KOR. JOUR. MICROBIOL, December, 1988 p. 283-290 Vol. 26. No. 4

Characterization of a Revertant that Restores the Export of Ribose-Binding Protein to the Periplasm in *Escherichia coli*

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리보스 결합 단백질을 페리플라슴으로 수송하는 복귀변이주의 분석

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ABSTRACT: A spontaneous revertant of mutation *rbsB103* that is ribose taxis-positive was characterized. This revertant was found to be export-competent in the export of ribose-binding protein shown by the disappearance of accumulated mutant precursor protein and the export of mature ribose-binding protein to the periplasm. The reversional change was shown to be in the region of *risB* gene that codes for the amino terminal portion of ribose-binding protein. Analysis by high-performance liquid chromatography of peptide patterns of ribose-binding proteins confirmed the relationship between the wild-type and the revertant proteins as shown for the mutant previously (Iida *et al.*, 1985). When the processing rate of presursor proteins from the wild type and the revertant strain *in vivo* was compared by pulse-chase experiment, it was found that processing is less efficient than normal in the revertant. Purified mature proteins from both wild-type and revertant were subjected to amino acid sequencing. The results confirmed the amino acid changes deduced from the DNA sequencing and showed that processing of the revertant precursor occurred in the correct position even though there are two different amino acids present in the signal sequence.

KEY WORDS □ *E. coli*, Protein Export, Ribose-binding protein, Revertant

The molecular mechanism of how exported proteins reach their final destination has been studied intensively during the past few decades by biologists working with many different proteins from numerous organisms. A lot of information were obtained and some of them were reviewed (Park, 1988).

We have been studying the mechanism of protein export in prokaryotes using ribose-binding protein as a model system. Ribose-binding protein (29kd) is a periplasmic protein of *E. coli* that functions in transport of ribose into the cell and in chemotaxis toward that sugar. Thus, this protein should be tranlocated across the cytoplasmic membrane. The gene *rbsB*, which codes for the ribose-binding protein, is part of an operon that contains genes coding for components of the Rbs transport system as well as *rbsK*, the gene for ribokinase (Iida *et al.*, 1985). It has been shown that ribose-binding protein is translocated through the cytoplasmic membrane posttranslationally (Randall, 1983).

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This work was supported by a grant from GM29798 to Linda L. Randall.

A ribose-taxis negative mutant was isolated and characterized as an export-defective mutant that accumulates unprocessed, precursor form of ribose-binding protein (32kd) in the cytoplasm, further as carrying a signal sequence mutation (*rbsB103*) in *rbsB*. The mutation was shown to cause a change of amino acid leucine to proline in the signal sequence (Iida *et al.*, 1985).

In addition, a revertant of *rbsB103* that restored the chemotactic response toward ribose was isolated from a strain carrying mutation *rbsB103* on a plasmid pACYC184. Here, We report several characteristics of this revertant using biochemical and recombinant DNA techniques.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this work are all derivatives of *E. coli* (Table 1).

Materials (Chemicals and Enzymes)

L-(^{35}S) methionine (> 1000Ci/mmol) was purchased from New England Nuclear Corp., Boston. MA. Chloramphenicol, lysozyme, protease type XI (proteinase K), and phenylmethyl-sulfonyl fluoride were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Enzymes for recombinant DNA techniques were purchased from New England Biolabs, Inc., Beverly, Mass. or Bethesda Research Laboratories, Inc., Gaithersburg, MD. Agarose for gel electrophoresis was purchased from Bio-Rad.

Media

LB medium and tryptone broth were prepared as described by Miller (1972). M9 minimal salts medium were prepared as described by Randall (1983) with supplement of 0.4% glycerol or 0.4% ribose. Minimal swarm plates containing ribose were made from 10 mM KPO_4 (pH 7.0), 1 mM MgSO4, 1 mM $(NH_4)_2SO_4$, 0.1 mM ribose and amino acid complements at 0.1 mM (Harayama *et al.*, 1982). Sugars were D form and amino acids were L form.

Biochemical Analysis

Labelling the cells with ^{35}S-methionine and chasing with cold methionine, immunoprecipitation and limited proteolysis, cold osmotic shock, spheroplast formation using Tris, EDTA and lysozyme, SDS-polyacrylamide gel electrophoresis and autoradiography (Randall, 1983), preparation of minicells were used as described.

Table 1. *Bacterial Strains and Plasmids.*

Strain/Plasmid	Relevant genotype, Source, Comment, Reference
Bacteria	
UH889	*minA minB rpsL mgl*(?) *rbsB102::Tn10 recA* (1)
AI179	UH889 (pAI12) (1)
AI287	UH889 (pAI27) (1)
HB735S	UH889 (pAI27S) (1)
SP110	UH889 (pSP106), this study
AI392	*minA minB rpsL mgl*(?) *rbsB102::Tn10* Kinase positive by a secondary mutation relieving polarity of Tn*10* on *rbsK* (1)
SP114	AI392(pSP104), this study.
Plasmids	
pACYC184	Chang and Cohen (1978) via K. Tanimoto
pAI12	*Hind*III fragment including *rbsC, rbsB* and *rbsK* cloned into pACYC184 (1)
pAI27	Like pAI12 but with *rbsB103* (1)
pAI24	Constructed by deleting *Pvu*II fragment from pAI12
pSP106	Like pAI27 but with *rbsB106,* this study
pSP104	Recombinant plasmid constructed from *Hind*III-*Bst*XI fragment of pSP106, this study

1. Described in Iida *et al.* (1985) in detail.

Recombinant DNA Technique

Isolation of DNA, enzyme reactions, agarose gel electrophoresis and transformation were done by the methods described by Maniatis *et al.* (1982).

In vitro Recombination

In vitro Recombination was carried out by exactly the same way as done for mutant characterization (Iida *et al.*, 1985).

DNA Sequencing

Sequencing was done by collaboration with J. Groarke (Purdue Univ.). The procedure of Maxam and Gilbert was used to determine the nucleotide sequence of isolated restriction fragments of DNA as described by Groarke *et al.* (1983).

Analysis of Tryptic Peptides

Bands of radiolabeled ribose-binding protein excised from dried gels were analyzed as described (Iida *et al.*, 1985).

Protein Sequencing

Protein sequencing was done with either purified proteins or proteins excised from gels. In the latter case, the protein was removed from the gel by electroelution (Hunkapiller *et al.*, 1983).

Sequence analysis of the amino-terminal portion of purified proteins were performed with a Beckman 890C sequencer according to the method described in Iida *et al.* (1985).

RESULTS

A Second Mutation in *rbsB* Suppresses the *rbsB103* Phenotype

We isolated a spontaneous Rbs-positive (taxis) derivative of an Rbs-negative strain harboring pAI27, the plasmid carrying *rbsB103*. This mutation has been shown to cause the export defect of ribose-binding protein to the periplasm (Iida *et al.*, 1985). The Rbs-positive derivative exhibited normal ability to form a tactic ring on a chemotactic swarm plate containing minimal salts and 0.1 mM ribose, indicating normal functioning of the high affinity transport and chemoreception systems for ribose (see below).

Comparison of Swarm Phenotype Among Wild-type, Mutant, and Revertant Strain

Strains, AI179, AI287, and HB735S, that are wild-type, mutant, and revertant, respectively, were grown to check their swarm phenotypes as described in Fig. 1 legend. As shown in Fig. 1a, the wild-type strain formed clear swarm ring around the place where a colony was initially inoculated. While the mutant strain did not make any ring (Fig. 1b), the revertant derived from this mutant formed clear swarm ring that looks almost like one formed by the wild-type strain (Fig. 1c). UH889 (Fig. 1d) that is a host strain for the plasmids did not make any ring.

Thus, this spontaneously isolated revertant strain was further characterized to map the reversional mutation.

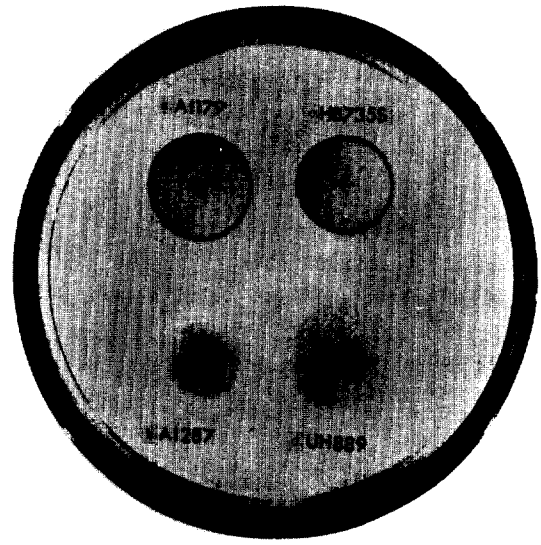

Fig. 1. *Tactic ring phenotype of wild-type, mutant, and revertant strain.*
Cultures of AI179, AI287 and HB735S were grown on the minimal medium plate supplemented with 0.4% glycerol and 0.4% ribose to get single colonies. Single colony from each strain was inoculated using a tooth pick on the minimal swarm plate by slightly touching the surface of the plate. The plate was incubated in the 37°C incubater overnight. The incubater was supplied with moist. a. wild-type swarm ring formed by strain AI179. b. mutant strain did not make a swarm ring. c. revertant swarm ring formed by strain HB735S. d. host strain, UH-889, for plasmids carrying *rbsB* derivatives.

Mapping The Reversional Mutation as a Mutation in *rbsB* Gene

The location of reversional change was first identified by isolation of the plasmid from the spontaneous Rbs-positive (taxis) derivative and by transformation of the Rbs-negative strain, UH889 with this plasmid. The plasmid conferred the Rbs-positive phenotype on this strain, thus the reversional change was located in this plasmid, designated as pSP106 (data not shown). The minicell strain transformed with this pSP106 was named as SP110. The ribose-binding protein produced in the minicell strain carrying the reverted gene resembled wild-type protein in its apparent molecular weight (Fig. 2). The culture carrying pSP106 that was grown in minimal medium supplemented with 0.4% ribose to induce the produc-

Fig. 2. *Ribose-binding proteins synthesized from minicells carrying plasmids, pAI12, pAI27, and pSP106.*
Cultures of strains, SP111, SP112 and SP113, were grown and minicells were isolated, labeled and analyzed as described by Iida *et al.* (1985). The positions for precursor and mature ribose-binding protein are indicated by p and m, respectively. 1. SP112 produced only precursor protein. 2. SP111 produced both mature and precursor proteins. 3. SP113 produced both mature and precursor proteins.

tion of ribose-binding protein was subjected to immunoprecipitation. Both 29 kd and 32 kd proteins from the revertant strain were crossreacted to antibody against ribose-binding protein suggesting these are ribose-binding proteins (data not shown).

Analysis by limited proteolysis using *Staphylococcus aureus* V8 protease revealed that the 29-kd polypeptide exhibited the same pattern of proteolytic fragments as authentic mature ribose-binding protein (Iida *et al.*, 1985).

Analysis of Tryptic Peptides from Proteins Produced by the Revertant Strain

The relationship of proteins produced in the revertant strain to the authentic mature and precursor forms of ribose-binding protein was investigated by the same way as for mutant precursor protein.

Strains, AI179, AI287, and SP110 carrying the wild-type, mutant, and revertant *rbs*B genes, respectively, were grown in M9 medium, long-term labeled with ^{35}S-methionine, processed for immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis as described (Randall, 1983). Both wild-type and revertant strain produced mature ribose-binding protein (29 kd) and precursor ribose-binding protein (32 kd), while the mutant strain synthesized only a 32 kd protein (data not shown).

Bands of radiolabeled ribose-binding protein were excised from dried polyacrylamide gels and processed for peptide analysis as described (Iida *et al.*, 1985). The pattern of ^{35}S-methionine-labeled peptides derived from the 29 kd species found in revertant cells (Fig. 3B) contained all peaks (labeled 1-8) characteristic of mature ribose-binding protein. There are four additional peptide peaks (p1-p4) characteristic of precursor ribose-binding protein. The apparent shift of peptides corresponding to peptides p3 and p4 of the authentic precursor to positions for less hydrophobic peptides and marked by asterisks in Fig. 2C in mutant precursor has been interpreted to be consistent with an amino acid alteration in the hydrophobic region of the signal sequence of the mutant protein (Iida *et al.*, 1985). In the revertant) precursor protein these two peptides were gone from the positions taken by mutant precursor derived peptides. Instead, it looked that they are eluted at the positions by circled asterisks for more hydrophobic peptides taken by the peptides in the authentic precursor. This suggested the hydrophobicity of the signal sequence in the precursor protein is important for normal export.

The peptide maps confirm the immunological identification of the 29 and 32- kd polypeptides as forms of ribose-binding protein and establish that the 32-kd species carries an uncleaved signal sequence. Thus, the revertant strain processed precursor ribose-binding protein to the mature form.

Fine Mapping of *rbsB106* Mutation by *in vitro* Recombination of the Gene

Instead of sequencing the entire *rbsB* gene on pSP106, we decided to map the relative position of the mutated site within the gene as done for mutant plasmid, pAI27. This mapping was done by

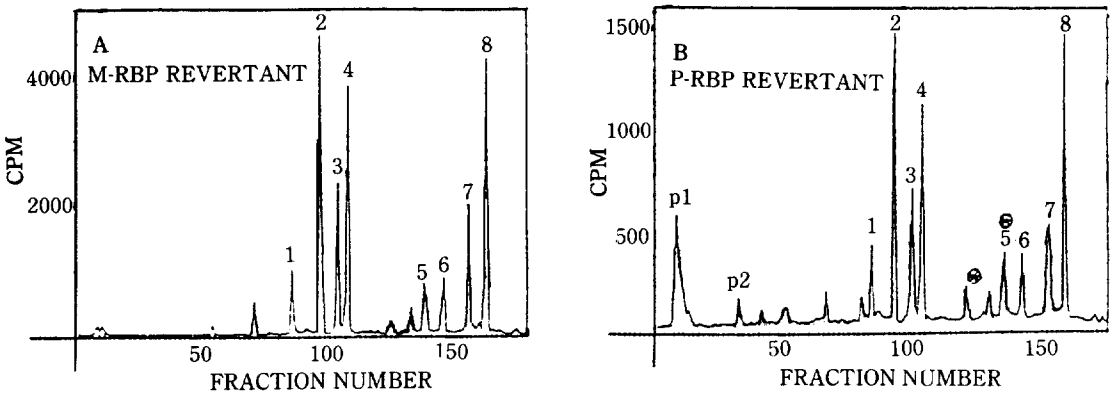

Fig. 3. *Peptide maps of ribose-binding protein from wild-type, mutant, and revertant strains.*
Bands of immunoprecipitated species of ribose-binding protein, labeled with ^{35}S-methionine, were excised from dried gels after SDS-polyacrylamide gel electrophoresis, digested with trypsin and the resulting peptides analyzed by reversed phase H.P.L.C. using a C-18 column. Peaks characteristic of the pattern derived from mature ribose-binding protein are numbered and additional peaks found in the pattern characteristic of precursor are labeled with a 'p' plus a number. Patterns derived from mature and precursor protein synthesized in UH889 harboring pAI12 which carries wild-type *rbsB* or pAI27 which carries *rbsB103* have been shown (Iida *et al.*, 1985) Panels A and B show the patterns obtained for 29 and 32 kd proteins from revertant proteins. These are analyzed as described for wild-type and mutant proteins with following modifications: 0-100% gradient, 0.5 m*l* fraction.

construction *in vitro* of a recombinant gene containing a *Hind*III-*Bst*XI segment which contains the first 30 codons of the *rbsB* gene from revertant strain joined to the rest of the gene from the wild type strain (Materials and Methods).

This constructed plasmid was transformed into AI392 and strain SP114 was obtained. The strain containing this plasmid (SP114) produced mature ribose-binding protein (data not shown). Thus, the *rbsB106* mutation was mapped to the *Hind*III-*Bst*XI fragment that contains the region encoding the amino-terminus of precursor ribose-binding protein.

Determination of the Mutational Change in *rbsB106*

The nucleotide sequence of the segment of the *rbsB* gene that is contained in the *Hind*III-*Bst*XI fragment of pSP106 was determined on both the coding and the complementary strands. The T to C transition that causes the *rbsB103* phenotype was still present and, in addition, there was a C to T transition that resulted in substitution of phenylalanine for serine at position −15 in the signal sequence (Iida *et al.*, 1985). The presence of the original mutation was confirmed by analysis using *Bst*NI restriction enzyme (data not shown). The serine to phenylalanine change should increase the hydrophobicity of the signal sequence, thus restoring the proper (critical) amount of hydrophobicity to the signal and thereby suppressing the mutant phenotype caused by the initial leucine to proline substitution. Tryptic peptides analysis confirmed this suggestion.

Localization of Ribose-Binding Proteins from Revertant Strain

Cells from wild-type and revertant strains were subjected to osmotic shock to determine the location of the mature and precursor forms of ribose-binding protein. Both wild-type and revertant strains were grown in minimal medium as described (Randall, 1983)

The matured form of the protein from the revertant was found in the periplasmic fraction like the authentic mature ribose-binding protein (Fig. 4), while precursor proteins from both strains were localized to the cytoplasm (data not shown). Thus, in the revertant, the second mutation in the signal sequence suppressed the export defect caused by the original mutation in the signal sequence, so that the precursor is processed

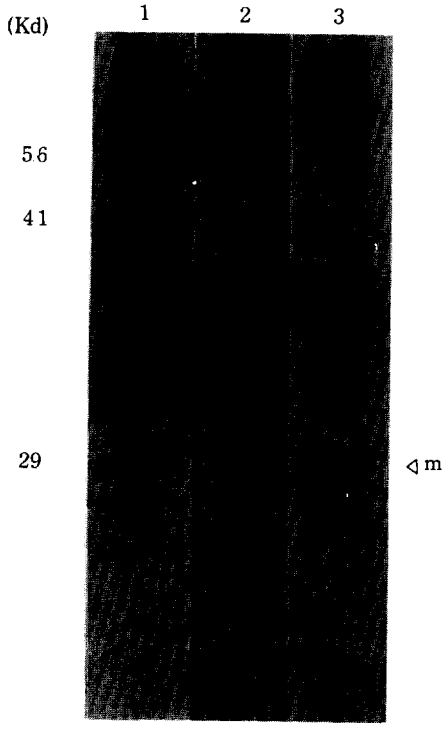

Fig. 4. *Cellular location of ribose-binding protein synthesized in the revertant strain.*
Strains, AI179 and SP110 carrying wild-type and revertant *rbsB* genes, respectively, were grown and subjected to osmotic shock to release periplasmic protein. Periplasmic fractions from these two strains were analyzed on SDS-polyacrylamide gels. This gel is stained with Coomassie blue. Lane 1: molecular weight markers. Lane 2,3: periplasmic fractions from wild-type and revertant strain, respectively. The position for mature ribose-binding protein is indicated by m.

and exported into the periplasm efficiently.

Comparison of the Processing Rate of Precursor Proteins from Wild-type and Mutant Strain

Wild-type and revertant strain were pulse-labeled and chased to compare the processing rate. The processing was less efficient than normal in the revertant as shown by the presence of precursor after chase (Fig. 5).

Amino Acid Sequences of Ribose-binding Proteins

We asked whether the revertant precursor ribose-binding protein was processed at the correct site to generate a mature species with the same amino terminus as that of the wild-type

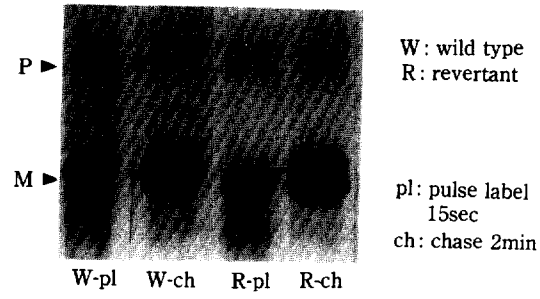

Fig. 5. *Comparison of processing rate between the wild-type and the revertant strain.*
Strains, AI179 and SP110 were grown and labeled with ^{35}S-methionine and chased with cold methionine. The positions for precursor and mature ribose-binding protein are indicated by P and M, respectively.

ribose-binding protein. It is known that the leader peptidase (which is coded by the *lep* gene and is responsible for the processing of M13 phage procoat protein) can process the signal sequences of various proteins *in vitro*. It has been suggested that this leader peptidase recognizes a specific conformation around the processing site, not the specific amino acids. We asked whether the enzyme that does process ribose-binding protein *in vivo* recognizes only the amino acids near the processing site.

It seems possible that two different amino acid changes in the signal sequence in the revertant precursor protein might affect the conformation around the processing site resulting in the incorrect processing. The sequences of the first several amino terminal amino acids from wild-type and revertant mature ribose-binding protein were determined. The results are shown in Fig. 6. The mature proteins from both the wild type and revertant begin with lysine and then asparatate, indicating that two different amino acids present in the revertant precursor does not affect the processing site, that is, the processing enzyme cleaves at the correct site. However, the efficiency of processing might be different as is implied by the fact that precursor protein accumulates more in the minicell strain producing revertant ribose-binding proteins and not the wild-type strain.

processing ↓ (between −1 and 1)

	-25								-17		-15		-1	1		3
1 DNA seq. :	ATG	AAC	ATG	AAA	AAA	CTG	GCT	ACC	CTG	GTT	TCC	....	GCA	AAA	GAC	ACC
2 A.A. seq.d :	MET	ASM	MET	LYS	LYS	LEU	ALA	THR	LEU	VAL	SER	....	ALA	LYS	ASP	THR
														1	2	3
3 W.T. seq.m :														LYS	ASP	GLU
4 REVERT.m:														LYS	ASP	GLU

Fig. 6. *Amino acid sequence analysis of purified ribose-binding proteins.*
Line 1: DNA coding for signal sequence of wild-type precursor protein and N-terminus of mature protein. Line 2: amino acid sequences deduced for signal sequence and N-terminus of wild type precursor protein, Line 3 and 4: amino terminal amino acid sequences determined for wild-type mature protein and revertant mature protein, respectively.

DISCUSSION

Hydrophobicity Change in the Revertant Signal Sequence Predicted Was Confirmed by Tryptic Peptides Analysis

We have reported the characteristics of *rbsB103* mutation and *rbsB106* reversional mutation partly (Iida *et al.*, 1985). There we predicted the amino-acid changes in the signal sequences deduced from DNA sequencing and discussed the amino-acid change in the revertant signal sequence would increase the hydrophobicity.

In this paper, we report the hydrophobicity of the revertant signal is actually changed to gain hydrophobicity in the tryptic peptides, p3 and p4, derived from signal sequence using H.P.L.C.

Even though p3 and p4 in the revertant were not eluted at the positions for mutant-derived p3 and p4, we noticed that their positions were not matched exactly to the positions for p3 and p4 that derived from wild-type signal sequence but even overlapping to peaks derived from mature part of protein. We think this reflects the minor differences in the hydrophobicity of tryptic peptides derived from signal sequences between functional wild-type and revertant by the presence of two different amino acids can be resolved by the high sensitivity of the H.P.L.C. system. The patterns shown were reproducible and characteristic of the respective polypeptides. However, more ^{35}S-labeled peaks are observed than predicted from the known amino acid sequence (Groarke *et al.*, 1983), presumably because of the resistance of ribose-binding protein to complete digestion by trypsin under the conditions used.

Mutations in the Revertant Signal Sequence Did Not Affect the Processing Site But Affected the Processing Rate Slightly

Determination of the amino acid sequence of N-terminus of mature ribose-binding protein, located in the periplasm of the revertant strain, showed that this protein had the same amino terminus as the wild-type protein thus its leader peptide had been processed correctly. Leader peptidase, product of *lep* gene, has been shown to cleave leader peptides of many precursor proteins thus its specificity for substrate seems broad. When amino acid sequences around cleavage site were sequenced, a common feature was shown such as the presence of amino acids having short side chains, specially alanine, glycine at −1 and −3 and helix-breaking proline at −6 positions related to the processing site are preferred. However, the actual sequences are not conserved. Thus, it was suggested that the secondary structure in this region is important. Mutations in the other regions other than these sites were shown not important (Emr and Bassford, 1982). As shown in the processing of revertant precursor ribose-binding protein, two amino acids change in the −15 and −17 positions did not affect the processing site implying that these mutations did not affect the secondary structure of this region. However, as shown in the comparison of processing rate to

wild-type precursor protein, these changes slowed the processing rate either by the slow translocation or by the inefficient processing.

적 요

리보스에 대한 화학주성이 결핍되고 리보스 결합 단백질의 수송 결핍으로 전구체 단백질이 세포질내에 축적된 *rbsB* 103 신호배열 돌연변이에 대해서는 이미 보고한 바 있다(Iida *et al.*, 1985). 본고에서는 이 변이주로부터 리보스 화학주성이 정상인 복귀변이주를 분리하여 분석한 결과를 보고하는 바, 이 복귀변이주에서 분리한 minicell에서 숙성 단백질이 합성되고 이 복귀변이가 리보스 결합 단백질의 구조유전자의 아미노말단을 코딩하는 부위에 일어났음을 보였다. DNA 염기서열 분석에 의해 원래 *rbsB* 103 신호배열 변이 이외에 또 하나의 변이가 일어나서 원래 돌연변이형을 상쇄한 pseudorevertant임을 확인하였다. 나아가 삼투압 충격분석으로 복귀변이주에서 합성된 숙성 리보스 결합 단백질이 페리플라즘으로 수송되었음을 보였다. 야생형에서 합성된 전구체, 숙성 리보스 결합 단백질과 복귀변이주에서 합성된 29, 32 kd 단백질의 펩티드 패턴을 H.P.L.C.로 조사하여 그 관련성을 확인하였으며, 전구체에 고유한 두 펩티드가 돌연변이주의 경우와 비교하여 복귀변이주에서 소수성이 더 큰 것을 확인하였다. 야생형과 복귀변이주에서 합성된 전구체 단백질의 생체내 신호배열 절단속도를 비교한 결과 복귀변이주에서 그 속도가 더 느림을 알 수 있었다. 그러나 야생형과 복귀변이주에서 숙성단백질을 순수 분리정제하여 아미노말단 아미노산 배열을 분석한 결과 복귀변이주의 신호배열내에 야생형과 다른 두 아미노산의 존재에도 불구하고 절단부위에는 변화가 오지 않았음을 보였다.

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(Received July 5, 1988)