

## ORIGINAL ARTICLE

## Expression of single-chain antibody against RgpA protease of *Porphyromonas gingivalis* in *Lactobacillus*

H. Marcotte<sup>1</sup>, P. Köll-Klais<sup>2</sup>, A. Hultberg<sup>1</sup>, Y. Zhao<sup>1</sup>, R. Gmür<sup>3</sup>, R. Mändar<sup>2</sup>, M. Mikelsaar<sup>2</sup> and L. Hammarström<sup>1</sup>

1 Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden

2 Department of Microbiology, Faculty of Medicine, University of Tartu, Tartu, Estonia

3 Institute of Oral Biology, University of Zurich, Zurich, Switzerland

### Keywords

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### Correspondence

Harold Marcotte, Department of Laboratory Medicine, Division of Clinical Immunology, Karolinska University Hospital Huddinge, Karolinska Institutet, SE-141 86, Stockholm, Sweden.  
E-mail: harold.marcotte@labmed.ki.se

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### Abstract

**Aims:** The monoclonal antibody 61BG1·3, recognizing the RgpA protease, has been reported to confer protection against recolonization by the periodontal pathogen *Porphyromonas gingivalis* in humans. The aim of this study was to express a functional scFv derived from the monoclonal antibody 61BG1·3 on the surface of *Lactobacillus paracasei* for potential use in the prevention or treatment of periodontal diseases.

**Methods and Results:** The scFv was fused to an E-tag and cloned in the *Escherichia coli*/*Lactobacillus* shuttle vector pLP501, which mediates surface expression of the scFv. FACS analysis using an anti-E-tag antibody revealed that the scFv was expressed on the surface of the transformed lactobacilli and binding of the scFv to RgpA was shown by ELISA. *Lact. paracasei* expressing the scFv against RgpA was able to agglutinate *P. gingivalis* whereas the *Lact. paracasei* expressing an irrelevant scFv fragment did not. Scanning electron microscopy demonstrated efficient binding of the lactobacilli expressing the scFv anti-RgpA to *P. gingivalis*.

**Conclusions:** We have expressed a functional scFv antibody directed against the RgpA protease of *P. gingivalis* in *Lactobacillus*.

**Significance and Impact of the Study:** These results suggest a potential of *Lactobacillus* expressing scFvs against *P. gingivalis* to be used to combat periodontal disease.

### Introduction

*Porphyromonas gingivalis*, a Gram-negative anaerobe present in subgingival plaque, has been identified as a major aetiological agent of chronic periodontitis (Slots *et al.* 1986; Slots and Ting 1999) where the proteolytic enzymes produced by this organism are involved in the pathophysiological process (Smalley *et al.* 1988; Andrian *et al.* 2004). *P. gingivalis* cysteine proteases, which are referred to as gingipains, are particularly implicated because of their capacity to cleave a broad range of host proteins at exposed arginyl peptide bonds (Travis *et al.* 1997). Gingipains are bound to the bacterial surface, associated with

extra-cellular vesicles, or secreted. The Arg-gingipains, derived from the RgpA gene, comprise a family of proteases in which the catalytic polypeptide is expressed as a monomer (RgpA<sub>cat</sub>), dimer or a heterodimer (HRgpA) (Curtis *et al.* 1999). HRgpA is a heterodimer in which the catalytic alpha chain is noncovalently associated with a haemagglutinin (beta chain), derived from the initial RgpA translation product, which is capable of mediating binding to the erythrocyte surface and host macromolecules such as haemoglobin, fibronectin and collagen type IV. Immunization with purified Arg-gingipain A or Arg-gingipain B protects against colonization and invasion by *P. gingivalis* in a mouse chamber model, suggesting that

antibodies to gingipains or gingipain-related proteins may protect against periodontal disease in humans (Genco *et al.* 1998a, 1999).

The anti-*P. gingivalis* monoclonal antibody 61BG1:3 is reactive with the adhesion associated epitope contained in the beta fragment (residues 748–1130) of RgpA and has previously been shown to inhibit haemagglutination of human red blood cells by *P. gingivalis* (Booth and Lehner 1997). This monoclonal antibody has also been used in passive immunization studies in humans and confers protection against recolonization with *P. gingivalis* for up to 9 months (Booth *et al.* 1996). These results suggest that passive immunotherapy could potentially be used to control *P. gingivalis* in subjects with progressive periodontal disease.

Lactobacilli are Gram-positive bacteria that are currently used in food fermentation and preservation. Some strains are normal constituents of the human microbiota and can colonize the oral cavity (Ahrné *et al.* 1998; Colloca *et al.* 2000). Engineered lactobacilli have been considered as delivery systems for both active and passive vaccinations (Krüger *et al.* 2002; Reveneau *et al.* 2002) and lactobacilli producing single-chain antibody fragments (scFv) against *Streptococcus mutans* derived from monoclonal antibody Guy's 13 have previously been administered orally and shown to offer protection against development of dental caries in rats (Krüger *et al.* 2002). In this paper, we describe the expression of scFv derived from the monoclonal antibody 61BG1:3 directed against the adhesion domain of RgpA of *P. gingivalis* in *Lactobacillus*.

## Materials and methods

### Construction of anti-*P. gingivalis* scFv-61BG1:3 expression vectors

Total RNA was extracted from an anti-RgpA monoclonal antibody secreting hybridoma (Mab 61BG 1:3) (Gmür *et al.* 1988). Variable region encoding sequences of both the heavy (VH) and light (VK) chains were amplified using a 5' RACE kit (5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Invitrogen Corporation, Carlsbad, CA, USA). The primers for the 5' RACE of the VH chain were ACRA1: 5'-CAGACTCAGGATGGGTAAC-3', ACRA2: 5'-CACTTGAATGATGCGCCACTGTT-3', ACRA3: 5'-GAGGGCTCCAGGTGAAGAC-3', while the primers mkRA1 (5'-TCATGCTGTAGGTGCTGTCT-3'), mkRA2 (5'-TCGTTCACTGCCATCAATCT-3') and mkRA3 (5'-TGGATGGTGGGAAGATGGAT-3') were utilized to amplify the variable region of the VL chain. The A-tailed resulting PCR product was cloned into a pGEM<sup>®</sup>-T easy vector with 3'-T overhangs and sequenced.

The VH and VK sequences were fused together with a linker gene encoding the amino acid sequence (G<sub>4</sub>S)<sub>3</sub>. Both chains were re-amplified from the cloned 5' RACE products using the primers CLA-RVSC-S (5'-TTTTATC-GATGTGCAGTTGGTGGAGTCTGG-3'), PGVHas-linker (5'-CGATCCGCCACCGCCAGAGCCACCTCCGCCTGA-ACCGCCTCCACCTGAGGAGACGGTGACTGAGG-3'), PGVKS-linker (5'-GGTGGAGCGGTTTCAGGCGGAGGT-GGCTCTGGCGGTGGCGGATCGCAAATTGTTCTCACCCAGTC-3') and EcoR-PGVKas (5'-TTTTGAATTCTTT-TATTTCCAGCTTGGTCC-3'). The resulting VH and VK PCR products were mixed together and used as a template for a fusion PCR using the primers CLA-RVSC-S (5'-TTTTATCGATGTGCAGTTGGTGGAGTCTGG-3') and EcoR-PGVKas (5'-TTTTGAATTCTTTTATTTCCAGCTT-GGTCC-3'). The fused PCR products were cloned into a pGEM<sup>®</sup>-T easy vector after addition of overhang A using *Taq* DNA polymerase. The fused scFv-61BG1:3 encoding sequence was finally cut out from the plasmids using *EcoRI* plus *ClaI* and subcloned into pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) containing an E-tag (pBS-E-tag) (Krüger *et al.* 2002). For generation of the surface-expressed antibody fragments, the scFv-E-tag fragment was amplified using primers antiPgClaS (5'-CCATC-GATGTGCAGTTGGTGGAGTCT-3') and antiPgEcoAS2 (5'-CGGAATTCTTTTATTTCCAGCTTGGT-3') with pBS-E-tag as a template. The PCR amplification product was cut using *ClaI* and *XhoI* and ligated into the *Escherichia coli/Lactobacillus* shuttle vector pLP501-scFv-long anchor, generating pLP501-scFv(61BG1:3)-long anchor. The pLP501 vector contains the constitutive promoter of the lactate dehydrogenase gene (*Pldh*) and an anchor sequence, encoding the last 244 amino acids of the protease P protein of *Lact. casei* (Krüger *et al.* 2002). *E. coli* XL10-Gold competent cells (Stratagene) were transformed by heat shock. Transformation of *Lactobacillus* was performed by electroporation as described previously (Krüger *et al.* 2002). Selection of plasmid positive clones was performed using MRS plates containing 3 µg ml<sup>-1</sup> erythromycin. At each step of the construction process, the gene expression cassette was sequenced (BIG DYE TERMINATOR v.2, Applied Biosystems, Foster City, CA, USA).

### Bacteria and culturing procedures

*Porphyromonas gingivalis* W83 was cultivated on Wilkins-Chalgren agar plates and brain-heart infusion broth supplemented with 0.5% yeast extract, 5 µg ml<sup>-1</sup> haemin (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 µg ml<sup>-1</sup> menadione (Calbiochem, San Diego, CA, USA) and incubation was made at 37°C under anaerobic conditions (BBL<sup>™</sup> Gaspak Plus<sup>™</sup> anaerobic system, Becton, Dickinson and Company, Sparks, MD, USA). *Lact. paracasei*

(previously considered as a plasmid free *Lact. casei* or *Lact. zeae* ATCC 393) (Acedo-Félix and Pérez-Martínez 2003) was obtained from TNO, Leiden, the Netherlands. Nontransformed *Lact. paracasei* were cultivated at 37°C on de Man–Rogosa–Sharp (MRS) agar under anaerobic condition or in MRS broth (Difco, Sparks, MD, USA) under aerobic condition. Lactobacilli containing construct pLP501–scFv(61BG1·3)-long anchor were cultured in MRS agar or broth containing 3 µg ml<sup>-1</sup> of erythromycin (Sigma-Aldrich). Lactobacilli containing construct pLP501–scFv-long anchor expressing a scFv derived from monoclonal antibody Guy's 13 directed against the SAI/II adhesin of *Strep. mutans* was used as a control (Krüger *et al.* 2005). These bacteria were cultivated in the same condition as the pLP501–scFv(61BG1·3)-long anchor *Lactobacillus* construct.

### Protein extraction

The lactobacilli containing the constructs pLP501–scFv(61BG1·3)-long anchor, pLP501–scFv-long anchor (irrelevant scFv derived from Guy's 13) or nontransformed lactobacilli were cultured as described above to an OD<sub>600</sub> of 0·8 (10<sup>8</sup> bacteria ml<sup>-1</sup>). After washing two times with 10 mmol l<sup>-1</sup> Tris–HCl, pH 8·0, the bacteria were resuspended in 500 µl of 10 mmol l<sup>-1</sup> Tris–HCl, pH 8·0, containing 10 mg ml<sup>-1</sup> lysozyme (Sigma-Aldrich) and incubated at 37°C for 1 h. The samples were disrupted by sonication (6 × 30 s on/off cycles) with a 60% duty cycle and output control put to 5 (Vibracell, Sonics & Materials, Danbury, CT, USA). Debris was removed by centrifugation for 10 min at 10 000 g.

### Quantification of scFv produced pLP501–scFv(61BG1·3)-long anchor *Lactobacillus* construct

The amount of scFv in the bacterial extract from the pLP501–scFv(61BG1·3)-long anchor *Lactobacillus* construct was estimated by densitometry using purified E-tag scFv as a standard. For purification of the standard E-tag scFv antibody fragments, *Lact. paracasei* expressing a secreted E-tagged (unrelated) scFv was grown in a 200 ml culture. The culture was centrifuged for 15 min at 10 000 g and the supernatant was saved. The pH was adjusted to 7·0 and filtered through a 0·45 µm filter (Schleicher & Schuell BioScience, Dassel, Germany). The E-tag scFv was subsequently purified using a HiTrap column prepacked with anti-E-tag sepharose according to the instructions provided in the RPAS Purification Module (Amersham-Bioscience, Little Chalfont, Buckinghamshire, UK). Dialysis against phosphate-buffered saline (PBS) was performed and the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) was used to determine the

protein concentration. Twofold dilutions of the standard scFv and the bacterial extract (from 2 × 10<sup>8</sup> and 1 × 10<sup>8</sup> bacteria) were loaded on precast polyacrylamide gels (Ready gel 4–15% Tris–HCl, Bio-Rad Laboratories, Hercules, CA, USA). After protein blotting, the nitrocellulose membrane (Hybond<sup>TM</sup>-ECL<sup>TM</sup>, Amersham-Bioscience) was blocked with PBS containing 0·05% Tween 20 and 5% milk powder (PBS–TM) overnight at 4°C. After washing, the membrane was incubated with anti-E-tag antibodies diluted in PBS–TM (2 µg ml<sup>-1</sup>) for 3 h at room temperature followed by horse radish peroxidase (HRP) labelled goat anti-mouse antibodies (1/1000) (DAKO A/S, Glostrup, Denmark) for 1 h at room temperature. The washed membrane was then developed with enhanced chemiluminescence (ECL) plus Western blotting kit (Amersham-Bioscience) according to the manufacturer's instructions. The amount of scFv in the extract was calculated by using the Gel Doc<sup>TM</sup> image analysis system and QUANTITY ONE<sup>®</sup> analysis software (Bio-Rad). This value was also used to estimate the amount of scFv produced per bacteria.

### Protein purification of RgpA

Plasmid pJFQ30β, encoding the RgpA adhesin domain (G<sup>721</sup>–R<sup>1262</sup>) fused to a (His)<sub>6</sub> tag at the N-terminus was a generous gift from Dr Michael A. Curtis, University of London, UK (Slaney *et al.* 2002). The recombinant plasmid was expressed in *E. coli* XL10-Gold competent cells (Stratagene). Transformed *E. coli* was grown overnight at 37°C in Luria–Bertani (LB) medium containing 50 µg ml<sup>-1</sup> ampicillin. Cells were harvested by centrifugation and resuspended in 4 ml of 6 mmol l<sup>-1</sup> guanidine–HCl (pH 8·0) and sonicated (3 × 10 s on/off cycles) with a 60% duty cycle and output control put to 5 (Vibracell). The lysate was centrifuged (10 000 g, 20 min) and the supernatant was mixed with Talon<sup>®</sup> metal affinity resins (Clontech Laboratories, Palo Alto, CA, USA), and proteins were subsequently purified according to the user's manual.

### ELISA

Ninety-six-well microtitre plates (Corning Incorporated, Acton, MA, USA) were coated with 4 µg ml<sup>-1</sup> of purified RgpA or 1 µg ml<sup>-1</sup> of purified *Strep. mutans* SAI/II antigen (negative control) in PBS and incubated overnight at 4°C. After washing with PBS containing 0·05% Tween 20 (PBS–T), the plates were blocked with 1% BSA in PBS–T for 3 h. After washing, 100 µl of the protein extracts (diluted 1/2) or the cell suspension (10<sup>8</sup> bacteria ml<sup>-1</sup>) of the nontransformed lactobacilli or the lactobacilli containing the constructs pLP501–scFv(61BG1·3)-long anchor

and pLP501–scFv–long anchor (anti-SAI/II) were added to plates coated with RgpA or SAI/II protein. Supernatant of the mouse hybridoma producing the monoclonal antibody 61BG1·3 (anti-RgpA) or Guy's 13 (anti-SAI/II) were added as positive controls. The plates were incubated overnight at 4°C. The plates were subsequently washed with PBS–T and 1 µg ml<sup>-1</sup> of an anti-E-tag antibody (Amersham Bioscience) was added. The plates were incubated for 1·5 h, the washing was repeated and alkaline phosphatase conjugated rabbit anti-mouse antibody (1/1000) (DAKO A/S, Glostrup, Denmark) was added. The incubation was repeated for 2 h at room temperature and then 1 mol l<sup>-1</sup> diethanolamine buffer (pH 10·0) containing 1 mg ml<sup>-1</sup> pNPP (*p*-nitrophenyl phosphate) (Sigma-Aldrich) was added to the wells. After 1 h incubation, reading was performed with a  $V_{\max}$  Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

#### FACS analysis

*Lactobacillus* transformants pLP501–scFv(61BG1·3)–long anchor and pLP501–scFv–long anchor (irrelevant scFv derived from Guy's 13) and nontransformed lactobacilli were cultured as described above to an OD<sub>600</sub> of 0·8. One hundred microlitres of each of the cultures were washed thrice in PBS, pH 7·4, by centrifugation at 10 000 g for 15 min before resuspension in 100 µl mouse anti-E-tag antibody (Amersham Bioscience) diluted 1/200. After incubation at 4°C for 1 h, the washing was repeated and the samples were resuspended in 100 µl PBS and mixed with 100 µl FITC conjugated rabbit anti-mouse antibodies (Dako A/S) diluted 1/500 (30 min at 4°C). The samples were washed once in PBS, resuspended in 2·25% formalin and incubated at 4°C for 20 min. The washing was repeated twice and the samples were finally resuspended in 1 ml of PBS and analysed in a FACS Calibur machine (Becton Dickinson, Stockholm, Sweden).

#### Agglutination assays

*Lactobacillus* transformants pLP501–scFv(61BG1·3)–long anchor and pLP501–scFv–long anchor (irrelevant scFv derived from Guy's 13) and the nontransformed lactobacilli were cultured as described above to an OD<sub>600</sub> of 0·8. Thirty microlitres of *P. gingivalis* suspension was placed on a glass slide (76 × 26 mm, Menzel-Gläser, Braunschweig, Germany), after which 30 µl of *Lact. paracasei* transformants pLP501–scFv(61BG1·3)–long anchor, pLP501–scFv–long anchor (irrelevant control) or nontransformed lactobacilli were added. The slides were then rotated vertically by hand and agglutination was arbitrarily scored after 1 min. Each strain was also evaluated in

the same way for autoaggregation. Each experiment was repeated five times. Agglutination was observed under a light microscope and by scanning electron microscopy (SEM) (JEOL JSM, Tokyo, Japan) at 15 kV.

#### Antagonism assay

Antagonistic activity of lactobacilli against *P. gingivalis* was assessed using a streak line procedure (Mikelsaar *et al.* 1987; Annuk *et al.* 2003) on Wilkins–Chalgren blood agar plates (Oxoid, Unipath, Basingstoke, UK). A single line of nontransformed lactobacilli or lactobacilli transformed with pLP501–scFv(61BG1·3)–long anchor was seeded in the middle of the agar plate. *Lactobacillus* strains were then cultivated for 48 h at 37°C in an anaerobic glove chamber (Sheldon Manufacturing, Shel LAB, Cornelius, OR, USA) with a gas mixture of CO<sub>2</sub>/H<sub>2</sub>/N<sub>2</sub> (5/5/90%). *P. gingivalis* was cultured in Wilkins–Chalgren broth for 48 h at 37°C in anaerobic conditions and seeded in quadruplicate perpendicular to the streak line of lactobacilli. Following incubation of the plates for 72 h at 37°C under anaerobic conditions, the width (mm) of the zone of inhibition of the target bacteria extending from the culture line of lactobacilli was measured (Mikelsaar *et al.* 1987).

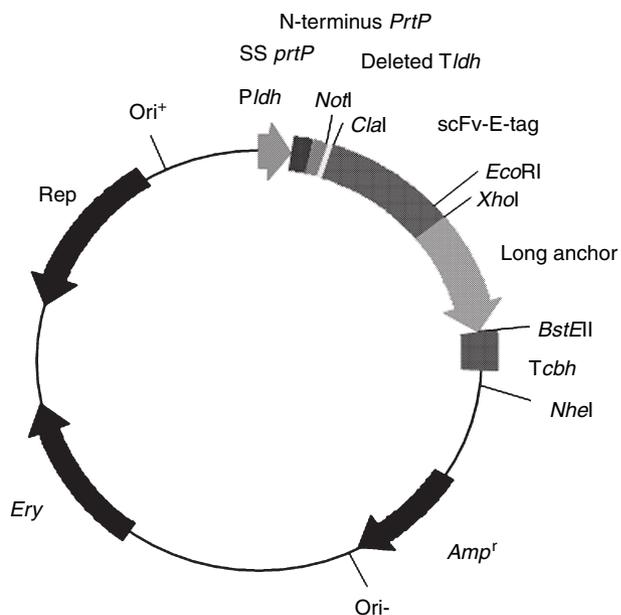
## Results

#### Construction of the *Lactobacillus* expressing surface scFv against RgpA

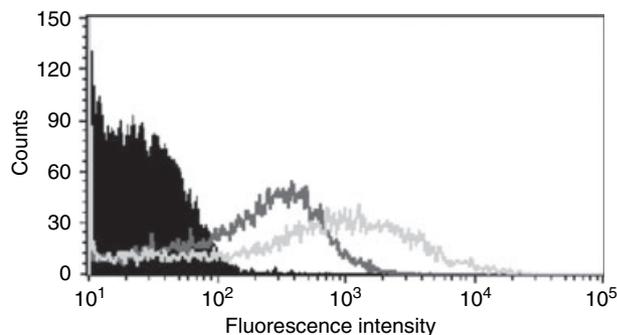
The scFv derived from the IgG1 monoclonal antibody 61BG1·3 was fused to an E-tag and cloned in the plasmid pLP501 (Fig. 1). The scFv-containing plasmid, named pLP501–scFv(61BG1·3)–long anchor, was introduced into *Lact. paracasei* in order to generate lactobacilli producing scFv antibody fragments against the RgpA gingipain on their surface. In pLP501, the scFv expression is under the control of the constitutive promoter of the *ldh* gene (Fig. 1).

#### Expression of the scFv

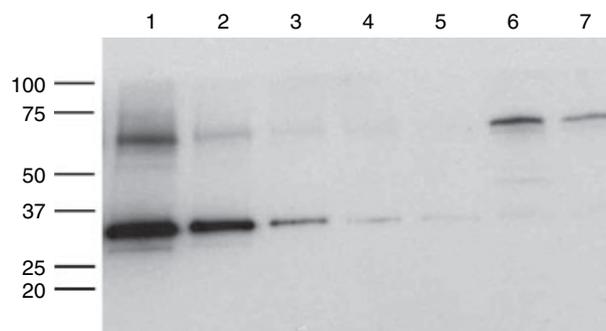
Surface expression of the scFv on lactobacilli transformed with pLP501–scFv(61BG1·3)–long anchor and pLP501–scFv–long anchor (scFv antibody directed against the SAI/II adhesin of *Strep. mutans*), was analysed by flow cytometry using an anti-E-tag antibody. The pLP501–scFv(61BG1·3)–long anchor transformed lactobacilli showed a positive signal when stained by the anti-E-tag antibody, but slightly lower than the lactobacilli transformed with pLP501–scFv–long anchor (Fig. 2). The amount of scFv anti-RgpA expressed on the surface



**Figure 1** Map of the *Lactobacillus* pLP501-scFv(61BG1-3)-long anchor vector. The vector mediates surface-anchored expression of scFv by fusion to the last 244 amino acids of *Lact. casei* proteinase P. *Pldh*, promoter sequence of the lactate dehydrogenase gene of *Lact. casei*; *SS prtP*, signal sequence of the *PrtP* gene (33 amino acids), N-terminus *prtP*, N-terminus (36 amino acids) of the *PrtP* gene; *Tldh*, transcription terminator of the lactate dehydrogenase gene of *Lact. casei*; deleted *Tldh*, remaining sequence after deletion of *Tldh*; scFv, single-chain antibody against RgpA proteinase; long anchor, anchor sequence from the proteinase P gene of *Lact. casei* (244 amino acids); *Tcbh*, transcription terminator sequence of the conjugated bile acid hydrolase gene of *Lact. plantarum* 80; *Amp<sup>r</sup>*, ampicillin-resistance gene; *Ery*, erythromycin-resistance gene; *Rep*, *repA* gene of plasmid p353-2 from *Lact. pentosis*; *Ori<sup>+</sup>*, origin of replication (*Ori<sup>+</sup>* = ori *E. coli*, *Ori<sup>-</sup>* = ori *Lactobacillus*).



**Figure 2** Flow cytometry analysis showing the expression of scFv (61BG1-3) on the surface of *Lactobacillus paracasei* by detection of the E-tag by a mouse anti-E-tag antibody. Black filled curve, non-transformed lactobacilli; dark grey line, *Lact. paracasei* expressing a scFv (61BG1-3) against RgpA; light grey line, *Lact. paracasei* expressing a scFv against the SAI/II adhesin.



**Figure 3** Western blot analysis of scFv expressed on the surface of *Lactobacillus paracasei*. Western blot was reacted with anti-E-tag antibody. Lanes 1–5: 50, 25, 12.5, 6.25 and 3.13 ng of purified secreted unrelated E-tagged scFv with molecular weight of 30 kDa. Lanes 6 and 7, extract from  $2 \times 10^8$  and  $1 \times 10^8$  *Lact. paracasei* expressing surface scFv against RgpA. The main protein band is about 70 kDa (theoretical molecular weight is 60 kDa).

of lactobacilli was estimated by densitometry using a standard curve of an affinity purified E-tagged (unrelated) scFv and showed approximately 8 ng per  $10^8$  bacteria or 850 scFv fusion molecules/bacterium (Fig. 3).

#### Biological activity of the scFv

The scFv extracted from *Lactobacillus* was added to plates coated with purified (His)<sub>6</sub> tag RgpA or *Strep. mutans* SAI/II antigen (negative control). The scFv anti-RgpA extracted from the transformed *Lact. paracasei* or intact recombinant bacteria bound to RgpA in ELISA but not to SAI/II antigens (Table 1). Extract or whole cells of *Lactobacillus* expressing scFv anti-SAI/II bound to the SAI/II antigen. The monoclonal antibodies 61BG1-3 and Guy's 13 bound to RgpA and SAI/II, respectively. The concentration of scFv anti-RgpA in the bacterial extract used in ELISA was about 200 ng ml<sup>-1</sup>. The reactivity of the scFv anti-RgpA was nearly 20 times lower than the corresponding anti-RgpA monoclonal antibody 61BG1-3. Considering that the molecular weight of the fusion protein is 2.4 times smaller than an IgG1 molecule, we estimate that the reactivity of the scFv is approximately 50 times lower than the parent bivalent monoclonal antibody.

Agglutination assay shows the presence of visible aggregates when mixing *Lact. paracasei* pLP501-scFv-(61BG1-3)-long anchor with *P. gingivalis* W83 whereas no agglutination was observed when mixing *P. gingivalis* with pLP501-scFv-long anchor (anti-SAI/II) or nontransformed lactobacilli. No autoaggregation was seen when testing *Lact. paracasei* pLP501-scFv(61BG1-3)-long anchor or *P. gingivalis* alone. These results were confirmed by Gram staining of the slides, which also revealed the

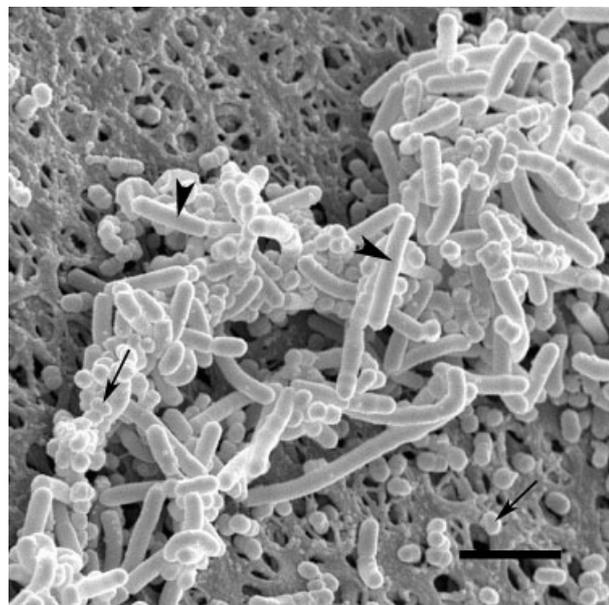
**Table 1** Binding activity of the scFv to RgpA

	OD 405 nm	
	RgpA coating	SA/II coating
Cell extracts*		
PLP501-scFv(61BG1·3)- long anchor (anti-RgpA)	0·195	0·084
Nontransformed	0·082	0·080
pLP501-scFv-long anchor (anti-SA/II)	0·085	0·205
Whole bacteria†		
pLP501-scFv(61BG1·3)-long anchor (anti-RgpA)	0·133	0·085
Nontransformed	0·087	0·083
PLP501-scFv-long anchor (anti-SA/II)	0·082	0·145
Monoclonal antibody 61BG1·3 (anti-RgpA)		
250 ng ml <sup>-1</sup>	1·508	0·078
25 ng ml <sup>-1</sup>	0·316	0·075
2·5 ng ml <sup>-1</sup>	0·122	0·079
Monoclonal antibody Guy's 13 (anti-SA/II)		
100 ng ml <sup>-1</sup>	0·076	1·875
10 ng ml <sup>-1</sup>	0·077	0·434
1 ng ml <sup>-1</sup>	0·079	0·138

\*Bacteria were disrupted, centrifuged and the supernatant subsequently added to RgpA or SA/II coated wells. The concentration of scFv anti-RgpA in extract was estimated to approximately 200 ng ml<sup>-1</sup>.

†Bacteria were added at a concentration of 10<sup>8</sup> bacteria ml<sup>-1</sup>.

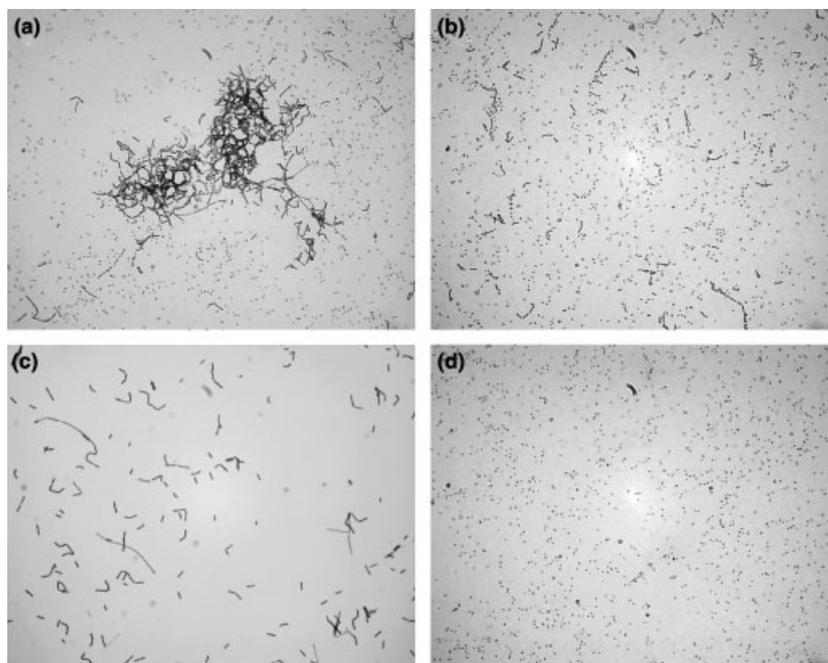
presence of aggregates containing *Lact. paracasei* pLP501-scFv(61BG1·3)-long anchor and *P. gingivalis* W83 (Fig. 4). SEM also shows binding of *P. gingivalis* to *Lactobacillus* expressing the scFv against *P. gingivalis* (Fig. 5).



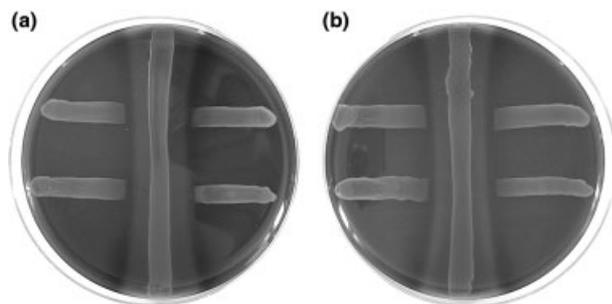
**Figure 5** Scanning electron microscope picture showing the agglutination of *Porphyromonas gingivalis* by *Lactobacillus paracasei* expressing a scFv against the RgpA of *P. gingivalis*.

### Antagonism

Nontransformed lactobacilli and lactobacilli transformed with pLP501-scFv(61BG1·3)-long anchor were both shown to inhibit the growth of *P. gingivalis* W83 on agar plates (Fig. 6). The mean width of inhibition zone was



**Figure 4** Gram staining of agglutination assay with (a) *Lactobacillus paracasei* expressing a scFv (61BG1·3) against RgpA and *P. gingivalis*, (b) nontransformed lactobacilli and *P. gingivalis*, (c) *Lact. paracasei* expressing a scFv against RgpA only, (d) *P. gingivalis* only



**Figure 6** Inhibition of *Porphyromonas gingivalis* growth by (a) *Lactobacillus paracasei* pLP501–scFv(61BG1-3)-long anchor and (b) non-transformed *Lact. paracasei*. Antagonistic activity of lactobacilli against *P. gingivalis* was assessed using a streak line procedure on Wilkins–Chalgren blood agar plates. A single line of lactobacilli culture was seeded in the middle of the agar plate and target bacteria were seeded in quadruplicate perpendicular to the streak line of lactobacilli.

7.3 ± 0.2 mm for the nontransformed lactobacilli and 7.6 ± 0.4 mm for the transformed one.

## Discussion

The monoclonal antibody 61BG1-3 has previously been reported to confer protection against recolonization with *P. gingivalis* in humans (Booth *et al.* 1996). In this study, we have expressed a functional scFv derived from monoclonal antibody 61BG1-3, which is directed against the RgpA protease of *P. gingivalis* on the surface of *Lactobacillus*.

When extracted from lactobacilli, the scFv binds to the RgpA antigen but at a lower level as the parent monoclonal antibody. This may be because of either the monovalency of the scFv resulting in low avidity or because of improper folding of the protein. However, lactobacilli expressing scFv anti-RgpA were shown to aggregate *P. gingivalis*. The numerous antibody fragments expressed on the bacterial surface probably compensate for the low affinity of the scFv and promote agglutination.

Both nontransformed and transformed *Lactobacillus* strains were shown to equally inhibit the growth of *P. gingivalis* on agar plates suggesting that expression of scFv by lactobacilli does not affect its bacteriostatic/bacteriocidal activities. It has been reported that antimicrobial activity of lactobacilli *in vitro* is principally related to the production of organic acids such as lactic and acetic upon fermentation of glucose with concomitant decrease in pH (Ouwehand and Vesterlund 2004). Our previous experiments have revealed that the antagonistic activity of *Lact. paracasei* strains against various pathogens is mainly associated with the production of high amounts of lactic acid secreted into environment (Annuk *et al.* 2003).

*Lactobacillus* expressing scFv against RgpA could potentially be used for protection against periodontal pathogens. Lactobacilli have been isolated from the saliva, the teeth, the gingival sulcus, the tongue and other soft tissues of the oral cavity (Ahrné *et al.* 1998; Colloca *et al.* 2000; Köll-Klais *et al.* 2005). Although *Lactobacillus* is not a common inhabitant of the gingival sulcus, lactobacilli expressing antibodies could potentially prevent colonization by periodontal pathogens that inhabit oral soft tissues (e.g., the tongue) (Mager *et al.* 2003). Alternatively, they could be applied directly into the periodontal pocket. The surface-expressed antibodies could act by aggregating *P. gingivalis* or act in synergy with antimicrobial substances produced by lactobacilli as shown by our experiments. We have also recently shown that some *Lactobacillus* species are associated with gingival and periodontal health, which could be related to a direct antagonist activity of some strains against periodontal pathogens (Köll-Klais *et al.* 2005).

Coaggregation also contributes to the formation and maturation of the biofilm, which is important in the initiation of caries and periodontal diseases. In this regard, coaggregation of *P. gingivalis* by Gram-positive bacteria may be important to the initial events in the formation of subgingival biofilm (Namikoshi *et al.* 2003). *Lactobacillus* expressing scFv could potentially even favour the attachment of *P. gingivalis*. However, we previously observed that *Lactobacillus* expressing scFv against *Strep. mutans* rather reduced the count of *Strep. mutans* and the development of dental caries in a rat model (Krüger *et al.* 2002). Although the mechanisms of action are not known, the target bacteria bound to the surface of transformed lactobacilli might be effectively killed by the high local concentration of antimicrobial substances produced by the latter.

These results suggest that *Lactobacillus* represents a good candidate organism to deliver antibodies against these micro-organisms. Oral *Lactobacillus* strains with enhanced antagonistic properties against periodontal pathogens could be selected for expression of scFv. Furthermore, scFv with higher affinity could be expressed on the surface of lactobacilli. However, animal models will be necessary to show that *Lactobacillus* expressing single-chain antibodies may effectively be used as a therapy against periodontal diseases (Genco *et al.* 1998b).

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