### 2·1 定性PCR法及びリアルタイム定性PCR法

定性PCR法の場合，鋳型となるゲノムDNAの抽出はPCR検出を可能にするのに重要である。ゲノムDNAと複合体を形成しやすいセチルトリメチルアンモニウムブロマイド（CTAB）等を利用した方法や市販のDNA抽出用のキット（シリカゲル膜タイプ法，イオン交換カラム法）を用いる方法が規定されている。

PCR反応を行う際には，コントロールとして対象作物に必ず含まれる内在性遺伝子のDNA配列のPCR（大豆であれば *le1* 遺伝子等，トウモロコシであれば *Zein*, *SSIIb* 遺伝子等）を行う必要がある。PCR反応後，反応液はアガロース電気泳動したものをエチジウムブロマイドで蛍光染色し，紫外線照射下において画像解析機器等で写真撮影する。設計したプライマー対に挟まれたDNAの長さと一致するバンドが検出される。違反の際の社会的な影響を考慮して，異なった標的領域を認識するプライマー対を2種類（検出と確認）用いて試験し，両方検出されれば陽性と判断する。リアルタイム定性PCR法は，近年リアルタイムPCR機器の普及により，広く応用されるようになっている。指数関数的な増幅曲線の有無で陽性と判断し，アガロースゲル電気泳動を行う必要がなく，多検体を迅速で簡易に判定可能である。

### 2·2 組換えタンパク質を検知する方法

組換えタンパク質を検知する定性検知法としては，ラテラルフロー法[7]，組織化学染色法等[5]，が採用されている。ラテラルフロー法については既にいくつかのキットが販売されている。定性PCR法と比較して，ラテラルフロー法および組織化学染色法は簡便である。

## 3 安全性審査済みのGM食品の検査法

我が国では，分別生産流通管理により，書類等により証明されている場合でも，非意図的な混入があることを考慮し，科学的な検知による行政上の判断の目安は重量割合（粒割合）で5%以下と考えている。そのため表示の妥当性を検証するためには5%を定量する必要がある。

### 3·1 ELISA法

GM食品に発現しているタンパク質を免疫抗体による特異的な結合を利用し，抗体に結合された酵素と基質により発色させて測定する酵素免疫測定法である。GMダイズ（ラウンドアップダイズ，GTS 40-3-2）の定量検査にELISA法（SDI社製）が採用されている[8]。しかしELISA法は，GMトウモロコシのように種々の系統に共通して発現しているタンパク質を検知する場合は，系統ごとの正確な定量は困難である。

### 3·2 定量PCR法

GM食品を定量PCR法により定量するためには，分析用の標準物質として，各GM系統を一定量だけ含むnon-GM作物の種子粉砕物またはそのゲノムDNAが必要になる。GM食品の混入率は，複数の混入率の標準物質から測定した検量線から計算することになる。このため，我が国の標準分析法はGM大豆，各GMトウモロコシに導入されている組換えDNA断片のPCR増幅産物をプラスミド上に連結した標準物質を採用している[9][10]。この標準物質では誰でもGM系統ごと，non-GM作物の種子を入手せず検査できる利点がある。

定量PCRは，該当する作物が必ず持っている内在性遺伝子に対する組換え遺伝子の存在比率からGM作物が何%存在するかを相対的に測定する方法である[11][12]。我が国の公定検査法に採用されている定量PCR法の原理（TaqMan Chemistry）を図1に示す。

定量PCR法では，一定のサイクルでの目的領域の生産量（プロダクト量）を比較するのではなく，一定のプロダクト量に達するサイクル数（Ct；スレッシュホールドサイクル）で比較する[13]。あらかじめ既知濃度の標準DNA（公定検査法ではプラスミド）を用いて定量PCRを行い，得られたCt値をもとに検量線を作成する（図2）。現状のGM食品定量検査法はGMダイズ（GTS 40－3－2）とGMトウモロコシ5系統(MON810, GA21, Bt11, E176, T25)法が示されている。GMトウモロコシの定量検査法は，GMトウモロコシ5系統のコンストラクト特異的な領域のDNA配列を定量する分析法を使用して，種子（F1世代）中の挿入

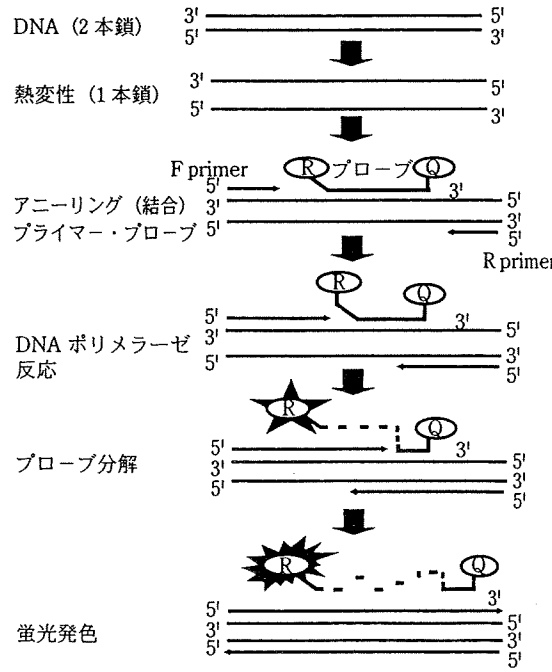
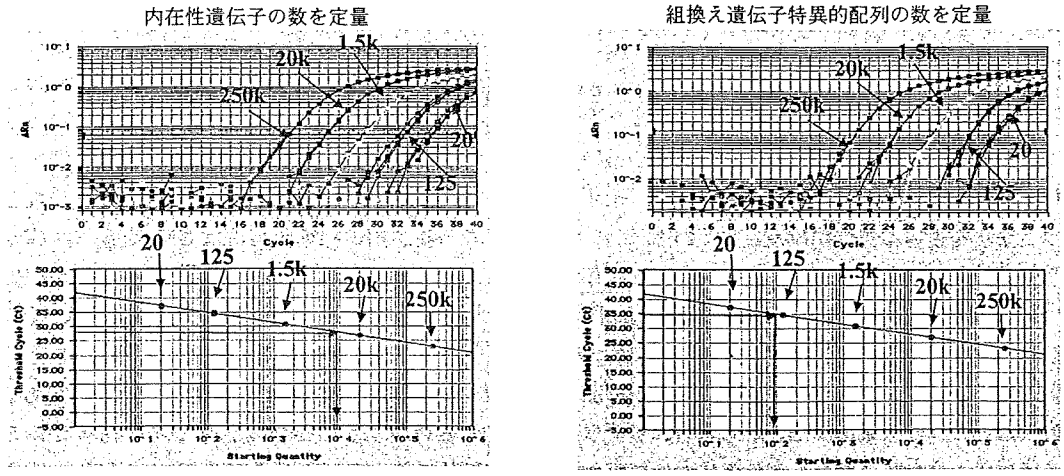



図1 定量PCRの原理（TaqMan chemistry）




$$\text{GM 混入率 (\%)} = \frac{\text{組換え遺伝子特異的配列の数}}{\text{内在性遺伝子の数}} \times \frac{1}{\text{内標比}} \times 100$$

(内標比は GM の系統及び増幅部位等による定数)

図 2　定量 PCR 法による遺伝子組換え体の定量

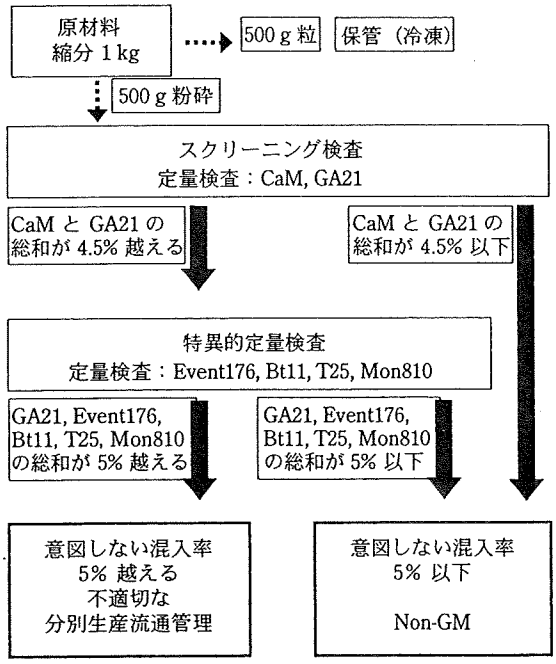



図 3　GM トウモロコシの定量検査法フロー

DNA 配列のコピー数を反映した内標比を測定し，これを使用して試料（穀物；F2 世代）中の GM 特異的配列のコピー数から種子の重量ベースとして GM 混入率（%）を表している（図 3）。しかし，トウモロコシ 5 系統を最初からすべて測定するのは煩雑なので，広く共通して使われている CaMV35S プロモーター（p35S）内の配列と GA21 系統のコンストラクト特異的配列の定量法を組み合わせたスクリーニング法をまず行い，求められた MON810 系統換算値が 4.5% を越える場合においては 5 系統ごとに個別に定量することになっている（図 3）。

## 4　最近の動向

安全性審査が終了した GM 食品が年々多くなり，さらに異なる系統由来の GM トウモロコシを交配させたスタック品種トウモロコシの開発が急激に進んでおり，我が国でも 18 種のスタック品種トウモロコシについて安全性審査が既に終了している（平成 20 年 2 月現在）。従来の単一系統のみのトウモロコシ系統では，重量換算で混合した試料を現在の通知検査法により粉砕物を定量 PCR で分析しても，科学的にはほぼ同じ値の測定値になる。しかしスタック品種トウモロコシが試料に混入している場合，粉砕物を定量 PCR で定量する方法では，1 穀粒中に複数の異なった GM 遺伝子が挿入されているため，コピー数の多重計測が起こり，測定混入率が重量割合で測定した値より常に高く見積もられる。この問題を解決するには粒ごとの検査法の必要が生じ，粒迅速検査法が確立された[14)15)]。この方法は現在までの表示閾値に則した考え方を遵守できる。また従来の定量 PCR 法の諸問題として，スタック品種混入による定量値のばらつき，トウモロコシにおける胚乳の核相（3n）と胚芽の核相（2n）が異なることから生じる定量値のばらつき，GM が父親由来か母親由来で種子（F1）の GM 遺伝子量が異なることから生じる定量値のばらつき，機種間による定量値のばらつき，DNA 抽出法による定量値のばらつき，ある一定企業の定量 PCR 機種のマーケットに依存する状況などがある。粒迅速検査法では，このような諸問題が大幅に解消される可能性があ

る。適切なサンプル数を行えば，粒単位でのGM混入率を求めることができ，またmultiplex PCRと併用することにより，単一系統かスタック品種の区別及びその系統判別が可能となる[16]。今後，スクリーニング法と粒迅速検査法を組み合わせた検査法システムの改正が望まれる。

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Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

# Display of heterologous proteins on the surface of *Lactococcus lactis* using the H and W domain of PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus* as an anchoring matrix

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**Keywords**
anchor, cell-surface display, delivery vehicle, *Lactococcus lactis*, PrtB.

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2007/1311: received 16 August 2007, revised 26 October 2007 and accepted 9 November 2007

doi:10.1111/j.1365-2672.2007.03690.x

## Abstract

Aims: The aim of this study was to develop a cell-surface display system for foreign antigens on the surface of a *Lactococcus lactis* strain using an H and W domain of PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus* as an anchoring matrix.

Methods and Results: To construct a cell-surface display pACL1 vector, a derivative of pSECE1 vector, we amplified the H and W domain of the cell-surface proteinase Prt B from *Lact. bulgaricus* using specific primers and then cloned it into a site downstream of the secretion signal sequence in the pSECE1 vector. The new system, designed for cell-surface display of recombinant proteins on *L. lactis*, was evaluated by the expression and display of the FliC protein of *Salmonella enterica* serovar Enteritidis as a reporter gene (pALC1:FliC). The expression of the FliC protein by the transformed cells was analysed by Western blot analysis, and the localization of FliC on the cell surface was confirmed by immunofluorescence microscopy and flow cytometry analysis. A specific band corresponding in size (approx. 110 kDa) to FliC plus the anchor residues was detected by anti-FliC antibody in the cell extract of *L. lactis* H61 harbouring pALC1:FliC, but not *L. lactis* H61 harbouring pALC1. In addition, flow cytometry and immunofluorescence microscopy revealed FliC-specific positive signals and a significant increase of fluorescence, respectively, in cells harbouring pALC1:FliC compared with that in control cells harbouring the parental pALC1 plasmid. These findings demonstrated that FliC was successfully displayed on the cell surface by the anchor domain of PrtB.

Conclusions: A pALC1 vector using the H and W domain of PrtB from *Lact. bulgaricus* as an anchoring matrix can be used to successfully display the FliC protein on the surface of *L. lactis*.

Significance and Impact of the Study: This novel way of displaying heterologous proteins on the cell surface of *L. lactis* using the PrtB anchor domain should prove useful for the delivery of antigens and other proteins.

## Introduction

*Lactococcus lactis* is widely used to produce various fermented foods, especially cheese and other dairy products. As a delivery vehicle, *L. lactis* has some desirable traits such that it is generally recognized as a safe organism and its low protease activities. In addition, because *L. lactis* is a noncommensal and transient bacterium in the digestive

tract, the risk of eliciting a tolerance response to the antigen delivered is diminished compared with persistent bacteria (Drouault *et al.* 1999).

For most pathogens, the initial infection occurs at the mucosa of the lungs and intestines. A great deal of research is currently focused on the development of mucosal vaccines that can elicit a local immune response at the mucosal surface. A variety of live, attenuated-bacterial strains have been used as carriers to deliver foreign antigens to mammalian hosts (Thole *et al.* 2000; Gentschev *et al.* 2001). However, they still retain invasiveness and virulence properties that limit their use in humans.

As mucosal delivery vehicles, recombinant lactic acid bacteria offer several practical advantages, including avoidance of culturing large quantities of pathogens, no need for purification of antigenic components or subunits, and the ability to express immunogens in their native conformation. In addition, because it is less invasive, mucosal immunization is more attractive for use in children and immunosuppressed patients (Robinson *et al.* 2004; Bermudez-Humaran *et al.* 2005).

Many heterologous proteins have been expressed in *L. lactis*, which is used as a delivery vehicle for mucosal vaccines. Several studies have demonstrated that oral or nasal administration of tetanus toxin fragment C-expressing recombinant *L. lactis* to mice stimulates secretory IgA against tetanus toxin fragment C and protects against a lethal challenge (Wells *et al.* 1993; Norton *et al.* 1997; Robinson *et al.* 1997, 2004). Other antigens expressed from *L. lactis* and delivered orally and nasally also elicit a mucosal immune response (Ribero *et al.* 2002; Dieye *et al.* 2003; Bermudez-Humaran *et al.* 2005; Pei *et al.* 2005).

The use of bacteria as vaccine delivery vehicles implies construction of recombinant strains that contain the gene cassette encoding the antigen (Detmer and Glenting 2006). Several delivery systems have been developed to target heterologous proteins to a specific cell location (i.e., cytoplasm, cell wall or extracellular medium) (Nouaille *et al.* 2003) and many studies have been carried out to evaluate the effect of antigen-presenting systems on the immune system. Even though spontaneous bacterial lysis can lead to local antigen release, extracellularly accessible antigens expressed on the surfaces of bacteria are better recognized by the immune system than those that are intracellular (Lee *et al.* 2000).

Thus, one strategy is to place a neutralizing antigen from a pathogenic bacterium on the surface of a live non-pathogenic bacterium. While surface proteins of Gram-negative bacteria are assembled in the outer membrane, Gram-positive bacteria predominantly utilize their cell wall as an organelle for anchoring and displaying adhesive molecules (Navarre and Schneewind 1999). Several mechanisms by which proteins anchor to the Gram-positive bacterial envelope are currently known. Each mechanism is characterized by specific structural features that can be identified in the sequences of the proteins and are involved in their specific properties (Jonquieres *et al.* 1999; Cossart and Jonquieres 2000).

PrtB, encoded by the *prtB* gene, is a cell-surface proteinase (CSP) of *Lactobacillus delbrueckii* subsp. *bulgaricus*. It is essential for *Lact. bulgaricus* growth in milk and is responsible for the first step of caseinolysis. The PrtB of *Lact. bulgaricus* strongly differs from other CSPs of lactic acid bacteria in its specificity of cleavage and the structure of its long C-extension domains. The different C terminus of PrtB raises the possibility of a mechanism of attachment to the cell envelope that is different from the covalent anchoring of lactococcal PrtPs to peptidoglycan via the LPXTG motif. In PrtB, a degenerated LPKKT motif is surrounded by two imperfect repeats of 59 amino acids with high lysine content. The high content of positive charges in the C terminus suggests interaction with the negatively charged teichoic acids of the cell wall (Germond *et al.* 2003).

In this study, a new cell-surface display vector for lactococci, pALC1, a derivative of the pSECE1 vector (Satoh *et al.* 1997), was developed using the H and W domain of the PrtB carboxy end from *Lact. bulgaricus* as an anchoring matrix. To verify its utility, we generated *L. lactis* expressing FliC, the flagellar antigen of *Samonella enterica* serovar Enteritidis, as a tool for the detection and demonstration of surface anchoring (Ogushi *et al.* 2001). We found that a heterologous anchor sequence can function in a highly efficient manner to expose functional units at the surface of the food-grade micro-organism *L. lactis*.

## Materials and methods

### Bacterial strains, plasmids and culture conditions

*Lactobacillus bulgaricus* JCM1002 came from the Japan Collection for Microorganisms *and L. lactis* H61 was obtained from the National Institute of Livestock and Grassland Science (Tsukuba, Japan). Bacterial strains *Lact. bulgaricus* JCM1002 and *L. lactis* H61 were grown, respectively, in MRS (deMan, Rogosa and Sharpe) broth (Difco Laboratories) at 37°C and in M17 broth (Difco) containing 1% glucose at 32°C. *Escherichia coli* JM109 for cloning was grown in LB medium containing 100 $\mu$l ml$^{-1}$ of ampicillin.

### Construction of pALC1 plasmid for cell-surface display

PCR amplification of the H and W domain of PrtB was performed with the An 377 and An 366 primers, using

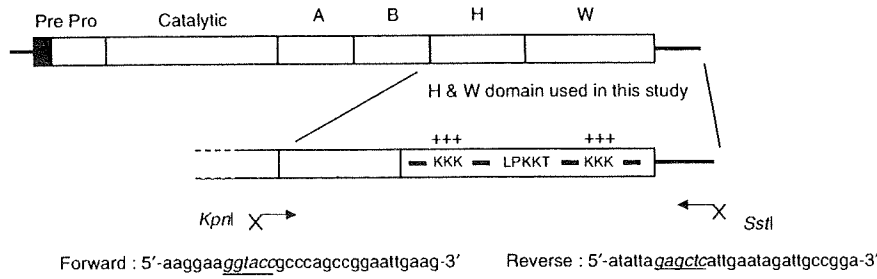


**Figure 1** Schematic representations of preproproteinase PrtB from *Lactobacillus bulagaricus*. Small bent arrows correspond to primers designed for gene amplification. The thick horizontal bars within the C-terminal region correspond to the two repeats of 59 residues surrounding the degenerated sorting signal LPKKT (Germond *et al.* 2003). Restriction enzyme sites are underlined. K represents lysine.

genomic DNA of *Lact. bulgaricus* JCM1002 as a template (Fig. 1). The oligonucleotides were designed to introduce a *Kpn*I and *Sst*I restriction site at the 5′ and 3′ end respectively. The restriction endonuclease recognition sites are underlined (Table 1).

PCR was performed as follows: samples were denatured at 95°C for 5 min and amplification was then carried out in 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. After the last cycle the reaction was held at 72°C for 10 min.

The amplified fragment was inserted into the pGEM-T Easy vector (Promega) and transformed into *E. coli* JM109. The recombinant plasmid pGEM-T-PrtB-H W was digested by *Kpn*I and *Sst*I, followed by ligation into the corresponding sites on the plasmid pSECE1, an expression/secretion vector for lactic acid bacteria.

The ligated plasmid pSECE1:PrtB-H·W, resulting in the pALC1 vector, was transformed into *L. lactis* H61 by electroporation. The preparation of competent cells and electroporation of *L. lactis* were carried out according to the modified method of Holo and Nes (1989).

Erythromycin-resistant transformants were selected and plasmids were extracted. To select transformants, erythromycin (Sigma, St Louis, MO, USA) was used at a concentration of 5 $\mu$g ml$^{-1}$.

Successful insertion was identified by restriction endonuclease digestion analysis and DNA sequencing. The pSECE1 and pALC1 vectors are presented in Fig. 2.

### Cloning of *fliC* gene into pALC1 vector

DNA fragments of the *fliC* gene were amplified from *Salmonella enterica* serovar Enteritidis (SE) #40 chromosomal DNA with two sets of primers (Table 1). As a *Kpn*I site exists within the *fliC* gene, two step PCR reactions were carried out using a overlapping primer with a T → C substitution at position 1023 (GenBank M84974). In the first step, two PCR products were amplified from SE #40 chromosomal DNA with the F 398, F 400 primer set and F 401, F 399 set respectively.

Finally, the *fliC* gene was amplified from the two fragments obtained in the first step as template by PCR with a forward primer (F 398) and a reverse primer (F 399) (Kajikawa *et al.* 2007). The amplified fragment was digested with *Eco*RV and *Kpn*I, and inserted into the same restriction enzyme sites of pALC1. The ligated plasmid was introduced by electroporation into *L. lactis*.

### Protein extraction and Western blotting

Transformed bacteria were grown overnight in GM 17 broth containing 5 $\mu$g ml$^{-1}$ of erythromycin. Bacterial cells were collected, washed twice with phosphate-buffered saline (PBS), and disrupted in 50 mmol l$^{-1}$ Tris–HCl buffer (pH 8·0) by a bead beater (Fast Prep, BIO 101). Each sample was resuspended by adding an equal volume of 2× SDS sample buffer. For protein denaturation, samples

**Table 1** Oligonucleotides used in this study

| Primer | Direction | Sequence (restriction enzyme)* |
|---|---|---|
| An 377 | Sense | 5′-AAGGAAGGTACCGCCCAGCCGGAATTGAAG-3′ (*Kpn*I) |
| An 366 | Antisense | 5′-ATATTAGAGCTCATTGAATAGATTGCCGGA-3′ (*Sst*I) |
| F 398 | Sense | 5′-GAAAAGGATATCGCACAAGTCATTAATACAAACAGC-3′ (*Eco*RV) |
| F 399 | Antisense | 5′-ATCGCCGGTACCACGCAGTAAAGAGAGGACGTT -3′(*Kpn*I) |
| F 400 | Antisense | 5′-TTTGGCTTCAGCGGTGCCAGCAGTAGATTT -3′ |
| F 401 | Sense | 5′-AAATCTACTGCTGGCACCGCTGAAGCCAAA -3′ |

*Restriction enzyme sites are underlined.

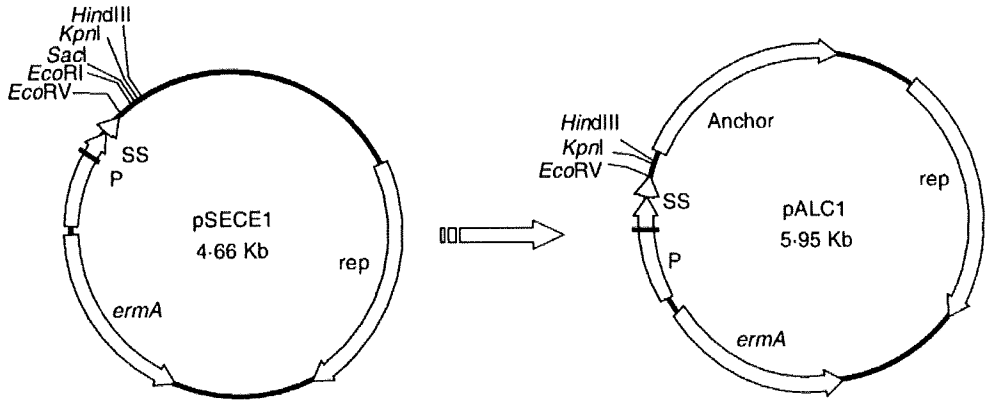


**Figure 2** Construction of pALC1 vector. pSECE1 vector was digested with *Kpn*I and *Sst*I, and then a fragment of the H and W domain of the *prtB* gene from *Lactobacillus bulgaricus* was inserted to yield the pALC1 vector. P, promoter of *amyA* gene; SS, secretion signal sequence of *amyA* gene; *ermA*, erythromycin resistance gene; *rep*, replication gene (Satoh *et al.* 1997); Anchor, H and W domain of *prtB* gene (Germond *et al.* 2003).

were heated for 5 min at 95°C. The cell debris was pelleted by centrifugation and the supernatants collected for analysis. Proteins were separated by SDS-PAGE and transferred onto PVDF (Immobilon™ –P; Millipore) by electroblotting. After blotting, nonspecific protein binding sites were blocked with a solution containing 1% bovine serum albumin (BSA) in 50 mmol $l^{-1}$ Tris–HCl (pH 8·0), 150 mmol $l^{-1}$ NaCl and 0·5% Tween 20.

The expression of FliC protein was detected using anti-FliC rabbit antibody as a primary antibody and goat anti-rabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase as a secondary antibody.

### Flow cytometry and immunofluorescence microscopy

Flow cytometry analysis was performed on a FACS-Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). *Lactococcus lactis* cells were grown to late exponential phase and harvested by centrifugation, washed with PBS, and resuspended directly in PBS + 1% BSA containing the primary antibody (anti-FliC rabbit antibody). After 30 min of incubation at 37°C, bacteria were washed twice in PBS and then resuspended in PBS + 1% BSA containing the secondary antibody conjugated with Alexa Fluor™ 488 goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR). After two final washes in PBS, the fluorescence intensity of the immunostained cells was then acquired with a FACSCalibur and the data were analysed using CELLQUEST software (Becton Dickinson).

For immunofluorescence microscopic observation, cells were prepared as for flow cytometry. The bacterial cells grown in GM17 broth were harvested and washed with PBS. Cells were then immunostained as described above and viewed under a fluorescent microscope (Nikon, Kawasaki, Japan) (Oggioni *et al.* 1999).

## Results

### Construction of pALC1 vector for cell-surface display

The 5·96 kb pACL1 vector, a derivative of the pSECE1 vector that allows expression of surface-located proteins, was constructed. Erythromycin-resistant transformants were selected, followed by plasmid extraction and restriction endonuclease digestion analysis. The 1·3 kb DNA fragment of the H and W domain region of the *prtB* gene that was inserted into the pSECE1 vector was sequenced. The fragment had 99% homology with the corresponding annotated gene from the CSP of *Lact. bulgaricus* NCDO 1489 (GenBank accession number L48487). Sequencing analysis of the recombinant constructs showed that the H and W domain of the C-terminal region of the CSP PrtB were successfully cloned in-frame downstream of the secretion signal sequence of the pSECE1 vector. Figure 2 shows a map of the newly constructed vector, pALC1.

### Expression of FliC-Anchor fusion protein in *L. lactis*

FliC was used as a reporter protein to facilitate detection and demonstration of surface anchoring. The *fliC* gene encoding the flagellin protein of SE #40 was amplified from template prepared by T → C substitution at position 1023 (GenBank M84974), by PCR with the primer F 398, which introduces an *Eco*RV restriction site at the 5′ end, and F 399, which introduces a *Kpn*I site at the 3′ end. The 1·5 kb of fragment was cloned into the pALC1 vector, resulting in the plasmid pALC1:FliC, and then

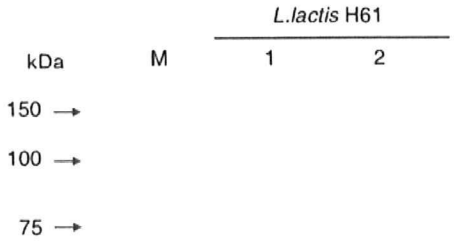


**Figure 3** Western blot analyses of FliC-Anchor fusion protein. Lane M, protein molecular weight markers (kDa); lane 1, *Lactococcus lactis* H61 harbouring pALC1:FliC; lane 2, *L. lactis* H61 harbouring pALC1.

transferred into *L. lactis* H61. Sequencing analysis of the recombinant constructs indicated that the *fliC* gene was successfully cloned in frame with the *prtB* anchor gene in the pALC1 vector.

To determine whether the FliC-Anchor (cell wall-binding domains of PrtB) fusion protein in *L. lactis* was expressed, Western blot analysis was conducted using antiserum directed against the flagellin protein. Western blot analysis of the cell extract of *L. lactis* H61 containing pALC1:FliC revealed a protein band reactive with anti-FliC, while extracts from the *L. lactis* containing H61 harbouring pALC1 were FliC negative. A specific band corresponding in size (approx. 110 kDa) to FliC plus the anchor residues was detected by anti-FliC antibody in the cell extract of *L. lactis* H61 harbouring pALC1:FliC but not *L. lactis* H61 harbouring pALC1, indicating that the FliC-Anchor protein was successfully expressed in *L. lactis* H61.

In the cell fraction, one major band (110 kDa), corresponding to the FliC-Anchor and several degradation products were also detected (Fig. 3). Cell wall-anchored FliC appeared as several closely spaced bands, a characteristic of cell wall-anchored proteins that seems to be attributed to cell surface proteolysis. Bands corresponding to smaller proteins may also reflect degradation of anchored FliC by cytoplasmic or surface housekeeping proteases (Ribero *et al.* 2002).

### Analysis of cell-surface display

To investigate whether the FliC-Anchor fusion proteins could be anchored and displayed on the cell surface, *L. lactis* was immunostained and the localization of the

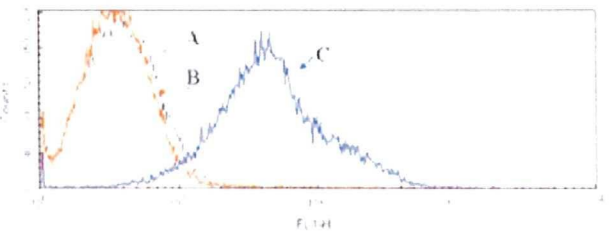


**Figure 4** Flow cytometric analysis of *Lactococcus lactis* H61 harbouring pALC1:FliC and pALC1. A, *L. lactis* H61; B, *L. lactis* H61 harbouring pALC1; C, *L. lactis* H61 harbouring pALC1:FliC. fluorescence levels (FL1-H) from bacterial cells are shown in histogram form.

proteins on the surface of *L. lactis* was verified by flow cytometry and fluorescence microscopy (Figs 4 and 5 respectively).

Immunofluorescence labelling of the cells was preformed using rabbit anti-FliC antibody as the primary antibody and Alexa Fluor™ 488-conjugated goat anti-rabbit IgG as the secondary antibody. Flow cytometry was used to quantitatively analyse the cell-surface display of the FliC-Anchor. Cells harbouring pALC1:FliC-Anchor were stained with primary and secondary antibodies and *L. lactis* cells harbouring the plasmid pALC1 were used as a control. As shown in Fig. 4, cells displaying the FliC-Anchor showed significantly more intense fluorescence signals compared with control cells harbouring the parental pALC1 plasmid. Analysis by immunofluorescence microscopy also revealed fluorescence only in the recombinant bacteria harbouring pALC1:FliC and not in control cells harbouring pALC1 (Fig. 5), indicating that FliC-Anchor was displayed on the cell surface of the *L. lactis* cells harbouring pALC1:FliC. This result was consistent with the flow cytometry data. These results confirmed the

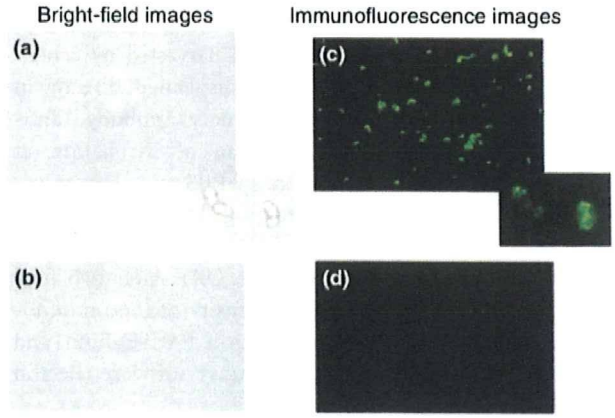


**Figure 5** Immunofluorescence labelling of *Lactococcus lactis* H61 harbouring pALC1:FliC and pALC1. (a) and (c) show *L. lactis* H61 harbouring pALC1:FliC, while (b) and (d) show *L. lactis* H61 harbouring pALC1.

successful cell-surface display of FliC-PrtB Anchor on *L. lactis*.

## Discussion

Cell-surface display in lactic acid bacteria has been studied using various anchor proteins. The most commonly used, the C-terminal cell wall-anchoring LPXTG motif, is covalently linked to the cell wall (Steidler *et al.* 1998; Turner *et al.* 2003) under the direction of a sorting signal made of an LPXTG motif followed by a hydrophobic domain consisting of about 20 amino acids and a tail of positively charged amino acids. After cleavage between threonine and glycine residues of the conserved LPXTG motif by sortase, the carboxyl group of the threonine is amide-linked to the free amino group of the peptide cross-bridge in the cell wall (Cossart and Jonquieres 2000). Differences in sortase activity between strains can cause problems in using the LPXTG motif for cell-surface display. In particular, deficient sortase activity causes an insufficient display of target protein using the LPXTG motif.

We tried to produce a cell-surface display using a PrtP anchor as an LPXTG motif anchor. However, the display of proteins on the surface of *L. lactis* was not successful (data not shown), possibly because of insufficient sortase activity in anchoring PrtP in our strain (Dieye *et al.* 2001; Narita *et al.* 2006).

In contrast to the LPXTG motif, the LPKKT motif of PrtB is surrounded by two imperfect repeats of 59 amino acids with high lysine content. The high content of positive charges in the C terminus suggests interaction with the negatively charged teichoic acids of the cell wall. Consequently, Germond *et al.* (2003) proposed that the mechanism of PrtB attachment to the cell wall probably involves electrostatic forces.

The combination of efficient secretion and a cell wall anchoring system provides the needed variability of antigen delivery in various lactic acid bacteria (Germond *et al.* 2003).

In this study, we developed a novel system of cell-surface display in *L. lactis* using an H and W domain of PrtB as an anchoring matrix. To show that the newly developed anchoring system could display immunogens on the *L. lactis* cell surface, we cloned the *fliC* gene of *Salmonella enterica* serovar Typhimurium into a site upstream of the PrtB anchor region in the pALC1 vector. As shown in Figs 3, 4 and 5, FliC was successfully expressed in a simple recombinant organism and can be displayed on the cell surface of *L. lactis*. Protective immunity induced by the oral vaccination of purified flagellin of *Salmonella* has been reported (Strindelius *et al.* 2004). Recombinant *L. lactis* expressing FliC may be applicable as an oral vaccine that can induce a protective immunity.

To confirm the efficiency of this anchoring system, we also attempted to clone the *hlyA* gene encoding listeriolysin O (LLO), the major virulence factor of *Listeria monocytogenes* (Tanabe *et al.* 1999), into the pALC1 vector. As we expected, LLO were displayed on the cell surface of *L. lactis* IL1403 (data not shown).

The successful expression of different antigens, such as FliC and LLO, on the surface of *L. lactis* indicates that it should be possible to clone immunogens into a site upstream of the anchor region of a plasmid and express the recombinant proteins on the cell surface of *L. lactis*.

In studies of cell-surface display, various peptides and antigen have been displayed in *L. lactis* using cell wall anchors of proteins A and M6 from *Staphylococcus aureus* and *Streptococcus pyogenes* respectively (Steidler *et al.* 1998; Dieye *et al.* 2001; Ribero *et al.* 2002). Such bacteria are considered pathogenic; however, and a system based on nonpathogenic bacteria may be more easily accepted by potential consumers.

Although the display of foreign proteins on the surface of *L. lactis* has been documented widely, use of the functional anchoring domain of PrtB from *Lact. bulgaricus* for cell-surface display has not been reported. Here, our results show that a heterologous anchor sequence can function in a highly efficient manner to expose functional units on the surface of the food-grade micro-organism *L. lactis*. As far as we are aware, this is the first report on a cell-surface display system in *L. lactis* that uses the H and W domain of PrtB as an anchoring matrix. However, the evaluation of effect fusion protein expressed on other properties of the organism needs to be further investigated. The development of *in vivo* inducible systems, as well as the replacement of the currently used antibiotic resistance markers by food-grade selection markers, is also necessary to improve the current system (Seegers 2002).

The success of the anchoring system developed here for surface display of heterologous proteins on the surface of *L. lactis* opens up possibilities of peptide and protein display using this organism. This novel way of displaying heterologous proteins on the cell surface of *L. lactis* should be very useful in the delivery of vaccines and other proteins.

## Acknowledgements

This work was supported by a grant from the Japan Health Sciences Foundation and the Brain Korea 21 programme from the Ministry of Education.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2009, p. 2727–2734 Vol. 75, No. 9
0099-2240/09/$08.00+0 doi:10.1128/AEM.01916-08


# Reduction of Tumor Necrosis Factor Alpha-Inducing Capacity of Recombinant *Lactobacillus casei* via Expression of *Salmonella* OmpC[▽]

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Received 18 August 2008/Accepted 23 February 2009

**The insertion of a heterologous gene into commensal bacteria is a common technique to develop a delivery agent for vaccination and therapies, but the pleiotropic effects of genetic modifications need to be investigated before its use in practical applications. Although supplemental properties provided by the expression of heterologous antigens have been reported, the negative or side effects on the immune-modulating properties caused by recombination are barely understood. In the present study, we fortuitously found that the secretion of tumor necrosis factor alpha (TNF-α) from murine macrophages was reduced by recombinant *Lactobacillus casei* expressing *Salmonella* OmpC compared to the stimulation of TNF-α secretion by nonexpressing *L. casei*. This reduction could not be attributed to OmpC as a purified protein. The main component of the OmpC-expressing strain included in the attenuation of TNF-α release seemed to be the cell wall, which exhibited higher sensitivity against *N*-acetylmuramidase than that of nonexpressing strains. These results suggest that the recombinant strain expressing a specific heterologous antigen might be digested rapidly in macrophages and lose immune-stimulating capability at an early time point.**

Many strains of lactobacilli and other lactic acid bacteria are safe symbiotic bacteria, which have been used for food fermentation or exist in our gastrointestinal tract commensally. Among their favorable health properties, the immune-modulating properties of lactobacilli have been investigated in many reports (11, 18, 25, 28). More particularly, several cell components of *Lactobacillus* strains, such as lipoteichoic acid and oligodeoxynucleotides, are known to elicit innate immune responses through Toll-like receptors 2 and 9 (24, 35). There is also evidence that lactobacilli can evoke immune responses associated with the nucleotide binding oligomerization domain-like receptor family that primarily recognizes microbial molecules of bacterial origin, such as muramyl dipeptide (10, 18). As a genetic factor of lactic acid bacteria, recent studies reported that a mutant of *Lactobacillus plantarum* NCIMB 8826 and *Lactobacillus rhamnosus* GG deficient in the D-alanylation of teichoic acid converted their capacity of immunomodulation (13, 29).

Recently, several studies demonstrated that genetically modified lactobacilli exhibited properties for the induction of supplemental immune responses in combination with heterologous proteins such as pathogenic antigens for vaccination, allergens for anti-allergic treatments, and other responses (2, 5, 6, 12, 14, 19, 21, 23, 27, 28, 30, 32, 33). Previously, it was also reported that recombinant *Lactobacillus casei* ATCC 393 expressing flagellin from *Salmonella enterica* serovar Enteritidis (SE) could induce protective immunity (17). In these studies, recombinant lactic acid bacteria showed additional immunological or physiological activities that the wild-type strains did not provide originally. On the other hand, the opposite case that a heterologous protein could negatively affect the functions of the host strain has scarcely been examined. Because the insertion of a heterologous gene into commensal bacteria appears to be a common technique to develop a delivery agent for vaccination and therapies, the pleiotropic effects of genetic modifications should be investigated before practical applications are put into therapeutic use. In the course of developing a recombinant vaccine based on lactobacilli, it was fortuitously found that heterologous protein-expression reduced an immunological property of the host bacteria.

In the present study, it was found that recombinant *L. casei* ATCC 393 expressing SE OmpC induced less tumor necrosis factor alpha (TNF-α) production by murine macrophage-like cells than a nonexpressing strain. OmpC refers to a major outer membrane porin of *Salmonella*, the function of which is the formation of a channel for the diffusion of nutrients and low-molecular-weight compounds across the outer membrane (26). OmpC is also known as a protective antigen for vaccination against *Salmonella* because OmpC-specific antibodies exert a bactericidal effect (15, 16, 20). In this context, the recombinant *Lactobacillus* producing OmpC was originally constructed to be applied for vaccination; however, the recombinant lactobacilli showed weaker immunogenic properties than the original strain. Interestingly, attenuation of the immunostimulating property of recombinant *L. casei* was not attributed directly to OmpC. We report here how OmpC expression reduced the TNF-α-inducing capacity of recombinant *L. casei*.

## MATERIALS AND METHODS

**Bacterial cells and culture conditions.** A frequently used plasmid-free strain, *L. casei* ATCC 393 (but probably different from the original strain of ATCC 393[T]), was used as a host strain for genetic modification (1). All recombinant *L. casei* strains were grown in de Mann-Rogosa-Sharpe (MRS) medium supple-

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▽ Published ahead of print on 6 March 2009.

mented with 5 μg of erythromycin/ml at 37°C. For heterologous-protein expression, recombinant lactobacilli were incubated in *Lactobacillus*-carrying medium (LCM) containing 1% mannitol and erythromycin at 37°C (22). As a negative control for the current experiments, *L. casei* carrying pLPEmpty, which was constructed in a previous study, was used (17). *Escherichia coli* JM109 (TaKaRa, Tokyo, Japan) was used as the cloning host and grown in LB broth containing 100 μg of ampicillin/ml. For the expression of $His_6$-tagged protein, *E. coli* M15 (Qiagen, Tokyo, Japan) was grown in LB broth supplemented with ampicillin, kanamycin (25 μg/ml), and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C. *S. enterica* SE strain #40 is a clinically isolated laboratory strain and was grown in LB medium (17).

**Preparation of recombinant SE OmpC and anti-OmpC anbibodies.** Histidine-tagged OmpC was prepared using *E. coli* M15 and vector pQE31 according to the manufacturer's instructions (Qiagen). The gene fragment encoding the signal peptide-deficient SE OmpC (C terminus of 315 amino acids) was amplified from SE chromosomal DNA by PCR using a pair of primers, IGM424 (5′-CCC CGG ATC CGG AAA CGC AGG TTA ACG ATC A) and IGM425 (5′-GGG GCT CGA GGA ACT GGT AAA CCA GAC CCA). The DNA segments were digested with BamHI and XhoI, followed by insertion into the BamHI-SalI site of pQE31. After the induction of protein expression, His-tagged proteins were purified under denaturing conditions. The molecular mass and purity of the prepared proteins were confirmed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. The concentration of the recombinant protein was determined by the Bradford protein assay (Bio-Rad, Tokyo, Japan). A high concentration (>1 mg/ml) of purified protein solution in phosphate-buffered saline (PBS) supplemented with 4 M urea was stored at −20°C until use. Protein renaturing was performed by rapid dilution in PBS or RPMI 1640 medium (Sigma-Aldrich Japan, Tokyo, Japan). Contamination with lipopolysaccharide (LPS) in the protein solution was detected using Endospecy (Seikagaku Corp., Tokyo, Japan) in accordance with the manufacturer's instructions. In order to prepare anti-OmpC antibodies, BALB/c mice were immunized intraperitoneally four times with 10 μg of His-tagged OmpC/mouse at 3-week intervals. Freund complete adjuvant was used only for the first injection. The care and use of experimental animals complied with local animal welfare laws and guidelines. Serum was prepared, and the titer of anti-OmpC antibodies was determined by enzyme-linked immunosorbent assay as described previously (17). The affinity of the antibodies to SE OmpC was evaluated by immunoblot analysis against a total cell extract of SE. The SE total cell extract was prepared by simple resuspension and boiling in Laemmli sample buffer.

**Construction of recombinant *L. casei*.** As an expression vector for the cell-surface anchoring of the heterologous antigens, pLP401::OmpC, was constructed from pLP401, which was developed by Pouwels et al. (31). The gene fragment encoding OmpC of SE was amplified by PCR using a pair of primers, IGM424 and IGM425. The DNA segments were digested with BamHI and XhoI, followed by insertion into the same restriction sites of pLP401. The resultant plasmid (pLP401::OmpC), the expression cassette for which consisted of the promoter plus the signal sequence of *amy*, *ompC*, and the anchor of *prtP*, was introduced into *L. casei* by electroporation as described previously (31). Expression of the heterologous antigen was confirmed by Western blot analysis. The recombinant *L. casei* were incubated in LCM for 8 h, and bacterial cells and culture supernatants were separated by centrifugation. For preparing a whole-cell extract, the bacterial cells were treated with 5 mg of lysozyme/ml and 20 U of mutanolysin/ml in Tris-Cl (pH 8.0) supplemented with 0.3 M sucrose at 37°C for 30 min. The enzyme-treated cells were washed, resuspended in water, and lysed in the same volume of 2× Laemmli sample buffer. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10 to 20% gradient gel and then electrically blotted onto a polyvinylidene difluoride membrane (Millipore, Kanagawa, Japan). For the quantification of OmpC expressed by recombinant *L. casei*, the specific signals were detected using an Alexa Fluor 488-labeled antibody (Molecular Probes). The specific bands were analyzed using Molecular Imager FX and Quantity One (Bio-Rad). The amount of OmpC produced by the recombinant strain was estimated by comparing the density of the band to purified OmpC standards. The surface location of OmpC on the bacterial cell was analyzed by using a FACSCalibur instrument and CellQuest software (BD, Tokyo, Japan). The bacterial cells incubated in LCM were collected, washed, and suspended in PBS supplemented with 0.05% Tween 20 and 1% bovine serum albumin. The cells were incubated with anti-OmpC antibodies and Alexa Fluor 488-labeled anti-mouse immunoglobulin G (IgG; Molecular Probes). Ten thousand events were analyzed by using a flow cytometer.

**Evaluation of cell viability of recombinant lactobacilli.** The cell viability of recombinant lactobacilli was determined by using a Live/Dead BacLight bacterial counting and viability kit (Molecular Probes). By staining with SYTO 9 and propidium iodide, "live" (intact cell membrane/wall) bacteria exhibit bright fluorescence, whereas "dead" (damaged cell membrane/wall) bacteria exhibit weak fluorescence. Logarithmically growing (8 h) cultures in LCM broth were stained with the two dyes and applied to flow cytometry. Ten thousand events were analyzed using a FACSCalibur instrument and plotted on a dot plot cytogram. The concentration of cell particles was calculated by comparison to microsphere standards. More detailed information is described in the manufacturer's protocol. Cell viability was also determined by the regular colony counting method. The CFU counts per cell particle were determined by using a flow cytometer. The percentage of colony-forming cells was calculated.

**Preparation of bacterial cells and cell components.** Heat-killed recombinant *L. casei* cells were prepared from fresh cultures. Prewarmed LCM/mannitol broth was inoculated with an overnight culture of recombinant lactobacilli in MRS broth and incubated for 8 h at 37°C. Bacterial cells were then collected, washed with PBS and distilled water, and heat killed at 80°C for 20 min, followed by lyophilization. Fluorescein isothiocyanate (FITC)-labeled lactobacilli were prepared with FITC-I (Dojindo, Kumamoto, Japan) as described by Shida et al. (34). Briefly, heat-killed bacterial cells were incubated in 50 mM carbonate buffer (pH 9), including 4.5 μg of FITC-I/ml, at 37°C for 1 h and then washed with PBS. A cell wall-removed fraction (CWRF) of recombinant lactobacilli was prepared from the heat-killed cells. The cells were treated with lysozyme (10 mg/ml) and mutanolysin (20 U/ml) in 50 mM Tris-Cl buffer supplemented with 0.3 M sucrose at 37°C for 16 h and washed sufficiently with the same buffer. An intact cell wall (ICW) sample was prepared as described previously (24). In short, heat-killed cells were boiled in 0.3% sodium dodecyl sulfate solution and washed thoroughly. The cell pellets were then treated with pronase, followed by delipidation with methanol, methanol-chloroform-water, and methanol-chloroform. Nucleotides were digested with benzonase, and the remaining cell particles were washed thoroughly with distilled water.

**Cell culture.** Murine peritoneal macrophages and the macrophagelike cell line RAW264.7 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (complete medium) at 37°C in a 5% $CO_2$ incubator. Murine peritoneal macrophages were isolated from female BALB/c mice (10–13 weeks old) that were injected intraperitoneally with 4% thioglycolate medium 3 days before sampling. Peritoneal lavage fluid in PBS was collected and washed with complete medium. The care and use of experimental animals complied with local animal welfare laws and guidelines. The murine macrophagelike cell line RAW264.7 was purchased from the American Type Culture Collection. Each cell suspension was seeded in a 96-well microplate ($2 \times 10^4$ cells/well) and incubated for 3 h to promote cell attachment. Nonadherent cells were removed, and then fresh medium, including purified protein (1 ng/ml or 1 μg/ml), heat-killed bacterial cells (0.5 to 10 μg/ml), or bacterial components, was added. For some experiments, an anticendotoxin reagent, polymyxin B nonapeptide (Sigma), or purified *E. coli* O55:B5 LPS (Sigma) was added to the medium at 100 μg/ml (7). After 24 h of incubation (or at other time points as indicated), cleared culture supernatants were collected by centrifugation and stored at −20°C.

**Cytokine quantification and cytotoxicity assay.** TNF-α and interleukin-10 (IL-10) released into the culture supernatants were detected with TNF-α and IL-10 OptEIA enzyme-linked immunosorbent assay sets (BD Biosciences, San Diego, CA), respectively. Appropriately diluted culture supernatants were assayed in accordance with the manufacturer's instructions. Concentrations of the cytokines were calculated using a standard curve. The cytotoxicity of killed bacteria during incubation with macrophages was determined with a CytoTox 96 nonradioactive cytotoxicity assay (Promega, Tokyo, Japan). In accordance with the manufacturer's protocol, free lactate dehydrogenase (LDH) leaked from the damaged cells was detected.

**Flow cytometry analysis of macrophages.** Preparation of macrophages for flow cytometry analysis was performed according to the methods described by Shida et al. (34). RAW264.7 cells ($10^5$ cells in 24-well plates) were incubated with FITC-conjugated bacteria in complete medium. Macrophages were dislodged by treatment with PBS supplemented with 10 mM EDTA, washed with PBS/EDTA, and fixed in 1.25% formalin-PBS. The cells were then analyzed by using a FACSCalibur cytometer and CellQuest software.

***N*-Acetylmuramidase treatment.** Heat-killed lactobacilli were suspended in 50 mM Tris-Cl buffer (pH 8) supplemented with 10 μg of mutanolysin/ml and incubated for 0, 10, 30, 60, and 120 min. The enzyme-treated cells were lysed in 2% sodium dodecyl sulfate solution, and the optical density was measured at 600 nm ($OD_{600}$).

**Statistical analysis.** Statistical significance was evaluated by using Student *t* test and one-way analysis of variance. Significant differences were defined as a $P$ value of $<0.05$.

## RESULTS

**Construction and evaluation of recombinant *L. casei*.** The expression of SE OmpC in recombinant *L. casei* was confirmed by immunoblotting. The specificity of anti-OmpC antibodies was validated by the detection of a specific appropriate-sized band in the cellular fraction of an SE culture (Fig. 1a). As shown in Fig. 1b, the OmpC-specific band of recombinant *L. casei* carrying pLP401::OmpC (LCO) was detected from its cell extract, while no specific band was detected from a control strain carrying pLPEmpty (LCN). The greater molecular mass of OmpC from LCO than purified recombinant OmpC (rOmpC) was appropriate because OmpC was fused to an anchor peptide provided from the pLP401 vector. The amount of heterologous antigen was also estimated by comparing to the signal intensity of purified recombinant OmpC. The expression efficiency of the antigen was approximately 25 ng per $2 \times 10^8$ CFU.

Because pLP401 provides the signal peptide and anchor, LCO cells were also examined for whether the heterologous protein was located on the cell surface. As a result of flow cytometric analysis, the fluorescence intensity of labeled LCO cells shifted slightly (Fig. 1c). This result indicated that at least part of the OmpC protein was exposed on the bacterial cell surface.

In order to determine whether OmpC-expression affected the cell viability of recombinant strains, the bacterial cells at the exponential phase (8 h) and the stationary phase (24 h) were tested by the plate culture method and using a Live/Dead BacLight bacterial counting and viability kit. By plate culture, 1.47 CFU/particle were detected from the LCO culture, and 1.31 CFU/particle were observed from the LCN culture. Both CFU values were greater than 1, indicating that almost 100% of the cells were viable. The flow cytometric analysis supported this result because 98.7% of the LCO and 99.3% of the LCN cells clustered in the ICW/membrane region. A slightly broader cluster of intact LCO cells than LCN culture cells was observed at this growth phase. Meanwhile, LCO formed fewer colonies (0.83 CFU/particle) than LCN (1.35 CFU/particle) at the stationary phase. A relatively high ratio of membrane/wall-damaged cells was detected in the cytogram of LCO cells (8.3%), whereas almost all LCN cells clustered in the intact cell region.

**OmpC-expressing recombinant *L. casei* induces less TNF-α release from RAW264.7 cells.** A murine macrophagelike cell line, RAW264.7, was stimulated with LCO cells, and TNF-α released into the culture supernatant was assayed. Cells incubated with heat-killed LCO cells released less TNF-α than did the nonexpressing *L. casei* strain in a dose-dependent manner (Fig. 2a). The lower cytokine induction was not caused by damage to the immune cells, because no difference was observed between LCO and LCN in the cytotoxicity assays (Fig. 2b). A regulatory cytokine, IL-10, was not detected in these cultures (data not shown). Similar results were obtained in the experiment using peritoneal macrophages instead of RAW264.7 cells (Fig. 2c).

**Purified OmpC does not inhibit the proinflammatory response.** The possibility that OmpC inhibited the proinflammatory response of RAW264.7 cells was explored. Purified rOmpC was added to the cell culture with or without heat-killed LCN. Because rOmpC was prepared from *E. coli*, the level of LPS contamination was determined. The results of the LPS detection assay showed an LPS contamination level of ~50 pg/ml in a 1-μg/ml solution of rOmpC, which could influence TNF-α production. In order to inhibit TNF-α induction by LPS, polymixin B nonapeptide was supplemented into the culture medium. The addition of 100 μg of polymixin B nonapeptide/ml eliminated <100 pg of LPS/ml completely but did not affect *L. casei*-induced TNF-α production (data not shown). As shown in Fig. 3, supplementing rOmpC did not inhibit LCN-induced TNF-α production, and no effect of LPS was observed.

**Importance of phagocytosed bacteria to TNF-α production.** The importance of the internalization of bacteria in RAW264.7 cells was evaluated using cytochalasin D. After 24 h of incubation with the phagocytosis inhibitor, the accumulation of TNF-α in the culture supernatants was decreased remarkably (Fig. 4a).

In order to investigate the efficiency of bacterial uptake by RAW264.7 cells, the cells exposed to heat-killed bacteria labeled with FITC were collected at different time points and applied to fluorescence-activated cell sorting (FACS) analysis. No remarkable difference between LCO and LCN was observed at any time point (Fig. 4b).

**Differences in *N*-acetylmuramidase sensitivity.** Internalized bacteria in macrophages are digested by phagolysosomal enzymes. If the bacteria resist such a digestive process, they may retain immunogenicity. To evaluate the sensitivity of recombinant lactobacilli to digestion, bacterial cells were treated with *N*-acetylmuramidase. As shown in Fig. 5, the lysis of LCO cells occurred more rapidly than that of LCN cells.

**Differences in the sustainability of TNF-α induction.** In order to determine the duration of cytokine production from RAW264.7 cells elicited by phagocytosed bacteria, culture supernatants from different time points were analyzed. After 1 h of uptake of bacteria, the same amount of TNF-α was released by macrophages stimulated with LCO and LCN until 4 h later; however, cells inoculated with LCO produced less TNF-α than with LCN after both 6- and 8-h incubations (Fig. 6).

**Cell wall involvement in differences in immunogenicity between LCO and LCN.** In order to identify the main component responsible for differences in immunogenicity between LCO and LCN, the ICW and CWRF of recombinant lactobacilli were prepared and added to the culture of RAW264.7 cells. As shown in Fig. 7, the cell wall of LCO elicited less inflammatory cytokine production than that of LCN, whereas both CWRFs stimulated macrophages weakly and equally.

## DISCUSSION

The heterologous expression of useful proteins in lactic acid bacteria has been trialed for medical applications. Many novel strains equipped supplemental function have been constructed by genetic modification. While the positive effects carried by the recombination were exhibited extensively in these studies, the negative effects caused by genetic modification have been scarcely remarked upon. The present study fortuitously found an unexpected negative effect of recombination caused by the expression of *Salmonella* OmpC in *L. casei*. As a purpose for which it was originally intended, a recombinant *L. casei* strain

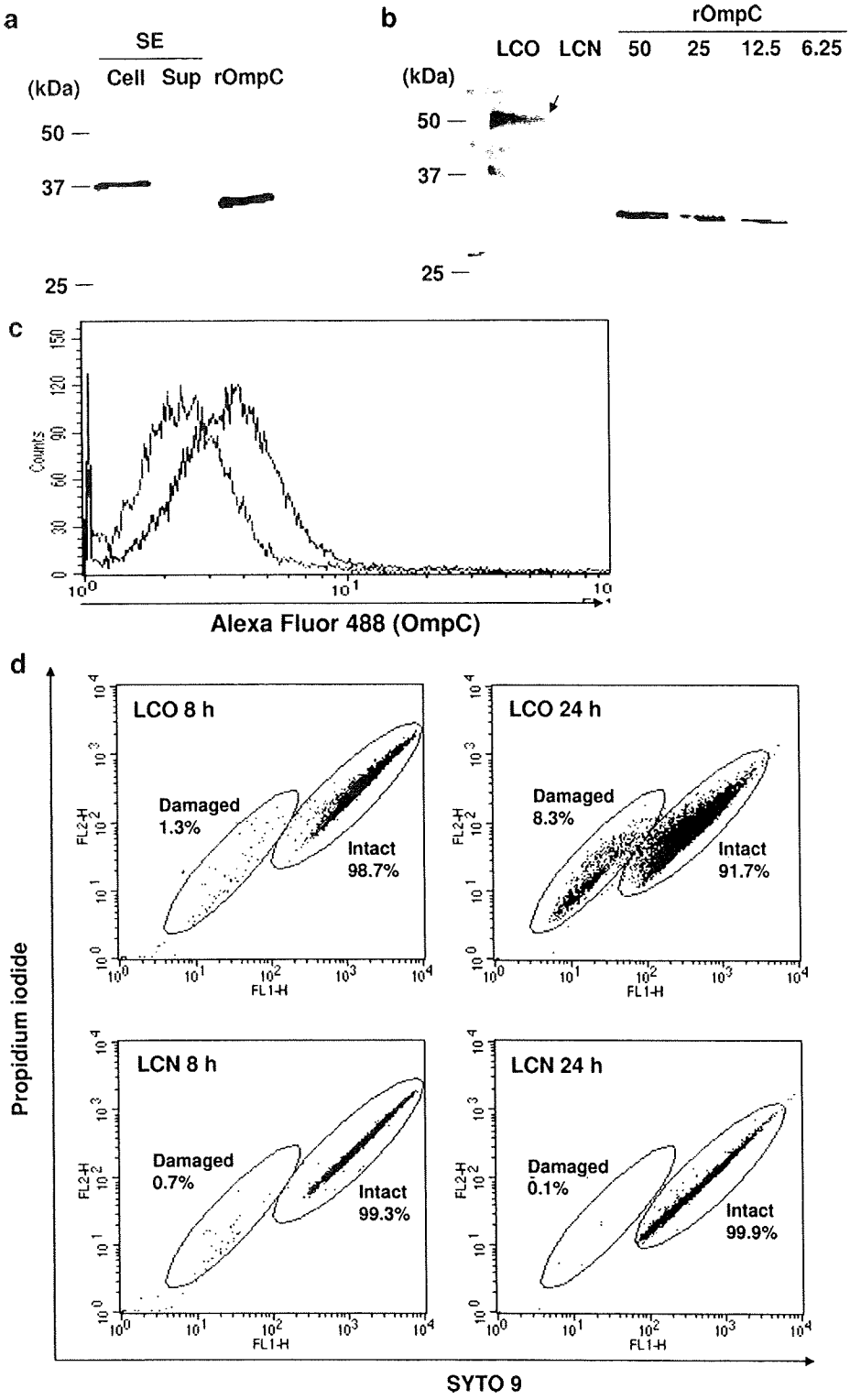


FIG. 1. Construction of recombinant *L. casei* expressing OmpC. (a) Specific antibody for the detection of OmpC. Purified recombinant OmpC and anti-OmpC antibodies were analyzed by immunoblotting. Approximately $10^8$ CFU of SE whole-cell extract (cell), the corresponding volume of culture supernatant (Sup), and 25 ng of purified OmpC (rOmpC) were applied. The sizes of the molecular mass markers are shown in the left margin. (b) Detection and quantification of OmpC expressed by recombinant *L. casei*. Whole-cell extracts of OmpC-expressing *L. casei* and a nonexpressing strain (corresponding to $2 \times 10^8$ CFU/lane) were applied to immunoblotting. The blot was conjugated with anti-OmpC antibody and Alexa Fluor 488-labeled IgG. An OmpC-specific band was visualized using Molecular Imager FX and analyzed with Quantity One (Bio-Rad). The sizes of the molecular mass markers are shown in the left margin. LCO, OmpC-expressing *L. casei*; LCN, nonexpressing *L. casei*. The values

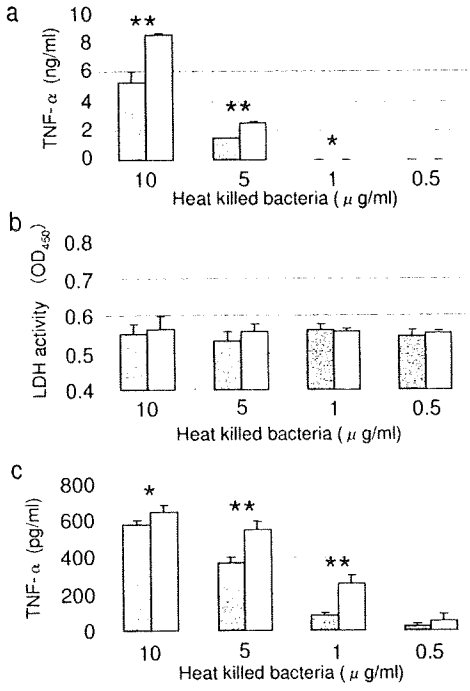


FIG. 2. TNF-α induction and LDH release caused by recombinant lactobacilli. (a) TNF-α released by RAW264.7 cells; (b) LDH release induced by recombinant *L. casei*; (c) TNF-α released by murine peritoneal macrophages. All three assays were performed using cell cultures incubated for 24 h. The concentrations of TNF-α in the culture supernatant or the $OD_{450}$ are described in the left margin. The concentrations of heat-killed bacteria added to the cell cultures are shown in the bottom margin (in μg/ml). Solid bars represent LCO cells, and open bars represent LCN cells. The data are presented as the means plus the standard deviations (SD) ($n$ = 3). The results shown are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

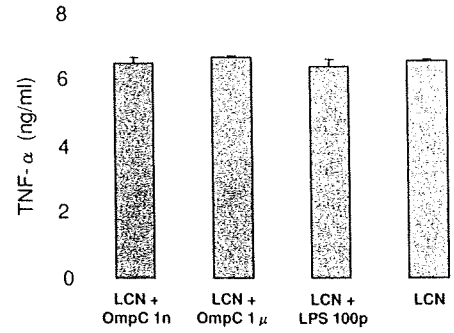


FIG. 3. TNF-α induction by LCN with or without rOmpC. A mixture of LCN (10 μg/ml) and rOmpC (or LPS) was added to a RAW264.7 cell culture. The concentrations of released TNF-α in the culture supernatant are described in the left margin. The data are presented as the means plus the SD ($n$ = 3). The results shown are representative of three independent experiments. No significant difference was shown. 1n, 1 ng/ml; 1μ, μg/ml; 100p, 100 pg/ml.

producing OmpC was established in order to develop a vaccine against *Salmonella*. Although the heterologous antigen was expressed clearly and exposed on the cell surface as a result of the use of an expression vector with a signal sequence and anchor, the efficiency of the surface localization seemed relatively low. Because *Salmonella* OmpC originally refers to a hydrophobic protein that forms channels in the outer membrane, it is understandable that the protein hardly passes through cytoplasmic membrane. Therefore, the result of FACS analysis for the detection of surface antigens led to speculation that only part of the produced antigens could pass through the cytoplasmic membrane, while the rest of the antigens were trapped around the membrane, or only the hydrophilic domain of the heterologous antigen was exposed on the cell surface, while the hydrophobic domain was left around the cell membrane. The expression of OmpC was also linked to the impaired cell viability of the recombinant lactobacilli. The data from flow cytometric analysis of recombinant bacteria stained with SYTO 9 and propidium iodide indicated that the cell membrane/wall of an OmpC-expressing strain was damaged. Because OmpC probably had high affinity to the cytoplasmic membrane, the protein might interrupt or affect cell membrane/wall formation. Among many genetically modified strains constructed in our laboratory, this is the only case in which such distinct damage to the cell envelope was observed using the pLP401 vector system (unpublished data).

The OmpC-expressing lactobacilli also exhibited side effects in their response to immune cells. It was found that the expression of SE OmpC reduced TNF-α production induced by *L. casei* from murine macrophages. Because none of the regulatory cytokines produced by macrophages, such as IL-10 (4, 8, 9), was detected during the incubation, this phenomenon may not occur by the downregulation of TNF-α expression. Subsequently, the study determined whether purified SE OmpC inhibited the release of TNF-α from RAW264.7 cells. Because 10 μg of OmpC-expressing *L. casei*, corresponding to approximately $5 \times 10^6$ CFU, was estimated to produce <1 ng of OmpC, 1 ng of OmpC/ml, along with a nonexpressing strain of *L. casei*, were added to the culture. A much higher concentration of OmpC (1 μg/ml) or higher contamination levels of LPS (100 pg/ml) were also supplemented; however, no significant differences were found. These results indicate that supplementation of purified recombinant SE OmpC into the cell culture did not prevent TNF-α induction by *L. casei*. This evidence does not support the phenomenon that the expression of SE OmpC by *L. casei* decreased the proinflammatory

50, 25, 12.5, and 6.25 refer to 50, 25, 12.5, and 6.25 ng of recombinant OmpC/lane, respectively. (c) Flow cytometric analysis of recombinant *L. casei*. Bacterial cells labeled with anti-OmpC antibody and Alexa Fluor 488-conjugated IgG. Ten thousand events were analyzed and are shown in histogram form. The gray-shaded area represents LCN cells, and the nonshaded solid line represents LCO cells. (d) Evaluation of cell viability by flow cytometry. Bacterial cells (LCO or LCN) at exponential phase (8 h) and stationary phase (24 h) were stained with SYTO 9 and propidium iodide. The percentages of damaged and/or intact cells were calculated by using CellQuest software. The result shown is representative of two independent experiments.

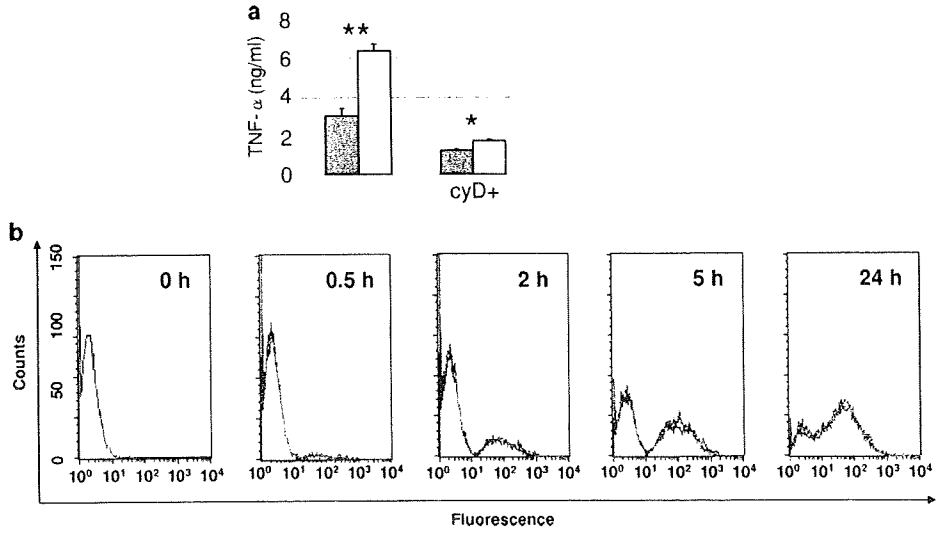


FIG. 4. Importance of phagocytosed bacteria in TNF-α induction and the frequency of phagocytosis. (a) TNF-α release elicited by recombinant lactobacilli with or without cytochalasin D (5 μg/ml) supplementation. Cytochalasin D was added to the RAW264.7 cell culture (described as cyD+) 30 min before the addition of 10 μg of LCO (solid bar) or 10 μg of LCN (open bar)/ml. The concentrations of released TNF-α (ng/ml) are indicated in the left margin. The data are presented as the mean plus the SD ($n$ = 3). (b) FACS analysis of phagocytosis of lactobacilli. RAW264.7 cells were cultured with FITC-labeled bacteria (10 μg/ml) for 0, 0.5, 2, 5, or 24 h and then collected. The bold gray line and thin black line represent LCN and LCO, respectively. The results shown are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$.

cytokine induction of RAW264.7 cells. The ICW prepared from OmpC-expressing *L. casei* still exhibited a different capacity from the nonexpressing strain to elicit TNF-α production by RAW264.7 cells. Because the ICW loses most protein during preparation, proteins may not affect this reaction. Taken together, OmpC, as a protein expressed by recombinant lactobacilli is not involved in the reduction of TNF-α release from RAW264.7 cells.

For further analysis, the present study evaluated the importance of phagocytosis in the immune responses of RAW264.7 cells stimulated with lactobacilli. Cytokine release by RAW264.7 cells was remarkably decreased by interference of phagocytosis by cytochalasin D, indicating that internalized bacteria mainly contribute to TNF-α induction. This evidence proposes a possibility in which the reduction in the number of internalized bacteria in macrophages could result in attenuation of the proinflammatory response. In order to determine the efficiency of phagocytosis, RAW264.7 cells internalizing FITC-labeled recombinant lactobacilli were analyzed by flow cytometry. However, the uptake frequency of recombinant bacteria was not different between OmpC-producing lactobacilli and the nonexpressing strain.

A phagosome including bacteria fuses to lysosomes and the bacteria are digested in the mature phagosome. In this process, *N*-acetylmuramidase, an enzyme that catalyzes the degradation of peptidoglycan, is one of the main agents for the digestion of bacteria (3). To evaluate the sensitivity to digestion, recombinant lactobacilli were treated with *N*-acetylmuramidase. As a result, the OmpC-expressing strain lysed more rapidly than the control strain. Consequently, the amount of TNF-α released

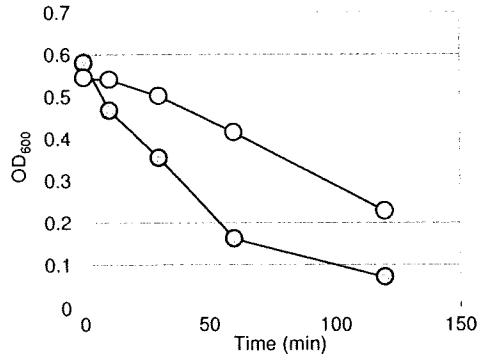


FIG. 5. Evaluation of *N*-acetylmuramidase sensitivity. The reduction of the $OD_{600}$ caused by cell lysis was measured at different time points. This result is representative of three independent experiments. Symbols: ●, LCO cells; ○, LCN cells.

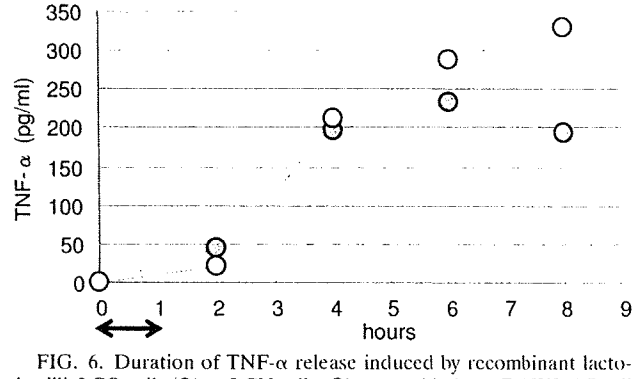


FIG. 6. Duration of TNF-α release induced by recombinant lactobacilli. LCO cells (●) or LCN cells (○) were added to a RAW264.7 cell culture, followed by incubation for 1 h (indicated by an arrow in the bottom margin) to allow phagocytosis. Excess bacteria that were not internalized were then removed by replacing the medium. Each culture supernatant was collected at 2, 4, 6, or 8 h. This result is representative of three independent experiments.

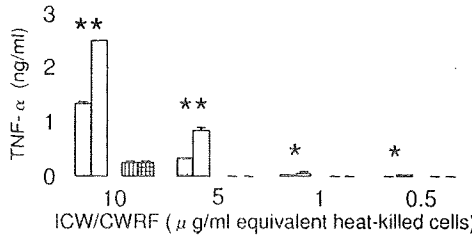


FIG. 7. TNF-α production induced by the ICW or CWRF of recombinant lactobacilli. Cell components were prepared from each concentration of heat-killed bacteria and added to RAW264.7 cell cultures. The concentrations of the added cell components described in the bottom margin are not the actual number but correspond to the concentration of heat-killed bacteria. The concentrations of released TNF-α are indicated in the left margin. The data are presented as the means + the SD ($n = 3$). The results are representative of two independent experiments. Bars: ■, ICW from LCO cells; □, ICW from LCN cells; ▥, CWRF from LCO cells; ▦, CWRF from LCN cells. *, $P < 0.05$; **, $P < 0.01$.

from RAW264.7 cells was measured, and it was shown that the cells phagocytosed a certain amount of bacteria at several time points. During the time course, proinflammatory cytokines elicited by OmpC-producing *L. casei* terminated earlier than that by the control strain. These results suggest that the recombinant strain expressing the heterologous antigen may be digested more rapidly in macrophages and lose its immune-stimulating capability at an earlier time point. In this context, Shida et al. reported previously that *Lactobacillus* strains exhibiting relatively low sensitivity to *N*-acetylmuramidase show high potency to induce IL-12 (34). Hence, the robustness of bacterial cells is probably an important factor for their immunogenicity. Based on the above findings, the involvement of the cell wall was speculated in the attenuation of the proinflammatory response by OmpC-expressing *L. casei*. As expected, the ICW evoked TNF-α release strongly, while a cell wall-digested fraction induced low levels of cytokine production. Moreover, a significant difference between the recombinant strain producing OmpC and the control strain was observed with the cell wall. This result suggested that the expression of OmpC in *L. casei* may cause some sort of conversion in the structure of the cell wall. The specific structure affected by OmpC expression will be determined in a further study. In conclusion, OmpC expression in *L. casei* affected the *N*-acetylmuramidase sensitivity of the cell wall, which resulted in attenuation of the property to elicit a proinflammatory response in RAW264.7 cells.

Recently, genetically modified lactobacilli have been developed for vaccines, anti-allergic agents, and other applications. Among these studies, reduction or negative effects brought about by heterologous gene expression have not yet been reported. The attenuation of immunogenicity due to OmpC expression is probably a unique and exceptional phenomenon; however, the present study provides important information about the pleiotropic effects of genetic modifications.

## ACKNOWLEDGMENTS

This study was supported by a grant from the Ministry of Health, Labor, and Welfare (Research on Food Safety) and partly by a grant from the Food Safety Commission of Japan.

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CLINICAL AND VACCINE IMMUNOLOGY, Jan. 2010, p. 43–48 Vol. 17, No. 1
1556-6811/10/$12.00 doi:10.1128/CVI.00337-09


# Adjuvant Effects for Oral Immunization Provided by Recombinant *Lactobacillus casei* Secreting Biologically Active Murine Interleukin-1β[▽]

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Received 20 August 2009/Returned for modification 17 September 2009/Accepted 9 November 2009

**Vaccine delivery systems using lactic acid bacteria are under development, but their efficiency is insufficient. Autologous cytokines, such as interleukin-1β (IL-1β), are potential adjuvants for mucosal vaccines and can be provided by recombinant lactic acid bacteria. The aim of this study was the construction and evaluation of recombinant *Lactobacillus casei* producing IL-1β as an adjuvant delivery agent. The recombinant strain was constructed using an expression/secretion vector plasmid, including a mature IL-1β gene from mouse. The biological activity of the cytokine was confirmed by IL-8 production from Caco-2 cells. In response to the recombinant *L. casei* secreting IL-1β, expression of IL-6 was detected *in vivo* using a ligated-intestinal-loop assay. The release of IL-6 from Peyer's patch cells was also detected *in vitro*. Intragastric immunization with heat-killed *Salmonella enterica* serovar Enteritidis (SE) in combination with IL-1β-secreting lactobacilli resulted in relatively high SE-specific antibody production. In this study, it was demonstrated that recombinant *L. casei* secreting bioactive murine IL-1β provided adjuvant effects for intragastric immunization.**

At present, a number of vaccines based on lactic acid bacteria (LAB vaccines) are under development (31). These commensals, including *Lactococcus lactis* and *Lactobacillus* strains, have been used as vaccine delivery vectors. Several LAB vaccines, such as tetanus toxin fragment C (TTFC)-producing LAB, exhibit high immunogenicity and can induce both mucosal and systemic immune responses, as well as protective immunity (11, 22). However, most LAB vaccines administered via the mucosal route, especially the oral or intragastric (i.g.) route, exhibit relatively low efficiency. As expected, a high dosage is required to elicit effective immunity. It is usually required that mice receive more than $10^9$ CFU of bacteria on three or more consecutive days with two or more boosts (4, 5, 16). In several cases, LAB vaccines were administered in combination with a supplemental adjuvant to induce sufficient immune responses (3, 32). In order to improve the efficiency of vaccination, several kinds of additional factors that assist LAB vaccines have been investigated. Steidler et al. carried out a pioneering study that demonstrated the adjuvant effect provided by *L. lactis* expressing TTFC intracellularly and also secreting either murine interleukin-2 (mIL-2) or mIL-6 (28). In another report, a single-chain murine IL-12 was produced by *L. lactis* and enhanced antigen-specific Th1 cytokine production (2). Recently, a unique strategy to accelerate bacterial uptake by dendritic cells (DCs) was developed by Mohamadzadeh et al. (18). They achieved effective oral vaccination using recombinant *Lactobacillus acidophilus* secreting a protective antigen of *Bacillus anthracis* in combination with a DC-targeted peptide. Although all these adjuvant molecules are promising, there are still only a few options for their use as mucosal adjuvants at present. Hence, exploration of other mucosal adjuvants that are applicable to LAB vaccines is important.

The present study investigated IL-1β as a mucosal vaccine. IL-1β is produced by activated monocytes and macrophages, etc., as a precursor that is proteolytically processed into a mature form by IL-1β-converting enzyme (ICE), also known as caspase 1 (6). This proinflammatory cytokine and other IL-1 family cytokines play important roles in modulating the adaptive immune response (7). In fact, a deficiency of IL-1β impairs T-cell-mediated cellular immune responses (26, 29). It was reported previously that IL-1β exhibited adjuvant effects in both mucosal and systemic immunization (27). In addition, Nakae et al. demonstrated that IL-1 enhanced T-cell-dependent antibody production (19). This evidence suggests that IL-1β may be a promising adjuvant for mucosal immunization if it is produced by recombinant lactic acid bacteria. Unlike other mucosal adjuvants, such as cholera toxin, IL-1β is an autologous protein and therefore nonimmunogenic. This may be a preferable feature, because adaptive immunity against adjuvant proteins induced by repeated doses may reduce the adjuvant effect in further immunizations. The aim of this study was to construct an IL-1β-secreting *Lactobacillus* and to evaluate its adjuvant effect.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *Lactobacillus casei* IGM393 and a nonexpressing control strain carrying pLPEmpty (LCN) were grown in de Mann-Rogosa-Sharpe (MRS) medium (Difco) at 37°C (14). Erythromycin (5 μg/ml) was used as a supplement for the culture of recombinant lactobacilli. Bicarbonate buffer (50 mM; pH 7.0 to 8.0) was also added to the bacterial culture in some experiments to control the pH of the media. *Salmonella enterica* serovar Enteritidis (SE) no. 40 was grown in Luria-Bertani (LB) broth (Difco) at 37°C under aerobic conditions (1).

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▽ Published ahead of print on 18 November 2009.