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Expression of SARS-coronavirus nucleocapsid protein in *Escherichia coli* and *Lactococcus lactis* for serodiagnosis and mucosal vaccination

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Abstract The nucleocapsid (N) protein of the severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) is an important antigen for the early diagnosis of SARS and the development of vaccines. It was expressed in *Escherichia coli* as a fusion with human glutathione S-transferase (hGST) and was confirmed by Western blotting analysis. This recombinant N protein (hGST-N) was purified and used to measure the SARS-CoV N-specific antibody in the sera of eight SARS patients by enzyme-linked immunosorbent assay. Specific antibody response to this purified recombinant N protein was 100% positive in the SARS patients' sera, while none of the control sera from 30 healthy people gave a positive reaction in the same assay. The SARS-CoV N protein was also expressed in *Lactococcus lactis* in the cytoplasm or secreted into the medium. The N-producing strain MG1363/pSECN and the purified hGST-N protein were respectively administered to mice, either orally or intranasally. Results indicated that orally delivered MG1363/pSECN induced significant N-specific IgG in the sera. In conclusion, our work provides a novel strategy to produce the SARS-CoV N protein for serodiagnosis and for *L. lactis*-based mucosal vaccines.

Introduction

The etiologic agent of the severe acute respiratory syndrome (SARS) emerging worldwide in 2003 has been identified as a novel type of coronavirus (SARS-CoV), which consists of a single-stranded positive-sense RNA genome of about 29,700 nucleotides, encoding a RNA-directed RNA polymerase and structural proteins including the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins (Marra et al. 2003; Rota et al. 2003; Ruan et al. 2003). The N protein is a 422-amino-acid alkaline protein with a short lysine-rich region suggested as the nuclear localization signal. It plays an important role in the process of virus particle assembly by enveloping the entire genomic RNA (Marra et al. 2003). The SARS-CoV N protein has been suggested as a potent antigen for the early diagnosis of SARS-CoV infection (Timani et al. 2004). Moreover, recent evidence indicates that the N protein can induce a T-cell response to SARS-CoV infection (Gao et al. 2003); and thus the N protein is also important in the development of an effective vaccine against SARS-CoV infection.

Because of their edibility and convenience, lactic acid bacteria (LAB) have been safely used in the food industry for centuries and are considered as potential mucosal delivery vehicles of vaccine antigens (Dieye et al. 2001, 2003; Gilbert et al. 2000; Robinson et al. 1997). LAB-based vaccines can effectively achieve mucosal and systemic immunization at mucosal sites, which are the entry routes of many pathogens including SARS-CoV and HIV. For instance, *Lactococcus lactis* has been used as a vaccine delivery system for the HIV Env protein. Oral administration of this recombinant vaccine antigen induces strong HIV-specific humoral and cell-mediated immune responses which significantly reduce the viral load following challenge with an Env-expressing vaccinia virus (Xin et al. 2003).

In this paper, we report the high-level production of SARS-CoV N protein in *Escherichia coli* and in the food-grade bacterium *L. lactis*. The potential applications of the purified human glutathione S-transferase (hGST)-fused

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Table 1 Strains and plasmids. pXN was obtained from the Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences, according to cooperation contract SIBS-SARS-IP-005. *Amp* ampicillin, *Cm* chloramphenicol, *Ery* erythromycin

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> JM109 and TG1	Host for cloning	Sambrook et al. (1989)
<i>E. coli</i> BL21DE3	Host for gene expression	Novagen
<i>L. lactis</i> MG1363	Plasmid-free strain	Gasson (1983)
<i>L. lactis</i> NZ9000	Derivative of MG1363 carrying regulatory genes <i>nisR</i> and <i>nisK</i>	Kuipers et al. (1998)
Plasmids		
pXN	Amp ^r ; SARS-CoV nucleocapsid gene (<i>n</i>) was cloned in pcDNA3.1(-)mychisA	SIBS
pGEX4T-1	Amp ^r ; <i>E. coli</i> expression vector; <i>P_{tac}</i> promoter	Amersham Biosciences
pGEXN	Amp ^r ; the <i>n</i> gene was inserted into the <i>Bam</i> HI- <i>Sal</i> I sites of pGEX4T-1	This work
pET23b	Amp ^r ; <i>E. coli</i> expression vector; <i>P_{T7}</i> promoter	Novagen
pET23bN	Amp ^r ; the <i>n</i> gene was inserted into the <i>Bam</i> HI- <i>Sal</i> I sites of pET23b	This work
pT7hGST	Amp ^r ; the <i>hGST</i> gene was inserted into the <i>Nde</i> I- <i>Bam</i> HI sites of pET23b	This work
pT7hGSTN	Amp ^r ; the <i>n</i> gene was inserted into the <i>Bam</i> HI- <i>Sal</i> I site of pT7hGST	This work
pNZ8048	Cm ^r ; <i>L. lactis</i> expression vector; <i>P_{NisA}</i> promoter	Kuipers et al. (1998)
pCYTN	Cm ^r ; the <i>n</i> gene was inserted into the <i>Nco</i> I- <i>Xba</i> I sites of pNZ8048	This work
pVE5523	Amp ^r ; Ery ^r ; <i>L. lactis</i> expression vector; <i>P₅₉</i> promoter; <i>sp_{Usp45}</i> signal peptide sequence	Dieye et al. (2001)
pSECN	Amp ^r ; Ery ^r ; <i>n</i> gene was inserted into pVE5523 at the <i>Sal</i> I- <i>Eco</i> RV sites	This work

N protein (hGST-N) in serodiagnosis and the N protein-producing *L. lactis* in mucosal vaccination were both experimentally evaluated.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown in Luria–Bertani medium at 37°C or 30°C (when induced) with shaking, while *L. lactis* strains were grown in GM17 medium at 30°C without shaking, as described by Dieye et al. (2003) and Xiang et al. (2003). When necessary, antibiotics and inducers were added as follows: for *E. coli*, ampicillin (100 µg ml⁻¹), erythromycin (150 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹) and isopropyl thiogalactose (IPTG; 1 mmol l⁻¹); and for *L. lactis*, erythromycin (5 µg ml⁻¹) and nisin (50 ng ml⁻¹).

DNA manipulations

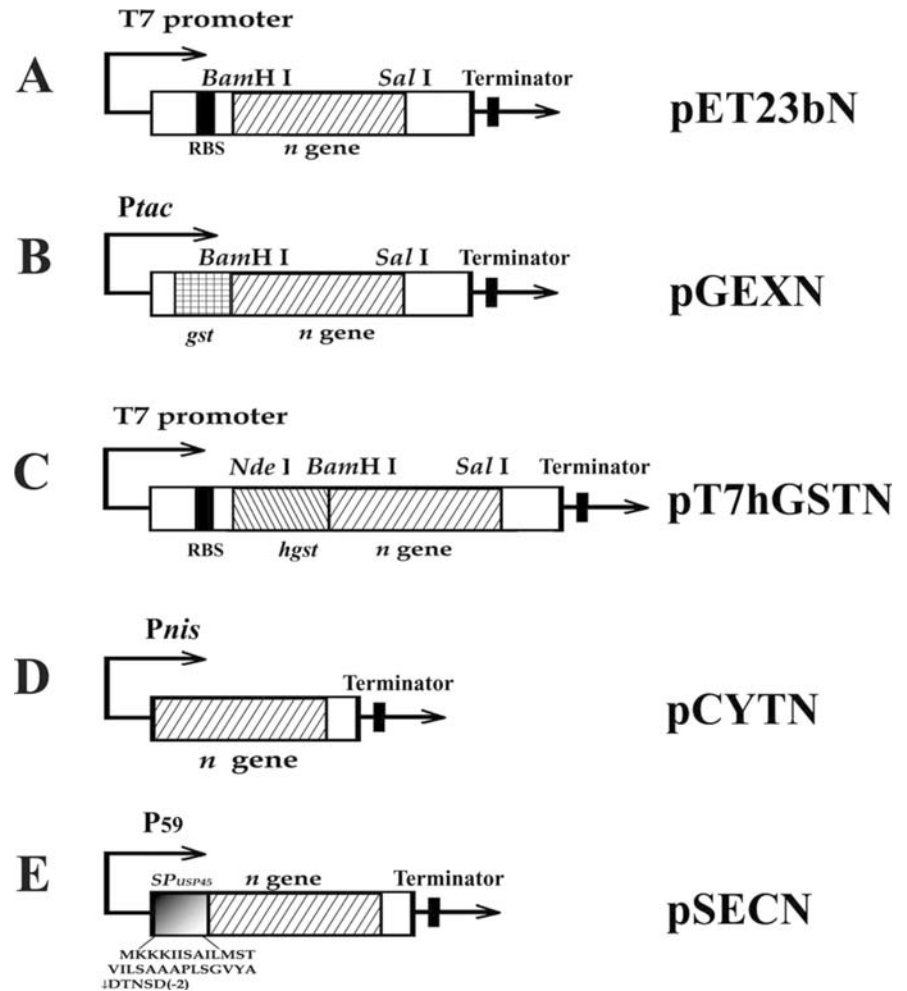
General molecular biological techniques were performed using standard methods (Sambrook et al. 1989). Unless otherwise indicated, the cDNA of the SARS-CoV nucleocapsid gene (*n*) was always amplified by PCR with *Pfu* polymerase, using plasmid pXN (see Table 1) as the template. The primers used in the following gene cloning procedures are listed in Table 2.

For expression and characterization of the N protein in *E. coli*, three different expression plasmids were constructed (Fig. 1). For the first one, the *n* gene was PCR-amplified with primers P1F/P1R. The PCR product was digested with *Bam*HI and *Sal*I and then cloned into the same sites of pET23b, yielding pET23bN (Fig. 1a). For the second one, the *n* gene was amplified with primers P2F/P2R and cloned into the *Bam*HI/*Sal*I-linearized pGEX4T-1, generating pGEXN (Fig. 1b). For the third one, the *hgst* gene was amplified from pET23b::hgst (Xiang et al. 2003) with primers P3F/P3R and digested with *Nde*I and *Bam*HI.

Table 2 Primers. Restriction enzyme sites are in italics

Name	Sequence (5'→3')	Restriction enzyme
P1F	TAGGATCCGATGTCTGATAATGGACCCCA	<i>Bam</i> HI
P1R	CTGTGACTTATGCCTGAGTTGAATCAGC	<i>Sal</i> I
P2F	TAGGATCCATGTCTGATAATGGACCCCA	<i>Bam</i> HI
P2R	CTGTGACTTATGCCTGAGTTGAA TC	<i>Sal</i> I
P3F	TCCATATGGCAGAGAAGCCCAAGC	<i>Nde</i> I
P3R	TAGGATCCAAACCTGAAAATCTTCCTTGC	<i>Bam</i> HI
P4F	TACCATGGCAATGTCTGATAATGGACCCCA	<i>Nco</i> I
P4R	CTTCTAGATTATGCCTGAGTTGAATCAGC	<i>Xba</i> I
P5F	TAGTCGACTGTCTGATAATGGACCCCA	<i>Sal</i> I
P5R	CTGATATCTTATGCCTGAGTTGAATCAGC	<i>Eco</i> RV

Fig. 1 Schematic diagram of plasmid constructs expressing SARS-CoV N protein in *E. coli* and *L. lactis*. **a** pET23bN, **b** pGEXN, **c** pT7hGSTN, **d** pCYTN, **e** pSECN. Detailed information on these expression plasmids is given in Table 1



At the same time, the *n* gene was generated from pGEXN by digestion with *Bam*HI and *Sal*I. These two DNA fragments were cloned into pET23b together at the *Nde*I and *Sal*I sites, resulting in plasmid pT7hGSTN (Fig. 1c). All these plasmids were transformed into *E. coli* BL21DE3 for N protein production and/or purification.

For expression of the N protein in the cytoplasm of *L. lactis* or secretion into the culture medium, two expression plasmids were also constructed. For the first one, the *n* gene was amplified with primers P4F/P4R. After digestion with *Nco*I and *Xba*I, it was cloned into pNZ8048 at the same sites, generating pCYTN for nisin-induced production of the N protein (Fig. 1d). For the second one, the *n* gene was PCR-amplified with primers P5F/P5R and cloned into plasmid pVE5523 (Dieye et al. 2001) at the *Sal*I and *Eco*RV sites, resulting in plasmid pSECN for secretion of the N protein (Fig. 1e). These two expression plasmids, pCYTN and pSECN, were first established in *E. coli* and then transferred by electroporation into *L. lactis* NZ9000 and MG1363, respectively, as described by Xiang et al. (2000).

Production and purification of SARS-CoV N protein in *E. coli*

E. coli BL21DE3 harboring the expression plasmids was cultured until the mid-exponential phase [optical density at 600 nm (OD_{600})=0.4–0.6] and then induced with IPTG (1 mM) for an additional 3–4 h at 30°C with shaking (180 rpm). The cells were collected and the pellets were resuspended in TES buffer (10 mM Tris-HCl, 1 mM EDTA, 25% sucrose, pH 5.8) and sonicated for 3–5 min until completely lysed. The fusion protein hGST-N (generated from pT7hGSTN) or GST-N (generated from pGEXN) was purified through a glutathione–agarose column (Amersham Biosciences), according to methods described by Xiang et al. (2000, 2003).

Preparation of antiserum against SARS-CoV N protein

For antiserum preparation, approximately 2 mg of purified GST-N mixed with Freund's complete adjuvant were injected into a healthy New Zealand rabbit three times,

once per week. Antiserum was collected from the marginal vein of the rabbit's ear at day 30 and was used for Western blotting analysis.

Immunoassay detection of N-specific antibody in the sera of SARS patients

Purified hGST-N protein (50 ng) dissolved in 100 μ l of coating buffer (16 mM Na₂CO₃, 34 mM NaHCO₃, pH 9.6), was added a 96-well microplate (50 ng/well) and incubated at 4°C overnight. Wells were then blocked with 7% non-fat milk in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3), incubated for 1 h at room temperature, washed three times with PBS and then dried at 37°C for 1 h. To detect the N-specific antibody in the sera of SARS patients, the sera were diluted (1:25) and loaded in the wells in triplicate and then incubated at 37°C for 30 min. Each well was washed with PBS and afterwards incubated with peroxidase-conjugated rabbit anti-human antibody (IgG) for an additional 30 min. The wells were washed again with PBS containing 0.5% Tween-20. The peroxidase reaction was performed at 37°C, using *O*-phenylenediamine as the substrate. After 10 min, the reaction was stopped by adding 50 μ l of 1 M H₂SO₄ to each well, and the OD₄₅₀ was read. For comparison, the same samples were assayed with a widely used commercially available enzyme-linked immunosorbent assay (ELISA) kit based on a SARS-CoV viral protein mixture prepared by the Beijing Genomic Institute, Chinese Academy of Sciences. The cutoff value was defined as the average OD₄₅₀ of control samples plus 0.1.

Expression and detection of SARS-CoV N protein in *L. lactis*

For expression of the N protein in the cytoplasm of *L. lactis*, cultures of NZ9000/pCYTN were induced with nisin (10 ng ml⁻¹; Sigma) at the mid-exponential phase (OD₆₀₀=0.6). After 3–6 h, the cells were harvested and resuspended in TES buffer for protein extraction by sonication. For secretion of the N protein by *L. lactis*, the recombinant strain *L. lactis* MG1363/pSECN was cultivated to the late-exponential phase (OD₆₀₀=0.8). Proteins in the medium and cells were separately prepared as described by Piard et al. (1997) and Dieye et al. (2003), with minor modifications. Briefly, The supernatant (medium part) of the culture was filtered (0.2- μ m pore size filter; Millipore) and then the proteins in the filtrate were precipitated with ice-cold trichloroacetic acid (16% final concentration). The resulting pellets from 4 ml of filtrate were dissolved in 80 μ l of water for analysis. In contrast, the cell pellets from the same culture were washed and resuspended in 500 μ l of TES for protein extraction by sonication. For Western blotting analysis, the protein extracts were subjected to SDS-PAGE (12% acrylamide) and the SARS-CoV N protein expressed in *L. lactis* was detected with the N-specific antisera

generated by GST-N in the above experiments. The N protein expressed by *E. coli* BL21DE3/pET23bN was used as a control. The protein electro-blotting, antibody reaction and detection were performed as described by Wei et al. (2002).

Mouse vaccination

Recombinant strain *L. lactis* MG1363/pSECN and control strain MG1363 were grown and harvested as described above. For MG1363, cells were resuspended in sterile PBS to obtain a concentration of 10⁸ cells ml⁻¹. For strain MG1363/pSECN, the supernatants of the culture (containing the secreted N protein) were collected and filtered as described above and proteins from 3 ml of the filtrate were concentrated into 0.5 ml by freeze-evaporation. Then, the cell pellets were diluted directly into PBS to achieve a concentration of 10⁸ cells in 0.5 ml. Then, the secreted N protein (0.5 ml) and the cells (0.5 ml) were combined into 1.0 ml. For mouse vaccination, six groups of BALB/c female mice (five mice in each group) were immunized orally or intranasally with the recombinant strains, the control strains, or the purified hGST-N resuspended in PBS, respectively. Oral doses of 15 μ g of hGST-N, or 10⁷ cells (110 μ l of *L. lactis* MG1363/pSECN or MG1363 cells, freshly prepared as described above), were administered orally on days 1, 2, 4 and 22. Intranasal doses of 1.5 μ g hGST-N, or 2.5 \times 10⁶ cells (25 μ l, as described above) were slowly administered to mice on days 1, 2, 4 and 22. Another non-immunized control group was maintained under the same conditions. The immune sera (collected by retro-orbital puncture) were collected 12 days after final immunization and used to evaluate the immunogenicity of both the recombinant *L. lactis* strain and the purified hGST-N, using ELISA as described above.

Results

Expression and purification of SARS-N protein in *E. coli*

The full-length fragment of the *n* gene was cloned and expressed in *E. coli* BL21DE3 using two different expression plasmids, pT7hGSTN and pGEXN, as described in Fig. 1 and Table 1. The inserts in both plasmids were sequenced and proved to be correct. After induction with IPTG, the hGST-N from *E. coli*/pT7hGST and the GST-N from *E. coli*/pGEXN were both overexpressed, with expected sizes of 73.5 kDa and 72.5 kDa, respectively (Fig. 2). When the supernatants and pellets of each extract prepared by sonication and centrifugation were respectively subjected to SDS-PAGE, it was also determined that most of the recombinant N proteins were soluble rather than forming inclusion bodies (data not shown). This allowed the convenience of purifying the recombinant proteins within the supernatants; and indeed the soluble GST-N and hGST-N were successfully purified

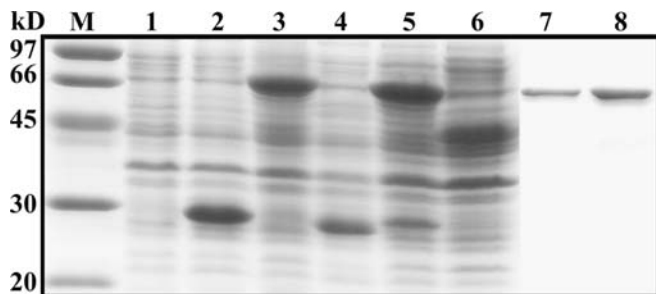


Fig. 2 Expression and purification of SARS-CoV N protein in *E. coli*. Equal amounts of protein extracts from the following strains (lanes 1–6) were separated by 12% SDS-PAGE: lane 1 BL21DE3/pET23b, lane 2 BL21DE3/pT7hGST, lane 3 BL21DE3/pT7hGSTN, lane 4 BL21DE3/pGEX-4T-1, lane 5 BL21DE3/pGEXN, lane 6 BL21DE3/pET23bN. The purified hGST-N and GST-N were loaded in lane 7 and lane 8, respectively. The sizes of molecular mass markers (M) are indicated on the left. kD KiloDaltons

through a single step of glutathione-based affinity chromatography (Fig. 2).

Preparation of N-specific antisera in rabbit with GST-N and detection of N-specific antibody in SARS patients with hGST-N

Polyclonal antibodies were prepared in rabbit using GST-N as the immunogen. To verify the specificity of these antibodies against the SARS-CoV N protein, the proteins hGST (from pET23b::hgst), hGST-N and GST-N were subjected to SDS-PAGE for Western blotting analysis. Both hGST-N (73.5 kDa) and GST-N (72.5 kDa) gave a strong signal, but hGST did not (Fig. 3a). These results clearly indicated that the N-specific antibody was successfully induced with the GST-N antigen, as it significantly recognized hGST-N but not hGST. Moreover, it implied that the hGST-N protein might be an effective antigen for detection of the N-specific antibody in SARS patients.

To investigate the possibility of using hGST-N in the diagnosis of SARS-CoV infection, an ELISA assay (see Materials and methods) was performed with the sera collected from eight SARS-CoV infected patients at 17–30 days after fever onset, using purified hGST-N as the antigen and the sera collected from 30 healthy people as controls. As shown in Fig. 3b, all the sera of the SARS patients had significant positive responses to the hGST-N protein, while none of the 30 control sera gave a positive signal. The same results were also achieved with a commercially available ELISA kit coated with a mixture of SARS-CoV viral proteins, indicating that the IgG antibodies to N protein are commonly present in the sera of SARS patients and can be detected at least as early as 17 days after fever onset. Moreover, our results provide a rapid and efficient strategy for the production and purification of the recombinant N protein for serodiagnosis of SARS-CoV infection.

Production and identification of SARS-CoV N protein in *L. lactis*

SARS-CoV N protein is also considered an important antigen for vaccine development, especially as an adjuvant vaccine antigen for the S protein, the major vaccine antigen that induces neutralizing antibody (Gao et al. 2003). For easy use of this protein in mucosal vaccination, we expressed this protein in the food-grade bacterium *L. lactis*. First, the production of the recombinant SARS-CoV N protein in *L. lactis* NZ9000/pCYTN was analyzed before and after nisin induction. The protein extracts from cytoplasm and the medium were both subjected to SDS-PAGE, followed by Western blotting assays with the anti-N antibodies prepared above. In the absence of nisin, no signal was detected, indicating that no recombinant N protein was produced (data not shown). After 6 h of induction with nisin, the results showed that recombinant N protein was present in the cell cytoplasm fractions as expected (Fig. 4a). However, the expression level of N

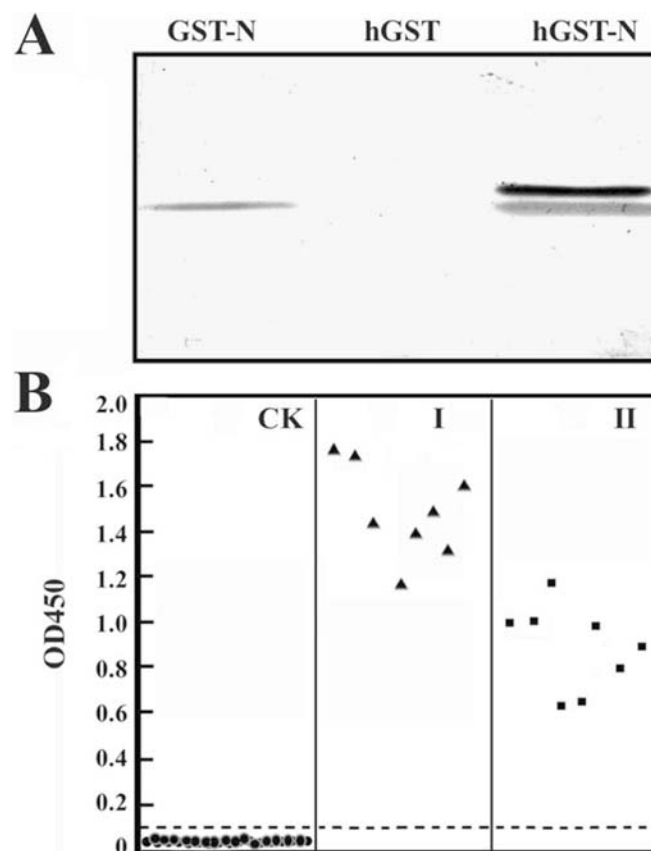


Fig. 3 a Western blotting assay with the specific polyclonal antibodies from GST-N. Note that GST-N from BL21DE3/pGEXN and hGST-N from BL21DE3/pT7hGSTN give positive signals, while hGST from BL21DE3/pT7hGST does not. b ELISA assay of N-specific antibody in SARS patients. CK Sera from 30 healthy people were detected using hGST-N as the antigen, I sera from eight SARS patients were detected using hGST-N as the antigen, II sera from eight SARS patients (the same as in I) were detected with a commercial kit using a SARS-CoV protein mixture as the antigen. The dashed line showed the cutoff value (0.135 = average value of healthy people detected by the commercial kit plus 0.1)

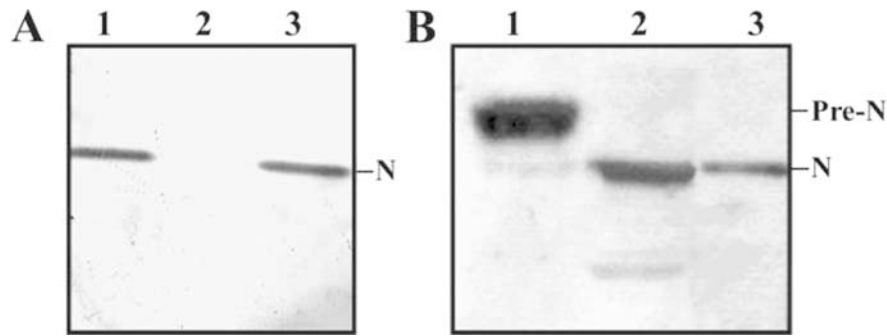


Fig. 4 Western blotting analysis of the expression SARS-CoV N protein in *L. lactis* strains NZ9000/pCYTN (a) and MG1363/pSECN (b). Lane 1 Proteins extracted from the cytoplasm, lane 2 proteins collected in the culture medium, lane 3 proteins extracted

from *E. coli* BL21DE3/pET23bN as a control. The expressed mature N protein (N) and the precursor of the secreted form (Pre-N) are indicated

protein in this strain was usually subject to variable induction efficiency. To overcome this inconvenience, we then constructed a novel recombinant strain, *L. lactis* MG1363 harboring an expression plasmid pSECN, in which the *n* gene was fused with the coding sequence of USP45 signal peptide and was controlled by the constitutive *L. lactis* promoter P_{59} . Western blotting showed that a prominent band of 46 kDa corresponding to size of the mature N protein was observed in the medium, indicating that the SARS-CoV N protein can be secreted by this recombinant *L. lactis* strain; and only a few secreted N proteins were degraded (Fig. 4b). However, nearly 60% of the N protein precursor was still in the cytoplasmic fraction (Fig. 4b). Thus, expression of the recombinant N protein in both cytoplasm and medium was realized by strain *L. lactis* MG1363/pSECN, rendering it suitable for both oral and intranasal administration.

Mucosal immunization of mice with MG1363/pSECN strain and hGST-N protein

Thirty mice were randomly divided into six groups and the five mice in each group were administered orally or intranasally with strain MG1363, strain MG1363/pSECN or purified hGST-N. The effect of immunization on the production of N-specific IgG was examined by ELISA 12 days after the final administration, using purified GST-N (not hGST-N) as the antigen to exclude the interference of hGST. Results indicated that orally administrated MG1363/pSECN induced a very significant response from the N-specific IgG in the sera ($P < 0.02$, *t*-test). However, oral administration of purified hGST-N did not seem to induce any N-specific response, as it just gave the same background signal in this ELISA inspection as the control strain MG1363 (Fig. 5), indicating that, while the MG1363/pSECN-carried N protein was protected in the gastric tract and could be released in the intestinal mucosal sites, the purified hGST-N might have been degraded in the gastrointestinal environment before reaching the mucosal site. Also, the intranasally administered MG1363/pSECN and hGST-N in this investigation just elicited a very weak N-specific immunization (not significant, $P > 0.05$, *t*-test, data not shown).

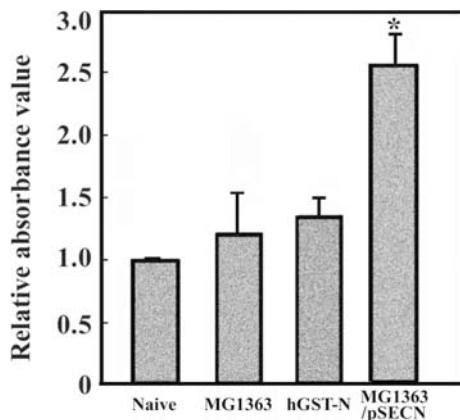


Fig. 5 N-specific antibody levels in groups of mice immunized orally with control strain *L. lactis* MG1363, purified hGST-N or recombinant strain *L. lactis* MG1363/pSECN. The sera of the mice in each group were combined and diluted (1:10, 1:100, 1:1000) when subjected to ELISA analysis. The ELISA results were expressed as relative OD₄₅₀ values, where the OD₄₅₀ of the sera from the naive mice (Naive) in each dilution was assigned the value of 1.0. * Note that oral administration of MG1363/pSECN induced a significant level of N-specific antibody ($P < 0.02$, *t*-test)

Discussion

Since SARS broke out in 2003, researchers have made great efforts in the development of fast and accurate analytical methods for its early diagnosis and the development of safe and efficient vaccines against its re-emergence. Although many ELISA diagnostic kits have been invented basing on a viral protein mixture, it does not seem convenient in its preparation, because it requires the cultivation of SARS-CoV. Thus, an alternative simple, sensitive and specific N protein-based ELISA has been investigated in many laboratories (Liu et al. 2004; Timani et al. 2004; Woo et al. 2004). All these investigations suggest that the N protein is immunodominant and is a perfect diagnostic antigen. For this purpose, we developed a sim-

ple way to express and purify the SARS-CoV N protein, by fusion with human GST protein. When the purified hGST-N protein was used as a coated antigen in the ELISA assay for serodiagnosis of SARS-CoV infection, it showed a high level of sensitivity (100% positive for the eight tested SARS patients) and specificity (100% negative for the 30 tested healthy people). Also, the ELISA signal was even better than that of the commercial kit (Fig. 3b). As the hGST-N protein can be purified in a single step and the fused human GST is believed not to generate antibodies in human sera, it thus provides a significant option for use in the serodiagnosis of SARS-CoV infection.

The SARS-CoV N protein is also reported to be important for the enhancement of antibody production and for the generation of specific cytotoxic T lymphocytes (CTL); and it is suggested that the N protein-induced CTL might play an important role in clearing the infected cells, thereby decreasing the rate of pathogen replication in the mucosal epithelium (Gao et al. 2003; Zhu et al. 2004). Since SARS-CoV infection is through the mucosal membrane, oral or intranasal vaccination might improve the protection efficiency. Thus, two strategies for mucosal vaccination of the N protein have been investigated in our work. One is using the purified hGST-N as the vaccine antigen and the other is using N-expression *L. lactis* as a live vaccine. For the first one, since hGST is safe for human beings, hGST-N should be acceptable for human vaccination. However, the efficient dosage of hGST-N and the means of administration remain to be investigated. For the second one, since *L. lactis* is a food-grade LAB and is also acid- and bile-resistant, it might be a good choice as a vehicle for N protein oral vaccination. It was revealed that oral administration of recombinant *L. lactis* MG1363/pSECN induces significant N-specific antibodies (Fig. 5) and is much more efficient than the oral or intranasal administration of the purified hGST-N, implying that *L. lactis* is a suitable vehicle for the delivery of N protein to intestinal mucosal sites. Thus, expression of SARS-Cov antigens in *L. lactis* might provide a novel vaccine. Of course, the immunogenicity of the MG1363/pSECN vaccine is still low. Its CTL response and protective function, including its use as an adjuvant antigen of the SARS-Cov S protein-based mucosal vaccines, remain to be investigated.

In summary, we successfully achieved the expression and purification of the SARS-CoV N protein as a fusion with human GST in *E. coli*; and this hGST-N showed potential as a useful option for the serodiagnosis of SARS-CoV infection. We also constructed a few recombinant *L. lactis* that expressed the N protein. Oral administration of N-producing *L. lactis* MG1363 induced N-specific antibodies in mouse sera. These results provide a novel strategy and crucial information for producing the SARS-CoV N protein as a diagnostic antigen and as a mucosal vaccine.

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