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Molecular Dissection of the *Selenomonas ruminantium* Cell Envelope and Lysine Decarboxylase Involved in the Biosynthesis of a Polyamine Covalently Linked to the Cell Wall Peptidoglycan Layer

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The wild type of *Selenomonas ruminantium* subsp. *lactilytica*, which is a strictly anaerobic, Gram-negative bacterium isolated from sheep rumen, requires one of the normal saturated volatile fatty acids with 3 to 10 carbon atoms for its growth in a glucose medium; however, no such obligate requirement of fatty acid is observed when the cells are grown in a lactate medium. This bacterium is characterized by a unique structure of the cell envelope and a novel lysine decarboxylase and its regulatory protein.

S. ruminantium possesses neither free nor bound forms of Braun lipoprotein which plays an important role of the maintenance of the structural integrity of the cell surface in general Gram-negative bacteria. However, *S. ruminantium* has cadaverine which links covalently to the peptidoglycan as a pivotal constituent for the cell division.

In this study, we did the molecular cloning of the genes encoding a major outer membrane protein of 45 kDa (45 kDa protein, *S. ruminanitum* lysine decarboxylase (LDC), and its regulatory protein of 22-kDa (22-kDa protein; P22) which has similar characteristics to that of antizyme of ornithine decarboxylase in eukaryotic cells, and the molecular dissection of these proteins for understanding the regulation of cadaverine biosynthesis.

(1) Cloning of the gene encoding of a major outer membrane protein of 45 kDa (45 kDa protein)

The nucleotide sequence of the gene encoding a major outer membrane protein of 45 kDa in *Selenomonas ruminantium* was determined. The structure gene of 44 kDa protein gene consisted of 1296 base pairs. An open reading frame that could encode a 44,990 dalton polypeptide consisting of 418 amino acid residues was assigned. Inspection of the amino acid sequence deduced from nucleotide sequence of 45 kDa protein gene and that from native 45 kDa protein clarified that pre-matured 45 kDa protein contains a typical signal sequence at the N-terminus. The amino acid sequence of the predicted matured 45 kDa protein correlated exactly with the known N-terminal 23 amino acid sequence of the native protein. The nucleotide sequence of the 5′-flanking region showed the presence of the consensus sequence of ribosome binidng site, Pribnow box and the RNA polymerase recognition site in *Escherichia coli*. *E. coli* Bla21(DE3) with the plasmid pOM44, which contains the 45 kDa protein gene expressed the gene to produce 45 kDa protein.

(2) Regulation of *S. ruminantium* LDC production

In mammalian cells and tissues, the turnover of ornithine decarboxylase (ODC) (EC 4.1.1.17) is very rapid and highly regulated. The degradation of mammalian ODC catalyzed by the 26S proteasome is accelerated by ODC antizyme, a trigger protein involved in the specific degradation of eukaryotic ODC. In prokaryote, S20, L26 and L34 ribosomal proteins were identified as antizymes in *Escherichia coli* whose overproduction decrease the level of ODC protein and its mRNA. However, the direct evidence on the existence of antizyme in *E. coli* has not been obtained. Previously, we found cadaverine covalently linked to the peptidoglycan in *Selenomonas ruminantium*, a strictly anaerobic bacterium, which is an essential constituent of the peptidoglycan to maintain the integrity of the cell envelope. We also found that cadaverine is synthesized constitutively from L-lysine catalyzed by LDC (EC 4.1.1.18) and transferred to the D-glutamic acid residue of the lipid intermediate for the synthesis of the peptidoglycan by lipid intermediate: cadaverine transferase. *S. ruminantium* LDC was purified and characterized and its gene(ldc) was cloned, and the following findings became evident. I) *S. ruminantium* LDC consists of two identical monomeric subunits with 45 kDa and decarboxylates both L-lysine and L-ornithine with similar Km and Vmax values and its decarboxylating activities toward both substrates were completely prevented by DFMO which had been known to be a specific inhibitor of eukaryotic ODC. II) The amino acid sequence of *S. ruminantium* LDC is 35% identical and 53 to 60% similar to those of eukaryotic ODCs and that 26 amino acid residues, all of which are implicated either in contributing to PLP- and substrate-binding domains or in formation of the homodimeric forms of eukaryotic ODCs, are conserved in *S. ruminantium* LDC. III) *S. ruminantium* LDC has a homologous sequence to that of mouse antizyme binding region in mouse ODC. IV) *S. ruminantium* LDC is

classified into fold type III to which eukaryotic ODCs but not bacterial ODC and LDC are belonging. In addition, *S. ruminantium* has no typical ODC with decarboxylase activity exclusively toward L-ornithine and our LDC involves in the synthesis of putrescine. These finding clarified that *S. ruminantium* LDC and eukaryotic ODC are resemble each other in both biochemical and biophysical characteristics except for the broad range of the substrate specificity in *S. ruminantium* LDC.

The production of LDC in *S. ruminantium* is highly regulated and is strictly linked to the growth phase of the bacteria, i.e. a drastic decrease in LDC activity occurred on entry into the stationary phase of cell growth, which was due to the rapid degradation of LDC. In the proceeding paper, we isolated a new protein of 22 kDa, which is induced in putrescine-grown cells as a regulating factor for the degradation of LDC in *S. ruminantium* cells by ATP-dependent protease. Take together with the characteristics of our LDC, 22-kDa protein is a possible direct counterpart of mammalian ODC antizyme. Here, we showed that the amino acid sequence of the protein is 47 identical and 60–66% similar to those of reported ribosomal L10 protein of bacteria. The recombinant 22-kDa protein, which was expressed in *E. coli*, was purified and the following findings were evident. I) The 22-kDa protein itself has no degradation activity toward LDC but it was required for the degradation of *S. ruminantium* LDC by ATP-dependent proteases in cell-free system. II) The mouse ODC was degraded in our cell-free system containing the 22-kDa protein preparation instead of mouse antizyme. III) *S. ruminantium* LDC was degraded in our cell-free system containing mouse antizyme preparation instead of the 22-kDa protein preparation. *S. ruminantium* LDC was degraded in ODC degradation system containing 22-kDa preparation or mouse antizyme preparation. IV) The production of 22-kDa protein is induced by putrescine but not cadaverine added in the culture. V) Both the 22-kDa protein and mouse antizyme preparations themselves inhibit LDC/ODC activities of *S. ruminantium* LDC preparation in the cell-free system. VI) Both 22-kDa protein and mouse antizyme bind to *S. ruminantium* LDC. However, they did not bind to *S. ruminantium* LDC mutant in antizyme binding region, in which three Lys residues K103, K123, K126 were replaced by Ala, resulting in null degradation of the mutant LDC in cell-free system. Thus, we conclude that the 22-kDa protein is a direct counterpart of mammalian ODC antizyme which is required for the degradation of *S. ruminantium* LDC by ATP-dependent protease. We propose to designate 22-kDa protein and its gene prokaryote antizyme (ProAZ) and proAZ, respectively.