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論 文 内 容 の 要 旨

Synopsis of thesis：

Display of proteins or peptides on the surface of bacterial cells by fusing them with various cell-surface proteins (anchors) has a broad range of potential biotechnological applications including vaccine development, bioconversion, biosensing, and biosorption. Lactic acid bacteria (LAB) constitute an important group of industrial microorganisms which have been used widely for fermentation and preservation of food products. Several types of anchoring domains have already been reported for LAB which are associated with the cell-surface either covalently or non-covalently. If interaction of the anchor protein with the cell surface is non-covalent, it can bind to cells when it is added from the outside (external mode of protein display), and the fusion of target protein to the anchor protein need not to be expressed in the display host. However, in the case of internal mode of protein display, the fusion protein is expressed in the display host. Use of the system based on the external mode offers several advantages over that of the internal mode including full exposure of target proteins outside of the cell wall, wide range of applicable host strains, more flexible control of display level, and keeping non-genetically modified status of the display host. In the present study, we investigated the efficiency of display hosts and expression hosts (*Escherichia coli* and *Pichia pastoris*) for construction of a surface display system based on the external mode of protein display using C-terminal repeat region of peptidoglycan hydrolase of *Lactococcus lactis* IL 1403 (CPH) whose interaction with the cell-surface of LAB is non-covalent. The content of this research is divided into three parts.

Part I：Bidirectional cell-surface anchoring functions of CPH

The display systems based on the internal mode are often associated with the limitations in the translocation

of target proteins and mislocalization of a target protein can affect its activity negatively. In contrast, a display system based on the external mode can ensure the full exposure of target proteins outside of the cell wall, and it is preferable for display of an enzyme with a huge substrate incapable of penetrating the cell wall. For efficient construction of these whole cell biocatalysts, three factors of cell-surface binding activity, enzymatic activity, and binding capacity of cells should be considered. Therefore, we investigated the capability of CPH for production of cell-surface adhesive enzymes in *E. coli* using α-amylase as a target protein. The effects of the fusion direction on the cell-surface binding activity of CPH and enzymatic activities of the fusion proteins were investigated and binding capacities of several LAB strains (display hosts) for the cell-surface adhesive enzymes were examined. Furthermore the binding stability of the adhesive enzyme to LAB strains was studied, and the effect of coexpression of chaperones on soluble expression of the adhesive enzyme in *E. coli* was investigated.

Part II : Expression of CPH in methylotrophic yeast *P. pastoris*

P. pastoris is an attractive host for production of cell-surface adhesive proteins since compared with prokaryotic hosts such as *E. coli*, this organism has a better capability for the correct folding and modifications of recombinant proteins. However, when a protein is expressed in this yeast, the influence of the post-translational modifications such as glycosylation on the protein properties should be considered. CPH contains several potential N-glycosylation sites, and attachment of glycoside chains at these sites may interfere with cell-surface binding activity of this domain. Therefore in this study, a CPH mutant devoid of potential N-glycosylation sites (CPHM) was constructed which was expressed extracellularly in *P. pastoris*. The cell-surface binding activity of the constructed domain (CPHM) was studied and compared with that of the original domain (CPH) produced intracellularly in *E. coli*. It was observed that binding of CPHM to the cells of *Lactobacillus casei* was more stable than that of CPH and its dissociation rate constant was 3.5 times lower than that of CPH.

Part III : A new strategy for enhancement of microbial viability in simulated gastric conditions based on the display of starch binding domain on the cell-surface

It is known that when bacteria are mixed with starch or grown in the presence of starch, starch exerts a protective effect on the bacterial survival in the intestinal tract conditions, and this fact is the basis for microencapsulation of bacteria within porous starch granules (bacterial core) which are then coated with amylose. However, if the bacteria can not adhere to starch properly, it is not easy to encapsulate them within the porous starch granules because they may leak out of the pores during the preparation procedure. The objective of this research is therefore, to enhance delivery of viable microorganisms to the intestinal tract through conferring starch adhesion ability on them. In this way, the bacteria are entrapped between starch granules to take the advantage of the protective effect of starch. Therefore, a cell-surface adhesive starch binding domain (CPH-SBD) was constructed and aggregation of bacteria displaying this protein with starch was examined as a different technique to provide a bacterial core for microencapsulation. When the aggregates were coated with amylose and subjected to the simulated gastric conditions, the survival was increased significantly compared with free cells.

論文審査の結果の要旨

細胞表層に吸着するタンパク質に融合させる形で新機能タンパク質を創成すれば、乳酸菌に新規な機能を持たせることができる。従来、宿主乳酸菌の細胞表層提示を支配するアンカータンパク質を利用する技術が展開されてきた。しかし、本研究では、宿主由来に限定せず、乳酸菌表層に吸着能を持つタンパク質を利用し、このタンパク質と融合する形の発現システムを開発し、応用を試みている。本論文はこれらの研究成果をまとめたものである。

まず、序論において、乳酸菌の表層提示系全般について現状調査し、問題点を述べている。

次に第一章では、乳酸菌吸着タンパク質として *Lactococcus lactis* IL 1403 のペプチドグリカン加水分解酵素の C 末繰り返し配列領域（CPH）を細胞表層接着タンパク質として利用することを提案している。このタンパク質の乳酸菌との結合は非共有結合であり、多くの乳酸菌に接着することを示し、応用範囲の広さが示されている。本章では CPH とモデル酵素であるα-アミラーゼとの融合タンパク質の発現に大腸菌を用い、連結方向が表層接着力、及びα-アミラーゼ酵素活性に及ぼす影響を調べている。その結果、α-アミラーゼの C 末側に CPH を結合させる方が、その逆に比べて、結合力、酵素活性何れも大きく、応用上有利であることを示している。また、大腸菌に成熟した融合タンパク質を効率よく発現させるため、シャペロン系の遺伝子群を発現させると効果があることも示している。

第二章では、融合タンパク質生産ホストとして酵母 *Pichia pastoris* を用いる場合を検討している。酵母を用いる場合は、翻訳後修飾の影響を受け、接着力が弱くなってしまうことが見いだされている。この場合、翻訳後修飾をできないように組み換えてやれば、改善されることを見いだしている。また、いくつかの乳酸菌への接着を調べ、*Lb. casei* に最も強く吸着することも明らかにしている。

第三章では、この系の応用として、腸で薬効を求められる経口薬を想定して、胃酸耐性のあるカプセル化を試みている。すなわち、デンプン結合領域（SBD）を CPH と融合発現（SBD-CPH）させ、これを表層に吸着させた乳酸菌（*Lb. casei*）に、デンプンを吸着させ、さらに 170℃で可溶化したアミロースと一晩インキュベートしたものについて、人工胃液に対する耐性を調べている。この結果、乳酸菌表層に SBD-CPH を提示させたものは、提示させていないものに比較して、優位に生存率が上昇し、このような応用が可能なことを示している。

最後に、結論として、本論文で扱っているような表層提示系と、従来良く研究されてきたもとの宿主乳酸菌のアンカータンパク質に融合させて表層提示させる系を比較し、その長所短所を明らかにし、本論文の位置付けを明らかにしている。

以上のように、本論文は、乳酸菌表層に吸着能を持つタンパク質を利用し、このタンパク質と融合する形の発現システムを開発し、その応用に成功している。

よって本論文は博士論文として価値あるものと認める。