Isolation and Characterization of an Escherichia coli Clone Overproducing Prolipoprotein Signal Peptidase*

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Based on the rationale that Escherichia coli cells
containing increased levels of prolipoprotein signal
peptidase would be highly resistant to globomycin, a
specific inhibitor of the prolipoprotein signal pepti-
dase, we have isolated a clone from the Carbon-Clarke
collection, plasmid pLC3-13, which is globomycin-re-
sistant and contains an increased level of prolipo-
protein signal peptidase activity. The plasmid pMT521, a
subclone of pLC3-13 in pBR322, conferred on its host
cells approximately 20 times overproduction of prolipo-
protein signal peptidase and an extremely high level
of resistance against globomycin. The overproduced
proliprotein signal peptidase was completely inhib-
ited by the presence of globomycin in the in vitro assay,
and the overproduced activity was found in the cell
envelope fraction. Several lines of biochemical and
genetic evidence suggest that the gene contained in
plasmid pLC3-13 and its derivative clones is most likely the
structure gene (isp) for prolipoprotein signal pepti-
dase.

Most, if not all, outer and periplasmic proteins in Esche-
richia coli are first synthesized as precursor forms containing
NH2-terminal signal sequences. These signal peptides are
removed by unique endopeptidase(s) called signal (or the)
peptidase(s) either during or immediately after trans-
location of nascent secretory proteins across the membrane.

There is a unique group of exported proteins in bacteria
which are lipoproteins containing covalently-linked lipids.
They include murein lipoprotein (1), the peptidoglycan-asso-
ciated lipoprotein (2), the membrane-bound form of penicil-
linase in Bacillus species (3,4), and the so-called new lipopro-
teins in the E. coli cell envelope (5).

We recently described an in vitro system for the assay of
enzymes which carry out the modification and processing of
prolipoprotein to form the mature lipoprotein (6, 7). We have
shown that (i) the processing of prolipoprotein by prolipo-
protein signal peptidase requires prior modification of prolipo-
protein by glyceride (6) and (ii) the prolipoprotein signal
peptidase is distinct from M13 procot protein signal pepti-
dase (7). Procoot protein signal peptidase has been purified
and extensively characterized by Wickner and his co-workers
(8–11). Although this enzyme can process the M13 procot
protein as well as several other precursor forms of outer
membrane and periplasmic proteins, it is unable to process
prolipoprotein.

The processing of prolipoproteins is specifically inhibited
by a cyclic antibiotic globomycin, and the accumulation of
modified prolipoproteins occurs in globomycin-treated cells
in vivo (12). Furthermore, prolipoprotein signal peptidase
activity is completely inhibited by globomycin in vitro (6).
We reasoned therefore, that E. coli variants containing in-
creased levels of prolipoprotein signal peptidase can be se-
lected by virtue of their increased resistance to globomycin.
Indeed, we have isolated one globomycin-resistant mutant of
E. coli K12 (strain SM31-2B4) which was found to contain 3–
4 times more prolipoprotein signal peptidase activity in the
crude extracts.1 We therefore employed globomycin selection
in an attempt to isolate E. coli strains containing multiple
copies of prolipoprotein signal peptidase gene encoded on
plasmids.

In this paper, we describe the isolation of a prolipoprotein
signal peptidase overproducing clone in the Carbon-Clarke
collection (13), the subcloning of the prolipoprotein signal
peptidase gene (isp) into pBR322, and preliminary character-
ization of E. coli strains containing the amplified isp gene.

MATERIALS AND METHODS

Bacterial Strains and Medium—Bacterial strains, JA200 (F-
ΔtrpE5 recA thr leu lac Y), SM31 (F' supE tonA thr leu rk' mk'-
recBC), KL320 (F' met pro his trp leu 1' 1' rpsL nalY) E609 (HfrC pps),
E610 (HfrC pps mlpA), and JE5505 (F' lpopps his proA argE thi gal
lac ytl mtl txs) were used. L Broth and M9 minimal medium con-
taining 0.2% glucose were used throughout this study. Ampicillin (30 µg/
ml) and tetracycline (10 µg/ml) were used for the selection or identifi-
cation of plasmid containing strains.

Screening of Globomycin-resistant Clones among the Carbon Collec-
tion—Individual clones of the Carbon collection (generous gift of H.
Tabor, National Institutes of Health) were grown in 50 µl of L broth
containing colicin E1 in microtiter plates at 37 °C overnight. Approx-
imately 107 cells of overnight culture were inoculated into 50 µl of L
broth containing 50 µg/ml of globomycin and incubated overnight at
37°C. Globomycin-resistant clones were further screened by the in
vitro prolipoprotein signal peptidase assay described below.

In vitro Prolipoprotein Signal Peptidase Assay—E. coli cells grown
in 300 µl of L broth containing colicin E1 were harvested in s
micro-uge and suspended in 30 µl of lysis buffer (0.1% trypsin, 10
mM EDTA, 20% sucrose, 50 mM Tris-HCl buffer (pH 8.0), and 10 µg
each of DNase and RNase/ml). After a 15-min incubation at 37°C,
270 µl of 50 mM Tris-HCl buffer (pH 7.4) containing 0.25% Nnokol
was added and the tube was mixed by brief vortexing and bath
sonication. The crude homogenate (7 µl) was then added to 3 µl of
a reaction mixture containing glyceride-modified prolipoprotein
(20,000 cpm) (prepared according to Ref. 7). 0.25% Nnokki, 50 mM
Tris-HCl buffer (pH 7.4) and 0.25% B-mercaptoethanol and the
incubation was continued for 60 min at 37°C. Termination of enzyme
reaction, gel electrophoresis, and calculation of prolipoprotein signal
peptidase activity were described previously (7).

Plasmid Preparation and Subcloning of Isp Gene—Plasmid DNA
purified by a NaOH-sodium dodecyl sulfate rapid method (14) was
utilized for plasmid screening, restriction endonuclease analysis, and
transformation. The procedures described by Maniatis et al. (15) were
employed for large-scale plasmid DNA purifcication by cesium chloride

1 M. Tokunaga, J. M. Loranger, and H. C. Wu, manuscript in
preparation.
Overproduction of Prolipoprotein Signal Peptidase

Strains Carrying pLC3-13 Overproduce Prolipoprotein Signal Peptidase—Since globomycin is a specific inhibitor of prolipoprotein signal peptidase, we assumed that overproduction of prolipoprotein signal peptidase would overcome the effect of globomycin and that E. coli strains with an amplified lsp gene would be globomycin-resistant. Among the approximately 2200 clones of the Carbon collection, the clone carrying plasmid pLC3-13 was found to be globomycin-resistant. This was confirmed by the in vitro prolipoprotein signal peptidase assay which revealed that this clone, JA200 (pLC3-13), indeed contained 4–5 times higher prolipoprotein signal peptidase activity (Fig. 1A, lane 1) as compared with the parental strain (Fig. 1B, lane 4). When strain JA200 was cured of the pLC3-13 plasmid, both globomycin sensitivity and prolipoprotein signal peptidase activity were restored to normal levels (Fig. 1A and B, lane 3). Other E. coli strains transformed by plasmid pLC3-13 became globomycin-resistant and contained increased levels of prolipoprotein signal peptidase (Fig. 1, A and B, lanes 2 and 5).

Subcloning of Prolipoprotein Signal Peptidase Gene into Plasmid pBR322—The restriction map of pLC3-13 is shown in Fig. 2. Plasmid pLC3-13 DNA was digested by restriction endonuclease EcoRV yielding two fragments (10.4 and 9.0 kb). EcoRV-digested pLC3-13 DNA was ligated with pBR322 DNA which had been digested with the same enzyme, and the ligated DNA was used to transform strain SM31. SM31 was used as the host for transformation with subcloned plasmids because of the high efficiency of transformation in this strain. Amp‘ Tet‘ transformants were selected and further screened for resistance to globomycin (50 μg/ml). One of these clones (containing plasmid pMT503) was found to have an insertion of the larger fragment (10.4 kb) of pLC3-13 into the EcoRV site of pBR322 (Fig. 2). The E. coli strain containing pMT503 showed the expected phenotypes, Amp‘, Tet‘, and Gmb‘ and contained increased levels of prolipoprotein signal peptidase activity as determined by the in vitro assay.

The plasmid pMT503 DNA was totally digested by NruI and religated by T4 DNA ligase. Although the religation at the NruI site was incomplete, about half of the Amp‘ Tet‘ Gmb‘ transformants obtained by transformation of strain SM31 with the religated DNA were found to contain plasmid DNA of a smaller size (8.5 kb) than that of pMT503. This smaller plasmid, designated pMT521, resulted from the deletion of 6 kb of the ColE1 region and 0.8 kb of pBR322 DNA from pMT503 (Fig. 2). The strains carrying pMT521 contained approximately 20 times higher prolipoprotein signal peptidase activity than the parental strains (data not shown). The prolipoprotein signal peptidase activity overproduced in strains carrying pMT521 was completely inhibited by globomycin in the in vitro assay (Fig. 3). Furthermore, all prolipoprotein signal peptidase activity in JE5505 (pMT521) was found in the cell envelope fraction (Fig. 4) which corresponds to the subcellular localization of prolipoprotein signal peptidase in wild type cells (16).

pMT503 DNA was totally digested by BamHI and religated by T4 DNA ligase resulting in the construction of plasmid pMT522 which contains further deletion of 2 kb of E. coli DNA (Fig. 2). The deletion of the 2-kb E. coli DNA fragment from pMT503 resulted in the loss of both the globomycin-resistance phenotype and increased levels of prolipoprotein signal peptidase. It is clear, therefore, that this 2-kb region must contain the promoter and/or part of the prolipoprotein signal peptidase structural gene.

Globomycin Sensitivity of E. coli Strains Harboring Plasmid

The abbreviations used are: kb, kilobases; PLP, prolipoprotein; LP, lipoprotein; Amp‘, ampicillin-resistant; Tet‘, tetracycline-sensitive; Gmb‘, globomycin-resistant.

**FIG. 1.** Globomycin sensitivity and prolipoprotein signal peptidase activity in the strains carrying plasmid pLC3-13. A, maximum globomycin concentration in which these strains can grow; B, prolipoprotein signal peptidase activity; lane 1, JA200 (pLC3-13) from Carbon collection; lane 2, JA200 transformed with plasmid pLC3-13 isolated from strain in lane 1; lane 3, plasmid pLC3-13 was cured from strain in lane 1; lane 4, JA200; lane 5, KL320 (pLC3-13); lane 6, KL320; lane 7, no enzyme control for signal peptidase assay.

**FIG. 2.** Restriction map of plasmid pLC3-13 and construction of the plasmids pMT503, pMT521, and pMT522. Col E1 DNA region; E. coli DNA region; pBR322 DNA region.

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2 The abbreviations used are: kb, kilobases; PLP, prolipoprotein; LP, lipoprotein; Amp‘, ampicillin-resistant; Tet‘, tetracycline-sensitive; Gmb‘, globomycin-resistant.
lethality is the accumulation of modified murein prolipoprotein in treated cells (12). However, while the murein lipoprotein is the most abundant lipoprotein in E. coli and is therefore the primary target of globomycin action, other relatively minor lipoprotein species also accumulate as their glyceride-modified precursor forms in globomycin-treated cells (5). The fact that JE5505 (pMT521) is much more resistant to globomycin than JE5505 strongly suggests that these secondary target(s) of globomycin action are responsible for the eventual globomycin-mediated lethality of JE5505. Furthermore, it provides further support that prolipoprotein signal peptidase is responsible for the processing of all these prolipoproteins. The same explanation can be applied to the results obtained with strains E610 (mplA) (18) and E610 (pMT521).

Strain SM31-2B4 was selected by globomycin treatment of SM31, and it presumably contained a spontaneous chromosomal mutation resulting in an increase level of prolipoprotein signal peptidase activity. Because of the apparent overproduction of prolipoprotein signal peptidase in mutant SM31-2B4 as compared to strain SM31, it is globomycin-resistant (120 µg/ml). Introduction of pMT521 into SM31-2B4 resulted in further overproduction of prolipoprotein signal peptidase and increased resistance to globomycin (>300 µg/ml).

**DISCUSSION**

We have identified among the Carbon-Clarke collection of E. coli genomic fragments a plasmid pLC3-13 which confers upon its host cell increased levels of prolipoprotein signal peptidase activity and a concomitant increase in globomycin resistance. Several lines of evidence suggest that the gene contained in pLC3-13 and its derivative clones is most likely the structural gene for prolipoprotein signal peptidase. The evidence supporting this conclusion is as follows. 1) The increased prolipoprotein signal peptidase activity in strains containing pMT521 is sensitive to globomycin in vitro (Fig. 3). 2) The gene product is a membrane-bound enzyme (Fig. 4). 3) The prolipoprotein signal peptidase activity in strains containing the cloned gene has the same mobility in nondenaturing gel as the enzyme present in the parental strain (data not shown). These properties are the same as observed for the prolipoprotein signal peptidase present in strains not harboring the plasmid (6, 16). 4) The crude extract of strains containing pMT521 does not contain any activity which would inactivate globomycin irreversibly (data not shown). 5) Genetic evidence indicates that the E. coli gene(s) in pMT521 is located approximately at 0.5 min of the E. coli map. This is the nearly the same location as the gene altered in the ls signal peptidase mutant reported recently (19). 6) Strains harboring an F'-plasmid (F'101) containing E. coli genes between thr and leu loci were found to contain 1.7 times prolipoprotein signal peptidase activity, as compared to the strains cured for the F'101 plasmid. The apparent gene dosage-prolipoprotein signal peptidase activity relationship observed among strains containing F'-plasmid, ColEl-plasmid, and pBR322 derivatives with the lwp gene strongly suggests that the cloned gene is the structural gene for prolipoprotein signal peptidase.

The availability of this plasmid will now permit a detailed study of the genetic organization of the lwp gene. In addition, the subcloning of the lwp gene into a runaway plasmid would facilitate the biochemical and physiological studies of this unique enzyme.

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**TABLE I**

Globomycin sensitivity of signal peptidase overproducing strains

| Strains | Globomycin sensitivity µg/ml |
|---------|-----------------------------|
| E609 (pMT521) | >300 |
| E609 | 20 |
| E610 (pMT521) | >300 |
| E610 | 40 |
| JE5505 (pMT521) | >300 |
| JE5505 | 120 |
| SM31-2B4 (pMT521) | >300 |
| SM31-2B4 | 120 |

*Maximum globomycin concentration in which these strains can grow.

pMT521—Plasmid pMT521 was used to transform E. coli strains E609, E610 (mplA), JE5505 (ipo), and SM31-2B4 (a spontaneous globomycin-resistant mutant of strain SM31). All transformants were extremely resistant to globomycin (>300 µg/ml) as compared to their parental strains regardless of the particular genetic background of these strains (Table I).

The murein lipoprotein structural gene is deleted in strain JE5505 (17). Accordingly, this strain is globomycin-resistant (120 µg/ml) since the major cause of globomycin-mediated
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REFERENCES
1. Braun, V. (1975) Biochim. Biophys. Acta 415, 355–377
2. Mizuno, T. (1979) J. Biochem. (Tokyo) 86, 991–1000
3. Lai, J. S., Sarvas, M., Brammar, W. J., Neugebauer, K., and Wu, H. C. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3506–3510
4. Chang, C. N., Nielsen, J. K., Izu, K., Blobel, G., and Lampen, J. O. (1982) J. Biol. Chem. 257, 4340–4344
5. Ichihara, S., Hussain, M., and Mizushima, S. (1981) J. Biol. Chem. 256, 3125–3129
6. Tokunaga, M., Tokunaga, H., and Wu, H. C. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2255–2259
7. Tokunaga, M., Loranger, J. M., Wolfe, P. B., and Wu, H. C. (1982) J. Biol. Chem. 257, 9922–9925
8. Zwizinski, C., and Wickner, W. (1980) J. Biol. Chem. 255, 7973–7977
9. Date, T., and Wickner, W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6106–6110
10. Wolfe, P. B., Silver, P., and Wickner, W. (1982) J. Biol. Chem. 257, 7898–7902
11. Silver, P., and Wickner, W. (1983) J. Bacteriol. 154, 569–572
12. Hussain, M., Ichihara, S., and Mizushima, S. (1980) J. Biol. Chem. 255, 3707–3712
13. Clarke, L., and Carbon, J. (1976) Cell 9, 91–99
14. Brinboim, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523
15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Tokunaga, M., Loranger, J. M., and Wu, H. C. (1983) J. Cell. Biochem., Suppl. 7B, 348
17. Hirota, Y., Suzuki, H., Nishimura, Y., and Yasuda, S. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1417–1420
18. Lai, J. S., Philbrick, W. M., Hayashi, S., Inukai, M., Arai, M., Hirota, Y., and Wu, H. C. (1981) J. Bacteriol. 145, 657–660
19. Yamagata, H., Ippolito, C., Inukai, M., and Inouye, M. (1982) J. Bacteriol. 152, 1163–1168
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