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Original Article

Expression of *Porphyromonas gingivalis* Gingipain Antigen Hgp44 Domain on Surface of *Lactococcus lactis*

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Abstract

Porphyromonas gingivalis, a pathogen involved in the development of chronic periodontitis, has a number of major virulence factors, among which are its surface cysteine protease gingipains. The purpose of this study was to investigate the feasibility of inducing protective antibodies against *P. gingivalis* by means of immunization with recombinant *Lactococcus lactis* expressing the 44-kDa gingipain adhesion/hemagglutinin domain (Hgp44). Part of the Hgp44 sequence encoding the first 314 amino acid residues, residues 188–251, and residues 354–393 was amplified and inserted into shuttle plasmid pSGANC332, with the resulting chimeric plasmids designated as pISTY210, pCOL, and pSHGRP44A, respectively. After confirming the clone sequences, expression of recombinant proteins was investigated by immunoblot. The results revealed that while pISTY210 and pCOL both expressed the Hgp44 antigen on the surface of *L. lactis*, the level of expression was quite low. To enhance expression of the protein on the surface of the cells, cysteine residues were changed to serine residues by site-directed mutagenesis. Replacement of 3 out of 5 cysteine residues (pISTY213) significantly increased expression of the recombinant protein on the surface of the bacteria. Interestingly, replacement of the 4th cysteine residue (pISTY215) reduced antigenicity of the recombinant protein. These results indicate that expression of Hgp44 on the surface of *L. lactis* cells requires the replacement of several key cysteine residues, and that *L. lactis* expressing this antigen could be a promising candidate for immunization against *P. gingivalis*-induced periodontitis.

Key words: Periodontitis — Vaccine — *Porphyromonas gingivalis* — Hgp44 — Gingipain

Introduction

Chronic periodontitis involves the inflammation of gingival tissue, which can lead to alveolar bone loss[29], and is a major public health problem in all societies[1]. Gram-negative anaerobic bacteria are involved in the development

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and progression of chronic periodontitis[32], and *Porphyromonas gingivalis*, in particular, is closely associated with this disease[16,31]. Recent reports have suggested that *P. gingivalis* is "a keystone microorganism" in the development of this periodontitis[14]. A number of virulence factors contribute to the pathogenicity of *P. gingivalis*, including fimbriae, lipopolysaccharide, hemagglutinin, hemolysin, and the cysteine protease gingipains[2,12,20,24,27]. Gingipains comprise arg-gingipains (RgpA, RgpB) and lys-gingipain (Kgp), which hydrolyze peptide bonds containing arg and lys residues, respectively. RgpA and Kgp consist of a prepropeptide, a catalytic domain, and an adhesion/hemagglutinin domain (HGP)[27]. The HGPs of RgpA consist of Hgp44, Hgp15, Hgp17, and Hgp27. Most gingipain activities are associated with the outer membrane[17], and they allow *P. gingivalis* to disrupt host defense mechanisms by degrading cytokines and immunoglobulins[18].

To reduce the incidence of periodontitis, it is essential to inhibit colonization of *P. gingivalis* and attenuate its virulence, and gingipains are candidates for vaccinations aimed at achieving this. The induction of immunoresponses to gingipains has been demonstrated to exert a protective effect against infection by *P. gingivalis*, reducing periodontitis in a *P. gingivalis*-infected mouse model[8,33,36].

Any vaccine capable of inducing mucosal immunity to colonization of *P. gingivalis* must also be safe. *Lactococcus lactis* is a noninvasive, nonpathogenic, Gram-positive bacterium, with a long history of use in the production of fermented milk products. When live *L. lactis* was fed to animals and human volunteers, it passed rapidly through the gastrointestinal tract, with no colonization[11,23]. *Lactococcus lactis* has been engineered to express several bacterial antigens because of its extraordinary safety profile[5,23]. Administration of recombinant *L. lactis* in mice generated antigen-specific immune responses[15]. Furthermore, genetically modified *L. lactis* has been effective in delivering antigens to the mucosal immune system and inducing a local immune response[34]. In an earlier study investigating various antibodies against each RgpA domain, we demonstrated that the anti-Hgp44 domain was the most effective in inducing antibody-mediated killing of invasive and non-invasive *P. gingivalis*[35]. The antigenicity of Hgp44 is stronger than that of the other domains, and the immunization of this domain reduced bone resorption[25]. In addition, the antigenicity of the N-terminal half of Hgp44 is stronger than that of the C-terminal half, and immunization of the former yielded a protective effect against alveolar bone resorption by *P. gingivalis* in a mouse infection model[26]. The purpose of the present study was to investigate the feasibility of developing a safe vaccine antigen for oral immunization against periodontitis by expressing *P. gingivalis* Hgp44 on the surface of *L. lactis*.

Materials and Methods

1. Microorganisms

P. gingivalis ATCC33277 was maintained in Tryptic soy agar (Becton Dickinson Microbiology System, Cockeysville, MD) supplemented with 5μg/ml hemin, 0.5μg/ml menadione, and 10% defibrinated horse blood at 37°C under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) in an anaerobic chamber (ANX-3, Hirasawa, Tokyo, Japan). *Lactococcus lactis* IL1403 was provided by Dr. Alain Chopin (INRA-CRJ, Jouy-en-Josas, France) and maintained on an M17 (Becton Dickinson Microbiology System) agar plate containing 0.5% glucose (GM17) at 30°C under aerobic conditions.

2. Construction of plasmid expressing Hgp44

Genomic DNA was isolated from *P. gingivalis* as described previously[19]. A fragment of Hgp44 was amplified by polymerase chain reaction (PCR) using three primer pairs (ISTY, COL, and 44A) with *Eco*RI or *Sal*I sites at the 5′ termini listed in Table 1. The fragments were digested with *Eco*RI and *Sal*I and then ligated with surface display vector pSGANC332, supplied by Yakult (Tokyo,

Table 1 Primer pairs used in this study

Sequence
ISTY
5′-GGGGGGAATTCTTAGCGGTCAGGCCGAGATTGTTCTTGAAGCT-3′
5′-GGGGGGTCGACCCAGGCAAATCCAATGCCGGTGTTATCAGATA-3′
COL
5′-GGGGGGAATTCTTGGCGTATCTCCGAAGGTATGTAAAGACG-3′
5′- GGGGGGTCGACTCTTCGAATGATTCGGAAAGTGTTGTTGTTCC-3′
44A
5′-GGGGGGAATTCGAGGCGTATCTCCGAAGGTATGTAAAGACGTT-3′
5′-GGGGGGTCGACACAGGTGCATCCCACTTGAGCGTTACTTTCTG-3′
1MS
5′-TGGCCGAACTCTAGTGTCCCGGCCAATCTGTTCGC-3′
1MAS
5′-GCGAACAGATTGGCCGGGACACTAGAGTTCGGCCA
2MS
5′-AATGCAGATCCTTCTTCTTCCCCTACCAATATGATAATGG
2MAS
5′-CCATTATCATATTGGTAGGGGAAGAAGAAGGATCTGCATT
3MS
5′-GGGCAATCATGAGTATTCCGTGGAAGTTAAGTACACAG
3MAS
5′-CTGTGTACTTAACTTCCACGGAATACTCATGATTGCCC
4MS
5′-CGTATCTCCGAAGGTATCTAAAGACGTTACGGTAGAAGGA
4MAS
5′-TCCTTCTACCGTAACGTCTTTAGATACCTTCGGAGATACG
5MS
5′-GGCTACAATAGCAATGGTTCTGTATATTCAGAGTCATTCG
5MAS
5′-CGAATGACTCTGAATATACAGAACCATTGCTATTGTAGCC

Mutagenesis of underlined residues was performed by replacing cysteine with serine.

Japan). In brief, pSGANC332 contains the 33 amino acid residues of the signal sequence of staphylokinase and cell-wall-anchor sequences of the C-terminal 332 amino acid residues of the cell-wall-associated protease of *L. lactis* NCDO763[22]. The anchor sequence contains sorting signals, including an LPXTG motif, a hydrophobic domain, and a positively-charged tail, which are commonly observed in Gram-positive bacterial cell wall-associated proteins[6]. The resulting plasmid was transformed into *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA), and the inserted sequence was confirmed by sequencing with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). *L. lactis* IL1403 was cultured in GM17 broth medium at 30°C overnight. The obtained plasmid harboring part of the Hgp44 coding sequence was transformed into *L. lactis* IL1403 by electroporation using a

Gene Pulser (BioRad, Hercules, CA) at 25 mF, 2.5 kV, and 200 Ω, with a 0.2-cm electrode cuvette (Bio-Rad)[34]. After electroporation, transformants of *L. lactis* IL1403 were inoculated onto GM17 plates supplemented with 20 μg/ml erythromycin.

3. Site-directed mutagenesis of plasmid expressing Hgp44

In *L. lactis* clones harboring the plasmids, expression of the protein on the cell surface was quite low. It is possible that expression of the recombinant protein was attenuated by its 3-dimensional structure, which involves disulfide bonds. To enhance expression of the recombinant protein on the surface of *L. lactis*, the cysteine residues of the Hgp44 coding sequence were replaced with serine residues by site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) according to the supplier's instructions. The five primer pairs (1MS and 1MAS, 2MS and 2MAS, 3MS and 3MAS, 4MS and 4MAS, and 5MS and 5MAS) used in this study are listed in Table 1. Mutagenesis was performed sequentially for the 5th, 3rd, 1st, 2nd, and 4th cysteine residues.

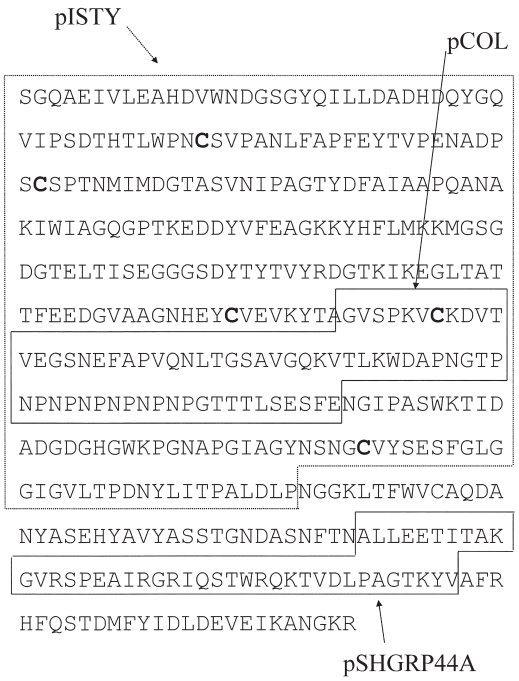

Fig. 1 Amino acid sequence of Hgp44 and expressed regions in shuttle plasmids in *L. lactis*

Fragments of 942, 192, or 120 bp in Hgp44 coding region were fused with surface-secreting signal and anchor sequences at N- and C-termini to produce shuttle vectors; they were designated pISTY210, pCOL, and pSHGRP44A, respectively. Expressed regions of each plasmid are indicated by rectangles.

4. Detection of recombinant protein in *L. lactis* by immunoblot

To confirm expression of the Hgp44 protein on the surface of the cells, *L. lactis* IL1403 harboring the expression plasmids was cultured on GM17 agar plates supplemented with 20 μg/ml erythromycin at 30°C overnight. The *L. lactis* cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C and the cells washed twice with phosphate-buffered saline (PBS, pH 7.4). To solubilize recombinant protein anchored on the surface of the cells, 500 μg (wet weight) of microorganisms was suspended in 1 ml SDS sample buffer (10 mM Tris-HCl buffer, pH 6.8, containing 1% SDS and 1% 2-mercaptoethanol) and boiled for 5 min. Supernatant fluid was obtained following centrifugation (15,000 × g for 5 min). The supernatant fluid (10 μl) was electrophoresed on a 10–20% gradient polyacrylamide gel and the proteins transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Merck Millipore, Billerica, MA) with a pore size of 0.45 μm for recombinant proteins larger than 15-kDa or 0.2 μm for recombinant proteins of less than 20-kDa with a Transblot Cell (BioRad). Expression of Hgp44 protein on the PVDF membranes was detected using polyclonal rabbit anti-HGP44 antiserum[35] followed by affinity-purified horseradish peroxidase-conjugated anti-rabbit IgG.

Results

1. Hgp44 expression by pSGANC332

Figure 1 shows the amino acid sequence of Hgp44 and the nucleotides corresponding

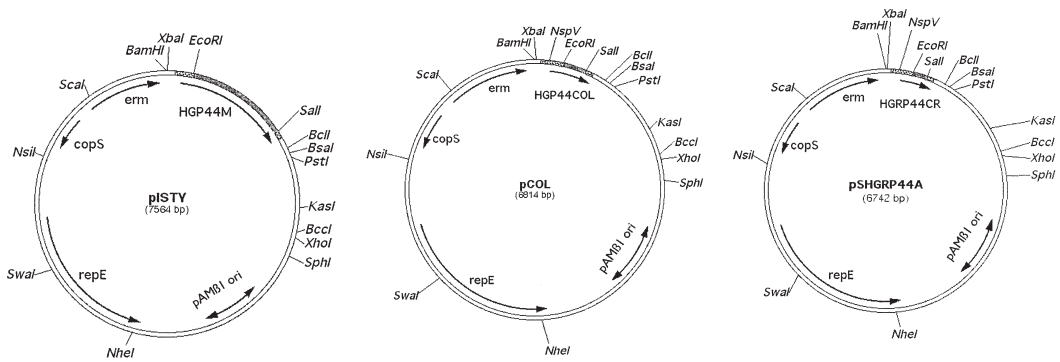

Fig. 2 Restriction map of shuttle plasmids for expression of Hgp44
A: pISTY210, B: pCOL, C: pSHGRP44A

to the sequences indicated were amplified by PCR. DNA fragments coding for the first 314 amino acid residues, residues 188–251, and residues 354–393 were amplified. Each amplified 942-, 192-, and 120-bp fragment was cloned into vector pSGANC332 and designated pISTY210, pCOL, and pSHGRP44A, respectively. Restriction maps of these plasmids are shown in Figs. 2A–2C.

The plasmids obtained were transformed into *L. lactis* IL1403 with an efficiency of transformation of approximately 0.1–1%. Growth rates of the transformants were similar to *L. lactis* IL1403. No recombinant protein bands were observed by Coomassie blue staining on SDS-PAGE. Immunoblot analysis did reveal an approximately 15-kDa band in *L. lactis* harboring pCOL, while none was evident with pSHGRP44A (Fig. 3A). The size of the band expressed with pCOL was comparable to the molecular mass deduced from the DNA sequence. Approximately 40-, 38-, and 12-kDa bands were also observed in the *L. lactis* clone harboring pISTY210 (Fig. 3B). The size of the second largest band was similar to the molecular mass of 37279.6 deduced from the DNA sequence.

The recombinant protein bands were detected in both pCOL and pISTY210, but at very low levels of expression. It is possible that the S-S bonds in the proteins had interfered with their secretion onto the cell surface. Therefore, the cysteine residues were replaced with serine residues by site-directed muta-

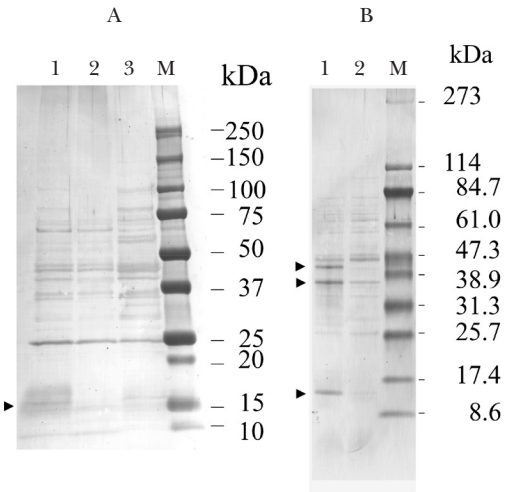

Fig. 3 Western blotting of *L. lactis* harboring expression vectors

Five hundred μg (wet weight) of microorganisms was suspended in 1 ml SDS sample buffer. After boiling for 5 min, supernatant fluids were isolated and 10μl sample applied to SDS-PAGE and immunoblot.

A: Lanes, 1: *L. lactis* harboring pCOL, 2: *L. lactis* harboring pSHGRP44A, 3: *L. lactis* harboring pSGANC332, M: Molecular size marker. Arrow indicates expressed protein.

B: Lanes, 1: *L. lactis* harboring pISTY210, 2: *L. lactis* harboring pSGANC332, M: Molecular size marker. Arrow indicates expressed protein.

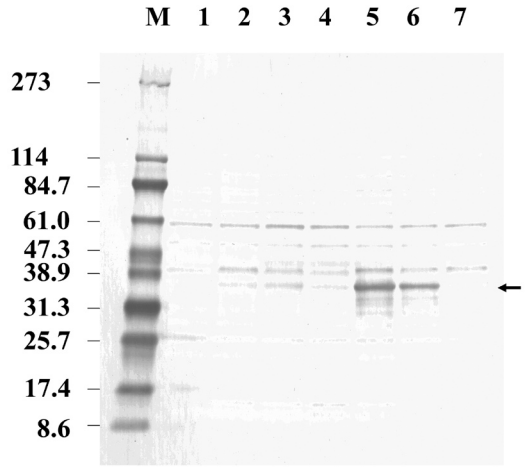

Fig. 4 Western blotting of proteins expressed by mutated derivatives of pISTY210

Five hundred μg (wet weight) of microorganisms was suspended in 1 ml SDS sample buffer. After boiling for 5 min, supernatant fluid was isolated and applied to SDS-PAGE and immunoblot.

Lanes, M: Molecular size marker, 1: *L. lactis* harboring pSGANC332, 2: *L. lactis* harboring pISTY210, 3: *L. lactis* harboring pISTY211, in which 5th cysteine was replaced with serine, 4: *L. lactis* harboring pISTY212, in which 3rd and 5th cysteines were replaced with serine, 5: *L. lactis* harboring pISTY213, in which 1st, 3rd, and 5th cysteines were replaced with serine, 6: *L. lactis* harboring pISTY214, in which 1st, 2nd, 3rd, and 5th cysteines was replaced with serine, 7: *L. lactis* harboring pISTY215, in which 5 cysteines were replaced with serine. Arrow indicates expressed protein.

genesis. The 5 cysteine residues of pISTY210 were replaced and designated pIS211–pIS215. Recombinant protein bands were not observed in all clones by Coomassie blue staining on SDS-PAGE (data not shown). However, the results of immunoblotting indicated that replacement of the first 3 residues together in the protein significantly enhanced expression of the 38-kDa band, whereas replacement of all 5 residues significantly reduced antigenicity (Fig. 4). The clone harboring pISTY213 showed the major 38-kDa antigenic band; however, the 40-kDa protein was very faint, and its expression level was not increased relative to pSTY210. These results indicate that expression of the recombinant protein on the surface of *L. lactis* was improved by replacement of specific cysteine residues.

Discussion

Expression of heterologous proteins on commensal or non-pathogenic bacterial cell surfaces has been extensively studied for a variety of applications, including epitope mapping, enzyme expression, and recombinant live vaccines[3,5,7,9,34]. In this study, we investigated the feasibility of using secretion-anchoring vector pSGANC332 to display a potential virulence factor, Hgp44, on the surface of an avirulent microorganism. The growth rate of the *L. lactis* clone expressing the protein was similar to that of the wild-type strain, indicating that the recombinant protein had no toxic effect on *L. lactis*. Segments of the Hgp44 domain were cloned in *L. lactis* harboring plasmids pISTY210, pCOL, and pSHGRP44A. Recombinant proteins were detected on the surface of *L. lactis* harboring pISTY210 and pCOL by anti-Hgp44 antibody. Both clones expressed recombinant proteins with molecular masses agreeing in size with that deduced from their amino acid sequences. The clone harboring pISTY210 showed weak 40-, 38- and 12-kDa immunogenic bands. The molecular mass of the deduced amino acid sequence of the cell-wall-associated protease of *L. lactis* is 2612.09. It is possible that the 38-kDa protein was a recombinant protein released from the junction between the cell wall anchor protein and Hgp44, although further analysis is required to confirm this. The results indicate that a portion of Hgp44 was expressed on the surface of the cell, as solubilization by SDS-PAGE buffer did not lyse the *L. lactis* cells.

The expressed proteins encoded by plasmids pCOL and pSHGRP44 were undetectable following Coomassie blue staining on SDS-PAGE. Sharma *et al.*[30] expressed *P. gingivalis* fimbriae in the Gram-positive coccus *Streptococcus gordonii*. They reported that neither full-length fimbrillin polypeptides (length, 337 amino acids), nor their peptide domains containing free thiol groups, were expressed on the surface of *S. gordonii*. Folding of the protein structure due to disulfide bonds or codon usage sometimes affects the expression

of recombinant proteins. The recombinant protein of the clone harboring pISTY210 contained 5 cysteine residues. Codon usage also affects expression of recombinant proteins and differs significantly between *P. gingivalis* Hgp44 and *L. lactis*[10,13]. Both of these factors may affect the expression level of *P. gingivalis* recombinant proteins on the cell surface of *L. lactis*. Replacement of the 3 cysteine residues significantly improved expression of the 38-kDa protein on the surface of *L. lactis*. These results suggest that expression was attenuated by the three-dimensional structure of the protein by disulphide bonds formed from cysteine residues.

Hgp44 was only detected in *L. lactis* harboring pISTY210 or pCOL. This may reflect localization of the Hgp44 epitope. These results agree with those of our previous report indicating that the antigenicity of the N-terminal half of Hgp44 was stronger than that of the C-terminal segment[26]. Booth *et al.*[4] showed that a monoclonal antibody against *P. gingivalis* inhibited recolonization by this organism. Kelly *et al.*[21] also revealed that the epitope of the antibody detected in the sera of patients with periodontitis was located in Hgp44 among 5 epitopes, and pISTY210 expressed a protein containing four of these. Furthermore, antibody directed to the sequence EGLATATTFEEDGVA protected against periodontal bone loss and inhibited binding of the RgpA-Kgp complex to fibrinogen, fibronectin, and collagen type V; while antibody directed to the sequence GTPNPNPNPNPNPNPGT protected against periodontal bone loss and inhibited binding to hemoglobin[28]. Both regions were included in the protein expressed from pISTY210, and the latter was also included in the pCOL product. The 4th cysteine residue, whose replacement decreased reactivity with anti-Hgp44, is located between these two sequences. It is possible that this amino acid replacement induced conformational changes which reduced the antigenicity of the protein. These results suggest that antibody against Hgp44 binds the epitopes of recombinant proteins expressed by *L. lactis* harboring pISTY210 and pCOL.

The expression of the Hgp44 peptide of *P. gingivalis* on the cell surface of an avirulent microorganism may serve as a suitable immunogen for oral vaccination against *P. gingivalis* to reduce the incidence of periodontitis. Additional studies will be necessary to determine if this would be practical.

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