allowing normal membrane assembly (Zhu and Dalbey, 1989), was found to impair the catalytic activity slightly (t^* = 22/10 s).

We also lengthened H2 by adding a repeat of the C-terminal residues Val-Leu-Ile-Val between positions 76 and 77 (mutant 76-VLIV-77), thus preserving the sequence at the C-terminal end while adding four residues to the overall length. Again, this has a small but measurable effect on the catalytic activity (t^* = 25/8 s).

The cytoplasmic domain is relatively unimportant for catalysis

Mutant Δ30-52 lacks most of the cytoplasmic P1 domain, yet has a fairly high activity (t^* = 20/7 s). A small deletion, Δ51-57, that removes three lysines and two prolines in front of H2, cannot be distinguished from the wild-type (t^* = 10/10 s). Both mutants assemble normally (Dalbey and Wickner, 1988; von Heijne *et al.*, 1988). Finally, a deletion that removes both H1 and most of P1 (mutant Δ4-50) also has a very high activity (t^* = 13/7 s). This mutant is known to assemble only slightly more slowly than the wild-type (Dalbey *et al.*, 1987; Dalbey and Wickner, 1987).

A critical region immediately downstream of H2

The observation that the double mutant (Δ5-22,L77) assembles in the correct orientation but has no measurable catalytic activity (see above) suggested that Arg77 may be critical for catalysis. Indeed, the L77 mutation alone almost completely inactivates the enzyme (t^* = 74/42 s).

Three further mutations at this position that we have analyzed also have low activities: K77 (t^* = 35/10 s), E77

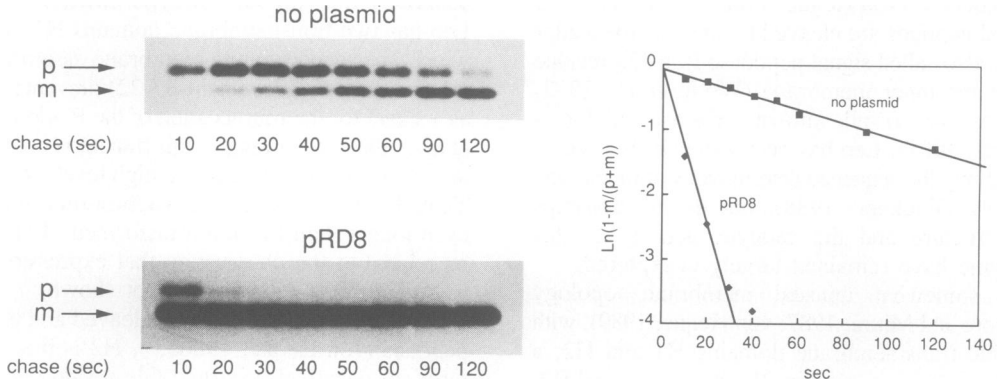

Fig. 2. *In vivo* processing of pro-OmpA in strain IT41 at the non-permissive temperature (42°C) with and without the pRD8 plasmid expressing wild-type Lep. The insert shows a quantitative kinetic plot (see Materials and methods), from which the characteristic reaction times t^*_{IT41} = 100 s, and t^*_{pRD8} = 7 s can be calculated. m = mature OmpA, p = pro-OmpA.

(t^* = 39/13 s), and N77 (t^* = 91/33 s). Interestingly, a double mutant (R74,N77) has a significantly higher activity *in vivo* than N77 (t^* = 45/16 s), whereas a double mutant (N77,R79) with the Arg positioned downstream of N77 is no better than the N77 parent (t^* = 91/33 s). Thus, Arg77 seems to be critical for catalysis, since even a conservative Arg→Lys substitution in this position leads to a major reduction in catalytic activity.

We also wanted to test whether the secondary structure of the region surrounding Arg77 might be critical. Since the membrane-spanning H2 segment is almost certainly α-helical (Jennings, 1989), and since the first strong helix-breaker downstream of H2 is a proline in position 83, we thought it likely that the α-helix might extend at least this far. Some support for the notion that a helical structure throughout this region is important for catalysis comes from the observation that a mutant with a helix-breaking Pro-Gly dipeptide inserted between Val76 and Arg77 (mutant 76-PG-77) has a very low activity *in vivo* (t^* = 68/38 s), much lower than e.g. the corresponding Val-Leu-Ile-Val insertion discussed above. Insertion of Asn, another helix-breaker, in the same position also has a strong negative effect (mutant 76-N-77, t^* = 44/12 s).

Table I. Catalytic activity of leader peptidase mutants

Mutant	*in vitro*	t^* (s) −arabinose	t^* (s) +arabinose	Growth at 42°C −/+ arabinose
Wild-type	+	8	7	++/++
No plasmid	−	90	94	−/−
Δ5-22	−	133	118	−/−
Δ5-22,V61	−	16	7	++/++
$(AL)_8$	−	44	30	+/+
Δ62-65	+	8	7	++/++
Δ62-68	+	22	10	++/++
76-VLIV-77	ND	25	8	++/++
Δ4-50	−	13	7	++/++
Δ30-52	−	20	7	++/++
Δ51-57	+	10	10	++/++
L77	−	74	42	−/−
K77	ND	35	10	++/++
E77	ND	39	13	+/++
N77	−	91	33	−/−
R74,N77	−	45	16	+/+
N77,R79	−	91	33	−/−
76-PG-77	ND	68	38	−/−
76-N-77	ND	44	12	+/+
4-D-5	+	10	7	++/++
4-K-5	−	67	18	−/−

Column 2 gives the results for *in vitro* processing of M13 procoat protein. Columns 3 and 4 give the characteristic reaction-times $t^* = 1/k_{app}$ for the processing of pro-OmpA to OmpA *in vivo* for uninduced and induced IT41 cells (see text). Column 5 gives the growth characteristics at 42°C of IT41 cells transformed with the appropriate plasmid, both with and without arabinose present. ND: not determined.

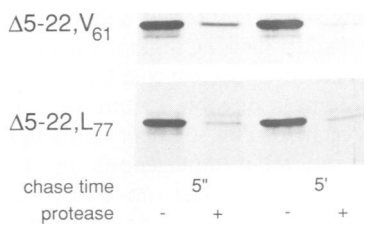

Fig. 3. Mutants (Δ5-22,V61) and (Δ5-22,L77) assemble normally into the plasma membrane. MC1061 cells expressing the mutants were labeled for 1 min with [^{35}S]Met and chased with non-radioactive Met, treated with lysozyme and EDTA in a high-sucrose buffer, and incubated with trypsin or trypsin + trypsin inhibitor + PMSF for 1 h. Samples were acid-precipitated, resuspended in Tris−SDS, immunoprecipitated with antisera to Lep and AraB (a cytoplasmic marker control, not shown), and analyzed by SDS−PAGE and fluorography.

Insertions at the N-terminus can affect the catalytic activity

Given the membrane topology of Lep and the likely positioning of the C-terminal end of a substrate signal peptide close to the periplasmic face of the inner membrane (Kuhn, 1987), it is conceivable that the N-terminus of H1 may be in the proximity of the active site. Of two insertions between Met4 and Phe5 tested so far, one with an aspartic acid inserted has wild-type activity (mutant 4-D-5; t^* = 10/7 s), whereas with a lysine inserted the activity is very low (mutant 4-K-5; t^* = 67/18 s). Both mutants assemble slightly slower than wild-type Lep, as judged by protease accessibility (not shown). In addition, the N-termini of both proteins are blocked to radiosequencing (not shown), suggesting that they are periplasmically located just as in wild-type Lep [Lep mutants with a cytoplasmically located N-terminus are unblocked, cf. von Heijne (1989)].

In vitro processing of M13 procoat protein

Since Lep is active *in vitro* (Dalbey and Wickner, 1987), we also wanted to test whether the activities of the different mutants as measured *in vivo* and *in vitro* correlate. Briefly, the *in vitro* assay is based on a lysate of MC1061 cells transformed with the same plasmids as used in the *in vivo* studies. Radiolabeled phage M13 procoat protein made from a pT712-derived plasmid by *in vitro* transcription/translation is used as a substrate. Typical results obtained with untransformed MC1061 (carrying only the chromosomal *lep* gene) and with arabinose induced MC1061 transformed with plasmid pRD8 (carrying a wild-type *lep* gene) are shown in Figure 5A. With no dilution of the lysate, ~80% of procoat is processed to the mature form under standard conditions when Lep is overproduced from pRD8. The background activity from wild-type Lep produced from the chromosomal gene is about 1/40 of this maximal activity.

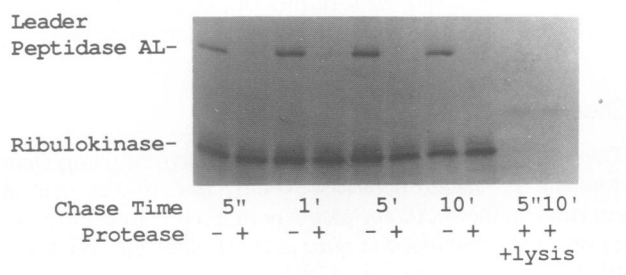

Fig. 4. Kinetics of membrane assembly of mutant $(AL)_8$. HJM114 cells expressing the mutant were labeled for 30 s with ^{35}S-translabel and chased for the indicated times with non-radioactive Met. Samples were transferred into a Tris−EDTA−high-sucrose buffer, incubated with or without trypsin for 1 h, acid-precipitated, resuspended in Tris−SDS, immunoprecipitated with antisera to Lep and AraB (a cytoplasmic marker), and analyzed by SDS−PAGE and fluorogrpahy.

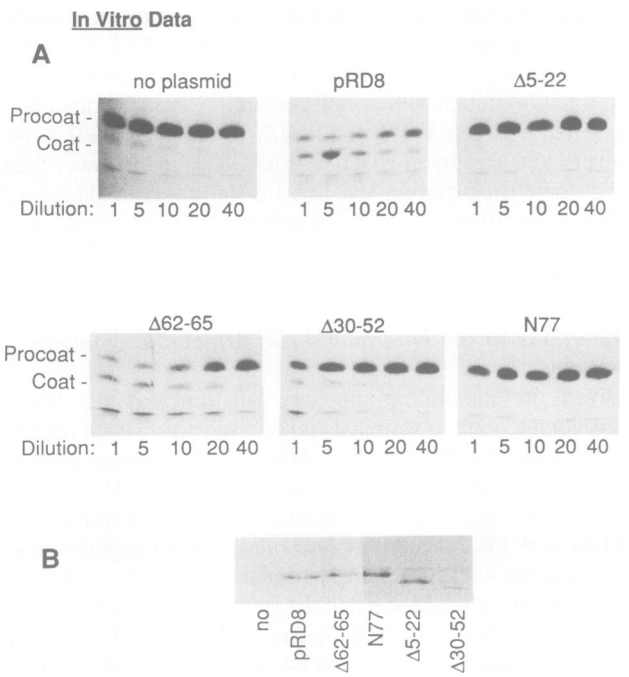

Fig. 5. Processing of phage M13 procoat protein by Lep *in vitro*. (**A**) Processing in crude extracts of *E.coli* MC1061 carrying plasmids expressing different Lep mutants. (**B**) Immunoblotting of MC1061 extracts containing different Lep mutants.

To control for possible differences in expression levels, the amount of each Lep mutant in the cell extract was assayed by immunoblotting (Towbin *et al.*, 1979) using an antiserum to Lep, Figure 5B. The levels of the mutants tested were found to be within a factor of four of each other.

Cell extracts containing mutants Δ5-22, Δ30-52, and N77 all have no more than background activities. An extract containing mutant Δ62-65, on the other hand, has almost full enzyme activity. The *in vitro* data for all the mutants are summarized in Table I. The only apparent differences between the *in vivo* and *in vitro* results are for two mutants where H1 has been deleted (Δ5-22,V61; Δ4-50) and one with a large deletion in P1 (Δ30-52), all three of which have high activities *in vivo* and grow well at 42°C in IT41, but have only background activity *in vitro*. Since all three are slightly but measurably less active than wild-type Lep *in vivo*, it is possible that they score as negatives simply because of the limited sensitivity of the *in vitro* assay. Another possibility is that the processing rates of pro-OmpA and M13 procoat are differently affected by these particular mutations.

Discussion

This study reports the first mapping of catalytically important domains in leader peptidase (Lep), one of the central enzymes in the secretory pathway of *E.coli*. In addition to a previously developed *in vitro* assay (Dalbey and Wickner, 1987), we have used a new, highly sensitive *in vivo* assay to assess the effects of a large panel of deletions, insertions and point-mutations on the catalytic activity of Lep (Table I). In general, the results from the two assays agree well, even though they measure processing of two different proteins (M13 procoat protein and pro-OmpA) under very different conditions. In addition, the growth characteristics of the temperature-sensitive IT41 strain when transformed with the different mutant plasmids (Table I) completely agree with the kinetics of pro-OmpA processing as measured *in vivo*, making us confident that the results obtained for M13 procoat and pro-OmpA are applicable to signal peptide processing in general.

Most of our results can be satisfactorily interpreted in terms of a model which places the active site of Lep in the periplasmic P2 domain, spatially close to the surface of the membrane and to the C-terminal end of the second transmembrane segment H2. While the first transmembrane segment and the cytoplasmic region are clearly not part of the active site, they nevertheless seem to be required for full catalytic activity. Removal of these regions from Lep has small but measurable effects on the enzymatic activity, both *in vivo* and *in vitro*. We suspect that such alterations may affect the distance or orientation of the active site with respect to the membrane and the substrate signal peptide. The observation that insertion of a lysine at the N-terminus of H1 (mutant 4-K-5) has a detrimental effect on signal peptide processing further suggests that this part of the molecule is also in close proximity to the active site.

The precise sequence of segment H2 seems to be more critical. A mutant where H2 has been replaced with an unrelated string of hydrophobic residues has very low activity [mutant $(AL)_8$]. Although the $(AL)_8$ substitution has a large effect on catalysis, a deletion removing the N-terminal half of H2 has only a minor effect, suggesting that the precise sequence of the C-terminal end of H2 may be important. It is possible that the hydrophobic central region of the substrate signal peptide interacts directly with this part of H2, although more experiments are needed to address this point. Earlier *in vitro* experiments have shown that a part of the hydrophobic region of the M13 procoat signal peptide is required for efficient recognition by Lep (Dierstein and Wickner, 1986), also consistent with a direct interaction between Lep and the apolar part of signal peptides.

An important role for residues closely following H2 is suggested by the effects of mutations in and around position 77. When Arg77 is replaced by leucine or asparagine, catalytic activity is almost completely lost (mutants L77 and N77). These mutations in essence extend the uncharged H2 segment from residue 77 up to Glu82, and may in fact pull residues 77–81 into the membrane, suggesting that the active site has to be held at a defined distance from the membrane surface and/or the cleavage site in the substrate signal peptide. Mutants 76-N-77 and 76-PG-77, with one and two helix-breaking residues inserted between the likely helical H2 segment and the flanking region, also have low activities, again pointing to an important role for residues immediately following H2 and a possible requirement for an α-helical structure throughout this segment. Although we have not carried out saturation mutagenesis in this region, the observation that even a conservative Arg→Lys substitution in position 77 is enough to seriously impair the catalytic activity is another indication that residues bordering on H2 are very important. We are currently testing whether the region surrounding Arg77 is primarily involved in the folding of the periplasmic domain, or whether it forms a part of the active site.

In summary, this first study of structure–function relationships in the leader peptidase enzyme points to H2 and its immediate downstream flanking region as being

critical for catalytic activity, consistent with the loop model for the insertion of signal peptides into the bacterial membrane (DiRienzo *et al.*, 1978). We have not yet attempted a similar dissection of the remainder of the periplasmic P2 region, since there are no obvious subdomains or stretches of residues that would serve as good targets for mutagenesis. Of course, catalytically defective mutations in this region can be searched for using classical genetic techniques (Inada *et al.*, 1989), but in the absence of a structural model for this part of the molecule such mutations will be hard to interpret.

Materials and methods

Materials

DNA modifying enzymes were from Promega, Bethesda Research Laboratories, and Pharmacia. [^{35}S]Met was from Amersham. Pansorbin was from Calbiochem.

Bacterial strains and plasmids

E.coli strain IT41, containing a temperature-sensitive amber mutation (*lep-9*) in the *lep* gene is described in Inada *et al.* (1989). MC1061 has been described elsewhere (Dalbey and Wickner, 1986). pING1 plasmid containing the *araB* promoter and the arabinose regulatory elements (Johnston *et al.*, 1985) and cloning of the *lep* gene into the pING1 to produce wild-type plasmid pRD8 were described by Dalbey and Wickner (1985).

Oligonucleotide-directed mutagenesis

Mutagenesis was performed according to the method of Kunkel (Kunkel, 1985) as modified by Geisselsoder *et al.* (1987).

Assay for the processing of pro-OmpA to OmpA in vivo

Overnight cultures, grown at 32°C, were diluted to 1/50 in fresh minimal medium (Miller, 1972) supplemented with 0.5% fructose, 4 μg/ml thiamine, 50 μg/ml of each amino acid except methionine and 150 μg/ml ampicillin. Cells were grown to early log phase at 32°C, whereupon the temperature was shifted to 42°C for 1 h in order to inactivate the temperature-sensitive chromosomally encoded Lep. Plasmid encoded Lep synthesis was induced by adding arabinose to a final concentration of 0.4% and incubating for 5 min. Cells were labeled with 15 μCi/ml [^{35}S]Met for 15 s and cold methionine was added to a final concentration of 0.5 mg/ml. 0.5 ml aliquots were taken at appropriate times and quenched with an equal volume of ice-cold 20% TCA. Precipitated proteins were collected by centrifugation, washed with ice-cold acetone, dried and dissolved in 100 μl 10 mM Tris−HCl pH 7.5 and 2% SDS at 95°C for 5 min. Immunoprecipitation was performed as described in (Wolfe *et al.*, 1982) using OmpA antiserum, immunoprecipitates were adsorbed on Pansorbin cells and washed. Proteins were separated on 12% SDS−polyacrylamide gels (acrylamide:bis,30:0.8) with 6% stacking gels using a discontinuous buffer system. Fluorography was with 1 M Na-salicylate (Chamberlain, 1979). With the help of autoradiograms, circles of 4 mm in diameter were cut from the gels at the center of each pro-OmpA and OmpA band. Gel pieces were dissolved in 150 μl water and 450 μl gel solubilizer (Protosol, DuPont), and counted in 2 ml of LSC-cocktail (Emulsifier Safe, Packard).

Calculation of kinetic rates

pro-OmpA processing rates, k_{app}, were obtained from the slope of $\ln(1-\frac{\text{mature Omp A}}{\text{pro-OmpA + mature OmpA}})$ versus time. The characteristic reaction-times t^* were defined as $t^* = \frac{1}{k_{app}}$.

In vitro synthesis and processing of phage M13 procoat protein

Procoat was synthesized in a cell-free transcription−translation reaction using the pT712 plasmid encoding procoat as a template with major modifications of the procedure of Zalkin *et al.* (1974). In our system, S150-2 was used instead of S-30, and T7 RNA polymerase, as well as Triton X-100 (0.4% final concentration), was added. Each reaction (30 μl) contained DNA template, 30 μCi of Trans [^{35}S]Met, Triton X-100, and S150-2 (Yamane *et al.*, 1987). The reaction was performed at 37°C for 30 min. After synthesis, extracts containing the various Lep mutants were added to the [^{35}S]procoat appropriately diluted in 50 mM Tris pH 8.0 (0.1% Triton X-100), and incubated at 37°C for 30 min. Procoat and coat proteins were resolved on a 23% polyacrylamide gel as described by (Boeke *et al.*, 1980).

Protease accessibility assay

E.coli MC1061 strains transformed with the pING1 vector carrying mutant leader peptidase (*lep*) genes under control of the arabinose promoter were grown at 37°C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose and all amino acids (50 μg/ml each) except methionine. Overnight cultures were diluted 1:35 in 1 ml fresh medium, shaken for 3.5 h at 37°C, induced with arabinose (0.2%) for 5 min, and labeled with [^{35}S]Met (150 μCi/ml). After 1 min, non-radioactive methionine was added (500 μg/ml) and incubation was continued for the indicated chase times or immediately stopped. Cells were chilled on ice, collected by centrifugation, resuspended in ice-cold buffer (40% w/v sucrose, 33 mM Tris pH 8.0), and incubated with lysozyme (5 μg/ml) and 1 mM EDTA for 15 min on ice. Aliquots of the cell suspension were incubated for 1 h on ice, either with 0.75 mg/ml trypsin or with 0.75 mg/ml trypsin + 0.8 mg/ml trypsin inhibitor + 0.33 mg/ml PMSF. Where indicated, cells were lysed by vortexing in 2% Triton X-100 before trypsin treatment. After addition of trypsin inhibitor and PMSF, samples were acid-precipitated, resuspended in 10 mM Tris−2% SDS, immunoprecipitated with antiserum of Lep and AraB (a cytoplasmic marker), washed, and analyzed by SDS−PAGE and fluorography.

Alternatively, HJM114 cells transformed with the appropriate plasmids were grown to early log-phase, induced with arabinose for 30 min, labeled with ^{35}S-translabel (Amersham) for 30 s, and chased for the indicated times with non-radioactive methionine. Samples were transferred to ice-cold buffer A (60 mM Tris pH 8.0, 20 mM EDTA, 40% sucrose) and incubated with or without trypsin for 60 min. Trypsin inhibitor and PMSF were added, samples were acid-precipitated, resuspended in Tris−SDS, immunoprecipitated with antisera to Lep and AraB, and analyzed by SDS−PAGE and fluorography.

Complementation of the ts-phenotype of strain IT41

IT41 cells transformed with the different plasmids were tested for overnight growth on L-broth−ampicillin plates at 32°C and at 42°C.

Radiosequencing

MC1061 transformed with the appropriate plasmid was grown overnight at 37°C in the same medium as above, backdiluted 1:35 into 0.5 ml medium, shaken for 3.5 h at 37°C, induced for 5 min with 0.2% arabinose, labeled for 10 min with 150 μCi [^{35}S]Met, acid- and immuno-precipitated as above, and purified by SDS−PAGE. The wet gel was exposed overnight, and the radioactive band was cut out, extracted with 0.1% SDS by gentle shaking overnight at 37°C, concentrated 10× by lyophilization, and sequenced on an Applied Biosystems Protein Sequencer.

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