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LACTOBACILLI EXPRESSING ANTIBODY FRAGMENTS AGAINST PATHOGENS

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To my family

Om du tänker för länge på nästa steg kommer du att tillbringa resten av livet på ett ben
Kinesiskt ordspråk

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ABSTRACT

Adherence to mucosal surfaces has been shown to be of great importance in the pathogenesis of various infections in the gastro-intestinal tract. Live recombinant bacteria can be used to deliver active or passive immunity at the mucosal surface, which is the site of entry for the majority of the pathogens. GRAS (Generally Regarded As safe) organisms with probiotic properties, such as *Lactobacillus*, are suitable candidates as vectors for delivery of foreign antigens or antibody fragments especially in the human gastrointestinal tract. The aims of these investigations have been to evaluate the potential of lactobacilli expressing antibody fragments against various types of pathogens in order to block adherence of these pathogens to their target sites.

Vectors, with a promoter inducible with mannitol, encoding a scFv antibody fragment, which recognizes the streptococcal antigen I/II (SA I/II) adhesion molecule of *S. mutans* (main etiological agent of dental caries), were constructed and expressed in lactobacilli. The expression and functionality of the single chain (scFv) antibody fragments, secreted into the supernatant or expressed on the surface of the bacteria, was verified *in vitro*. Oral administration of scFv-expressing bacteria in desalivated rats resulted in decreased number of *S. mutans* bacteria in the oral cavity and lower development of dental caries (Paper I). In order to evaluate if continuous expression of antibodies *in situ* in the oral cavity might be therapeutically superior to intermittent production, we compared the anti-cariogenic effect of lactobacilli expressing the scFv against the SA I/II adhesin under the control of an inducible or constitutive promoter. Both lactobacilli expressed equal amounts of scFv on the surface and agglutinated *S. mutans* bacteria expressing SA I/II to a similar level. In a rat caries model, transformed lactobacilli could be detected in the oral cavity throughout the duration of the study. Transformants containing the constitutive promoter were slightly more protective than those containing the inducible promoter suggesting that *in situ* expression might increase protection (Paper II).

Selected llama IgG subclass antibodies are composed of an elongated heavy chain but no light chain. The variable domain of llama heavy chain antibodies (VHHs) which are formed by a single polypeptide are markedly stable and can be easily engineered more easily, while retaining the correct functional conformation. These properties make them suitable for therapy against gastrointestinal infections. We first analysed the possibility of expressing functional model VHH fragments under the control of an inducible promoter. Two VHH fragments, VHH2 (directed against the capsid of the phage) and VHH5 (directed against the receptor-binding protein of the phage tail) against a lactic acid bacteriophage, p2, were respectively expressed as an anchored and secreted product. Both VHH fragments were efficiently expressed and shown to inhibit phage infection of lactococci in a neutralization assay. This may be of future use for prevention of lysis of starters and destruction of cheese batches caused by bacteriophages (Paper III). Subsequently, a VHH directed against rotavirus (VHH1) was expressed constitutively by lactobacilli, both in secreted and in cell surface anchored forms. Expression of VHH fragments in lactobacilli conferred significant reduction of infection in cell cultures in a neutralization assay. When administered orally, lactobacilli producing surface expressed VHH markedly shortened disease duration, severity and viral load in a mouse model of rotavirus-induced diarrhea both when given fresh or in a freeze-dried form. Such "lactobodies" may form the basis of a novel form of therapy against rotavirus infections and other diarrheal diseases worldwide (Paper IV).

Our experiments suggest that lactobacilli are potent expressers of functional antibody fragments against various pathogens, thus making them future candidates as delivery vehicles of passive immunity.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I. **Single chain producing lactobacilli: a new tool for *in situ* delivery of passive immunity.**
Krüger, C., Hu, Y., Pan, Q., Marcotte, H., **Hultberg, A.**, Delwar, D., van Dalen, P. J., Pouwels, P. H., Leer, R. J., Kelly, C. G., van Dolleweerd, C., Ma, J. K. and Hammarström, L.
Nature Biotechnology. 2002, 20:702-706.

II. **Passive immunization by lactobacilli expressing single-chain antibodies against *Streptococcus mutans*.**
Krüger, C., **Hultberg, A.**, Marcotte, H., van Dollenweerd, C. and Hammarström, L.
Molecular Biotechnology. 2005, 31;221-231.

III. **Lactobacilli expressing llama VHH fragments for prevention of phage infection in lactococci.**
Hultberg, A., Tremblay, D. M., de Haard, H., Verrips, T., Moineau, S., Hammarström, L. and Marcotte, H.
2006, *Manuscript.*

IV. **Lactobacilli expressing VHH antibody fragments from llama (lactobodies) confer protection against rotavirus-induced diarrhea.**
Pant, N.*, **Hultberg, A.***, Zhao, Y., Svensson, L., Pan-Hammarström, Q., Johansen, K., Pouwels, P. H., Ruggeri, F. M., Hermans, P., Frenken, L., Borén, T., Marcotte, H. and Hammarström, L.
The Journal of Infectious Diseases. 2006, E-pub ahead of print.

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ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion media or agar
bp	basepair
CFU	Colony Forming Unit
ELISA	Enzyme-linked Immunoabsorbent assay
Ig	Immunoglobulin
kDa	kilo Dalton
LAB	lactic acid bacteria
LCM	Lactobacillus carrying medium
LPS	lipopolysaccharide (endotoxin from gram-negative bacteria)
MRS	lactobacilli media, developed by de **M**an, **R**ogosa and **S**harpe
ND	not detected
Nm	nanometer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RRV	Rhesus rotavirus
SA I/II	surface adhesion antigen I/II
scFv	single-chain variable antibody fragment
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron microscopy
VHH	variable heavy chain from *Camelideae*

POPULÄRVETENSKAPLIG SAMMANFATTNING

För att en mikroorganism ska kunna orsaka sjukdom behöver den bland annat kunna vidhäfta till en yta (adhesion) i tex munhålan eller mag-tarmkanalen. Vidhäftningsmolekylerna kallas för adhesiner och sitter på mikroorganimens yta. Dessa adhesinerna binder i sin tur till receptorer på en yta i munhålan eller mag-tarm kanalen. Genom att blockera denna bindning av adhesinet till receptorn kan man hindra mikroorganismen från att orsaka sjukdom, vilket är målet med vaccination. Antikroppar som är en viktig del av vårt immunförsvar har förmåga att binda till olika delar av patogenerna (mikroorganimser som orsakar sjukdom) t ex adhesinet och kan därmed hindra den från att vidhäfta och orsaka sjukdom. Antikropparna är ganska stora molekyler, men man kan med olika metoder dela upp den så att man bara får den antigen bindande delen (antikroppsfragment).

Laktobaciller är probiotiska, hälsofrämjande bakterier, som är en del av vår normala mikroflora. De hjälper till att upprätthålla en balans mellan ”bra” och ”dåliga” bakterier i munhålan och mag-tarm kanalen. Man kan ganska lätt modifiera bakterier, däribland laktobaciller, och få dem att uttrycka genetisk material som egentligen inte tillhör bakterien själv och det är detta som min avhandling handlar om.

Vi har arbetat med att få laktobaciller att producera antikroppsfragment mot olika patogener i mun hålan och mag-tarm kanalen. När de uttrycker antikroppsfragment på sin yta binder de till allt som antikroppsfragmentet är riktat mot t ex en adhesin och hindrar därmed vidhäftning av patogenen. En laktobacill kan uttrycka cirka 1000 antikroppsfragment på sin yta och kan teoretisk binda till lika många patogener, så de fungerar ungefär som en kardborre. När de har bundit så många patogener de kan går de ut den naturliga vägen. Det kallas för passiv immunisering och det måste ges kontinuerligt så länge man vill ha ett skydd eftersom det inte ger något minne hos immunförsvaret.

Streptococcus mutans är en bakterie som återfinns i munhålan. Den anses vara den främsta orsaken till karies. Vi har tillverkat laktobaciller som uttrycker antikroppsfragment på sin yta mot denna bakterie och de har visat sig effektiva i minskningen av karies i en djurmodell.

Det finns speciella antikroppar i kameler och lamadjur som är lite mindre och mer tåliga mot syra och höga temperaturer än vad våra ”vanliga” antikroppar är. De är bättre på att motstå en färd genom mag-tarmkanalen än vad de ”vanliga” antikropparna är och därför har vi valt att arbeta med dem för vårt passiva vaccin mot patogener i tarmen. Rotavirus orsakar diarré hos spädbarn och i tredje världen är det många barn som dör av uttorkning till följd av denna infektion. Vi uttryckte

antikroppsfragment från lamadjur i laktobacillerna och när vi testade dem på våra möss så kunde vi se att de minskade infektionen och därmed diarrén. Tanken är att barnen ska kunna få dessa laktobaciller tillsammans med vatten och salt och därmed klara infektionen bättre.

Vi hoppas att våra antikroppsproducerande laktobaciller ska kunna användas för att behandla olika sjukdomar i mag-tarm kanalen och i munhålan. För att nå dit måste vi anpassa dem till de regulatoriska krav som finns (”food grade”) och få dem att och producera högre mängder av sina antikroppar.

INTRODUCTION

Adherence to mucosal surfaces has been shown to be of great importance in the pathogenesis of various infections in the gastro-intestinal tract. The majority of the pathogens enters and gives rise to an infection at mucosal sites, thus making live bacteria potential candidates as carriers of genetic material that will confer immunity against selected pathogens. GRAS (Generally Regarded As safe) organisms with probiotic properties, such as *Lactobacillus*, are suitable candidates as vectors for delivery of foreign antigens or antibody fragments in especially the human oro-gastrointestinal tract.

PASSIVE IMMUNIZATION AGAINST MUCOSAL PATHOGENS

Passive immunization is the administration of preformed exogenous antibodies for treatment of disease. Antibodies have long been considered as a powerful tool to recognise and target almost any molecule with a high degree of specificity and affinity. Purified human immunoglobulins have been given orally against mucosal pathogens, but this is not cost efficient since only low titres of antibodies are available in current preparations. There is also a risk of transmitting infectious diseases when human plasma is used as starting material. Alternative sources of antibodies have therefore been sought and include antibodies from immunized animals such as cows, chickens and a number of additional sources.

Oral administration of hyperimmune polyclonal bovine colostrums derived IgG or chicken egg yolk IgY have been shown to reduce oral diseases caused by *Streptococcus mutans* and *Candida albicans* [Ma et al., 1987, Weiner et al., 1999, Tollemar et al., 1999]. They have also shown to reduce gastrointestinal diseases caused by rotavirus, *Helicobacter pylori*, *Campylobacter jejuni*, *Escherichia coli* and rotavirus [Weiner et al., 1999, Casswall et al., 1998, Casswall et al., 2002, Hammarström et al., 1999, Tsubokura et al., 1997, Sarker et al., 1998, 2001]. IgY antibodies from eggs and antibodies from colostrum are polyclonal in origin and do not give as high titres as monoclonal antibodies.

Local passive immunization is safe, but the production and purification of the antibodies is also very costly, making it necessary to find other approaches. The use of selected monoclonal antibodies or antibody fragments is therefore increasing.

ANTIBODY FRAGMENTS

A conventional antibody is a bivalent, Y-shaped molecule that consists of two heavy chains and two light chains (Fig. 1A). The variable domains of the heavy and the light chain make up the antigen binding part of the antibody. Complete immunoglobulins are big and complex molecules that are difficult to produce in bacteria and strategies are therefore being developed for secretion and cell-wall anchoring of single-chain polypeptides, which comprise only the binding domain of the immunoglobulins, single-chain antibody fragments (scFv).

Single-chain antibody fragments, scFv

Single-chain variable fragment (scFv) is a genetically engineered antibody fragment that consists of the variable heavy chain (VH) and light chain (VL) of an immunoglobulin joined together by a flexible peptide linker (Fig. 1B). The first scFv molecules were developed independently in 1988. [Huston et al., 1988, Bird et al., 1988]. These scFv were derived from genes isolated from murine hybridoma cell lines and could specifically bind to their target antigens with affinities ranging up to those of their parent monoclonal antibodies. Nowadays, large libraries with scFvs expressed on the surface of filamentous phage can easily be made. The genes for antigen-specific scFv can be readily isolated by panning against the antigen of choice and then the desired scFv can be expressed in *Escherichia coli* [Schier et al., 1995].

There has been extensive work on the design of antibody fragments to get the most stable scFv with best affinity and specificity for the chosen target. There are many reports about how to achieve that, for instance by changing the scFv linker length [Arndt et al., 1998], site directed mutagenesis of the variable regions [Adams et al., 1999] and expression in various organisms such as bacteria, yeast, plants and even transgenic animals [Chambers, 2005]. *Escherichia coli* is the most popular choice for expression of scFv since they produce them at a high rate, give a high yield and allow easy purification.

VHH antibody fragments

Camelidae (camels, dromedaries, alpacas and llamas), nurse shark and wobbegong shark contain antibodies that lack the light chains. In camelids, these heavy chain antibodies constitute 50-60% of the IgG found in sera and are functional in antigen binding [Hamers-Casterman et al., 1993]. There are three or four different heavy chain antibody isotypes in dromedary and llama, respectively [Vu et al., 1997]. The cDNA sequences lack the exon coding for the first constant domain, CH1 (Fig. 1C). The absence of the CH1 domain explains

the absence of the light chain in the heavy chain-only antibodies, as this domain is the anchoring place for the constant domain of the light chain [Padlan, 1994].

There is a pathological disorder in humans or mice, known as heavy chain disease, characterised by the presence of heavy chain antibodies in their sera [Seligmann et al., 1979]. These truncated antibodies result from a somatic event that removes various parts of the VH and CH1 region from the expressed Ig gene making them dysfunctional in antigen binding.

The variable domain of llama heavy chain antibodies (VHH) consists of a single immunoglobulin domain and constitutes the smallest naturally occurring antigen-binding molecule known to date, thus making it an alternative to using conventional antibodies or antibody fragments. The VHH exhibit several advantages over scFvs as they are smaller, markedly more acid- and heat-resistant, are formed by a single polypeptide, and are thus easier to express in recombinant form with an intact spatial structure [Frenken et al., 2000, Vu et al., 1997, van der Linden et al., 1999]. Expression of VHH in yeast is highly efficient and results in the secretion of functional antibody fragments (VHH) in the growth medium [Frenken, et al., 2000]. Yeast produced VHH have been shown to prevent phage lysis of lactococcus lactis [Ledeboer et al., 2002] and decrease morbidity of rotavirus induced diarrhea in mice [van der Vaart et al., 2006].

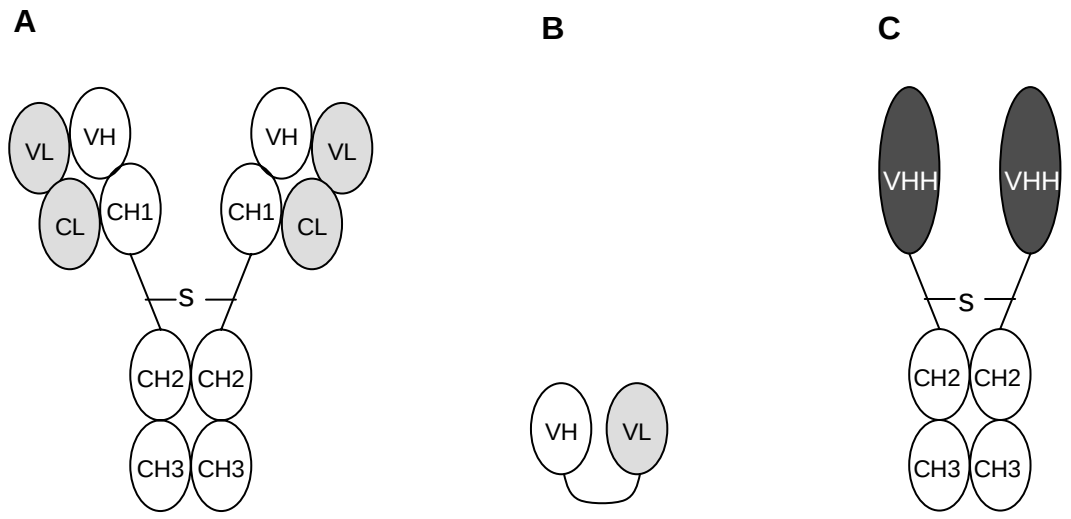

Fig. 1. Schematic drawings of (A) conventional IgG, (B) single-chain antibody fragment (scFv) and (C) *Camelidae* heavy chain antibody

PROBIOTICS

Over the past several years the relationship of the health of the intestinal tract to the overall health of the body has become increasingly appreciated. The human gastrointestinal (GI) tract is home to a vast and complex bacterial ecosystem, hosting over 400 different species. In terms of sheer numbers, the human intestinal tract contains ten times as many bacteria as there are tissue cells in the entire body.

The gut microbiota play a vital role in human health and perform important metabolic functions that support the digestive system. We are completely reliant upon the activities of the bacteria for the manufacture of key vitamins, the assimilation and distribution of nutrients, and for the suppression of pathogenic and putrefactive bacteria. Consequently, maintenance of a proper balance of bacteria in the gut is vital to good health.

In 1905, the Russian scientist, Elie Metchnikoff, proposed that lactic bacteria in fermented milk could promote the development of a healthy intestinal microbiota. Probiotics can be described as "mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora" and can consist of *Lactobacillus* species, *Bifidobacterium* species and yeasts [Havennar et al., 1992]. The dietary use of live microorganisms has a long history. Mention of cultured dairy products is found in the Bible and the sacred books of Hinduism. Soured milks and cultured dairy products, such as kefir (fermented milk from Caucasus with kefir grains i. e. grains of bacteria, yeast, lipids and sugars) and dahi (South Asian youghurt), were often used therapeutically before the existence of microorganisms was recognized. The use of microorganisms in food fermentation is one of the oldest methods for producing and preserving food.

LACTOBACILLI

Lactobacilli are members of the lactic acid bacteria (LAB), a broadly defined group characterized by the formation of lactic acid as a sole or main end product of carbohydrate metabolism. Lactobacilli are gram-positive, non-spore-forming rods or coccobacilli with a G + C content usually below 50 % [Hammes et al., 1995]. Eighty species of lactobacilli are recognized at present [Satokari et al., 2003]. They are normal constituents of our microbiota and in the Swedish population, *L. plantarum* and *L. rhamnosus* are the most frequently isolated species from the rectal and oral mucosa [Arhne et al., 1998], and *L. gasseri* from the female

vaginal tract [Vasquez et al., 2002]. Variations in the lactobacillus microbiota are found in different countries depending on food habits and breast feeding of infants [Mikelsaar et al., 2002, Satokari et al., 2002].

Lactobacilli are strictly fermentative and by using glucose as a carbon source, they may be either homofermentative (producing more than 85% of fermentative products as lactic acid) or heterofermentative (producing lactic acid, carbon dioxide, ethanol, and/or acetic acid in equimolar amounts). The nutritional requirements of lactobacilli are reflected in their habitats, which are rich in carbohydrate-containing substrates: they are found on plants or material of plant origin, in fermented or spoiled food, or in animals [Hammes et al., 1995].

Lactobacilli are important in the production of foods that require lactic acid fermentation, notably dairy products (yogurt and cheese), fermented vegetables (olives, pickles, and sauerkraut), fermented meats (salami), and sourdough bread. They are GRAS (Generally Regarded As Safe) organisms, non-pathogenic and are suggested to have probiotic, health promoting properties. These include control of intestinal infections [Sullivan et al., 2005], improvement of lactose metabolism and even anti carcinogenic activity, but the mechanisms of these effects are not fully understood [Marteau et al., 1993, Huis in´t Veldet al., 1994]. Some strains of lactobacilli, *L. casei* and *L. plantarum*, also exert strong adjuvant effects on the immune response [Perdigon et al., 1998, Isolauri et al., 1995]. Most lactobacilli are quite acid resistant and certain strains are able to survive passage through the stomach. Since they lack LPS in the cell wall there is no risk of endotoxic shock. A suitable lactobacilli strain that should be used as a vaccine carrier requires some important properties, just as those strains used for probiotics. These criteria are; they should be non-pathogenic, genetically amenable and good colonizers of the mucosa i. e. they should be able to adhere to the mucosa and persist for a certain amount of time [Havenaar et al., 1992].

There have been several reports of the effect of probiotic strains of lactobacilli on pathogens of the oro-gastrointestinal tract. Lactobacilli have been shown to have both prophylactic and therapeutic properties against rotavirus infection in controlled clinical trials [Mastretta et al., 2002, Isolauri et al., 1994]. The amount of dental caries in day-care children was decreased using *Lactobacillus rhamnosus* GG [Näse et al., 2001] and side-effects during anti-*Helicobacter pylori* treatment in children could be reduced by *Lactobacillus reuteri* [Lionetti et al., 2006].

LIVE VACCINE VECTORS

Live vaccines are potential candidates as carriers of genetic material that will induce neutralizing immune responses against selected pathogens without direct contact with the pathogen. This type of vaccine have another advantage in that they can be delivered and induce immunity the mucosal surface which is the site for the majority of the pathogens to enter and give rise to an infection. Live vaccines consist of a vector such as bacteria that are able to induce neutralizing immune responses. Live vaccine vectors are delivered at the mucosal surface, the place where the first line of defence is set and the onset of the infection. Different bacteria, both attenuated pathogens and commensal, have been used throughout the years as carriers for antigens of pathogens for which an immune response is desired [Medina et al., 2001]. The risk of using attenuated pathogens as carriers, especially in elderly, infants and other immunocompromised individuals has made the use of GRAS organisms as carriers an attractive concept. There have been several reports of the use of lactic acid bacteria as vaccine vectors such as *Lactococcus lactis*, *Streptococcus gordonii* and *Lactobacillus* [Seegers, 2002]. *Lactobacillus* have been employed as carriers for vaccine against tetanus [Shaw et al., 2000, Grangette et al., 2001], anthrax [Zegers et al., 1999] and pneumococcal surface antigen A [Oliveira et al., 2003, 2006].

Lactobacilli for delivery of antibody fragments

GRAS (Generally Regarded As safe) organisms with probiotic properties such as *Lactobacillus* are suitable candidates as vectors for delivery of antibody fragments in especially the human gastrointestinal tract. They are normal constituents of the human oro-gastrointestinal tract and have been suggested to have health promoting (probiotic) effects. The lactobacilli expressing antibody fragments can bind and thereby neutralize the pathogens and inhibit their binding and interaction to their target sites. They work as biological beads and can be referred to as "lactobodies". *Lactobacillus paracasei* expressing antibody fragments derived from monoclonal antibodies (resulting in scFv) or llama antibodies (resulting in VHH) against various pathogens have been reported and some of them are included in this thesis (Paper I-IV) [Krüger et al., 2002, Krüger et al., 2005, Pant et al., 2006]. ScFv expressed by *Lactobacillus* against rotavirus VP8 [Monedero et al., 2004] have been reported as well as against RgpA protease of *Porphyromonas gingivalis* [Marcotte et al., 2006] and against intercellular adhesion molecule 1 (ICAM-1) to block HIV infection [Chancey et al., 2006].

The major advantage of delivering antibody fragments instead of antigens is that it provides an immediate protection. It may be difficult to induce a sufficient local immune response on the mucosa using local administration of antigens.

THE TARGETS OF THE *LACTOBACILLUS* DELIVERY SYSTEM

Mucosal pathogens, such as *S. mutans* and rotavirus are suitable targets for lactobacilli expressing antibody fragments against them, blocking the adherence to the tooth pellicle (*S. mutans*) or the enterocytes of the small intestine (rotavirus). Lactobacilli can work as a "biological bead" by expression of the antibody fragments on the surface or secrete the antibody fragments.

Streptococcus mutans

Mutans streptococci are gram positive cocci found in the oral cavity. They are transferred from mother to child early in life by vertical transmission [Aaltonen et al., 1990]. *S. mutans* is the major pathogen involved in caries development and virulence factors include acidurance, acidogenicity and the ability of the bacteria to adhere to and accumulate on tooth surfaces [Marcotte et al., 1998]. There are seven *S. mutans* species, which can be subdivided into eight serotypes: a-h. *S. mutans* serotype c is the most commonly isolated from the dental plaque [Masuda et al., 1979]. *S. mutans* serotype c and *S. sobrinus* serotype d have been implicated as the primary causative agents of dental caries in humans [Loesche et al., 1986].

SA I/II is a 185 kDa protein expressed on the surface of *S. mutans* and has been implied to be important for colonization, involved in the initial adherence to the tooth pellicle [Jenkinson et al., 1997]. It has previously been called Ag I/II, B, IF, P1, SR, MSL-1 and Pac, [Koga et al., 1995, Jenkinson et al., 1997]. It is an immunodominant antigen that has been shown to induce both an antibody response [Lehner et al., 1981] and T-cell proliferation [Lehner et al., 1984].

Topical oral administration of antibodies against *S. mutans,* derived from animals or plants has been investigated [Ma et al., 1987 and 1998, Krüger et al., 2004] and may be an advantageous strategy to prevent caries in individuals with hyposalivation. Severe dental caries is a major problem among individuals with hyposalivation due to adverse effects of certain pharmaceutical products, systemic diseases such as Sjögren´s syndrome or head and neck tumor patients receiving heavy local irradiation therapy [Brown et al., 1978, Spak et al., 1994]. Recently, there has also been a report about the use of llama VHH antibody fragments against SA I/II to decrease caries caused by *S. mutans* in a rat model [Krüger et al., 2006].

Rotavirus

Rotaviruses belong to the Reoviridae family. Seven major groups have been identified, three of which (groups A, B, and C) infect humans. Group A is the most common and widespread one. The virus infects enterocytes of the villi of the small intestine, which leads to structural changes of the epithelium and diarrhea, thereby causing vomiting and diarrhea. It is the most common cause of severe diarrhea in children, resulting in close to 1 million deaths annually in children below 5 years of age in developing countries [Parashar et al., 2003]. Different strategies, including both active and passive immunotherapy, have been employed in efforts to develop a safe vaccine against rotavirus infection [Weiner et al., 1999, Kirkwood et al., 2003]. Recently, two new rotavirus vaccines, a live pentavalent human-bovine reassortant virus and an attenuated human monovalent virus vaccine, were shown to reduce the rate of severe rotavirus gastroenteritis in infants [Vesikari et al., 2006, 2006, Ruiz-Palacios et al., 2006]. However, issues regarding the rate of intussusception in children over 3 months of age or efficiency in malnourished children in the developing world, frequently suffering from concomitant parasitic or other enteric infections, still need to be resolved [Glass et al., 2006, Wood et al., 2005]. Passive immunization is currently the only available intervention that provides immediate protection and may thus represent the prophylaxis of choice for selected groups of children and immunocompromised patients [Sarker et al., 1998, 2001].

Delivery of passive immunization using lactobacilli as the delivery vehicle has been used in a mouse pup model and showed reduction in severity and duration of rotavirus induced diarrhea and is included in this thesis (Paper IV) [Pant et al., 2006]. *L. casei* expressing scFv against Vp8 has been developed, but not tested *in vivo* [Monedero et al., 2004].

Lactococcus lactis bacteriophage p2

Lactococcal bacteriophage p2 belongs to 936 phages and has a double-stranded DNA genome, a long noncontractile tail and a small isometric head. *Lactococcus lactis* is a grampositive lactic acid producing bacteria used as a starter in fermentation processes, such as cheese production. Bacteriophage infection results in lysis of the bacteria, causing delays in the production, variations in the taste and texture of the products, or even complete failure of fermentation, which in turn causes major economical losses [Bissonnette, et al., 2000]. A VHH fragment, VHH5 directed against a receptor-binding protein located on the tip of the non-contractile tail of the bacteriophage [Spinelli et al., 2006], has been selected and shown to inhibit bacteriophage infection of *L. lactis* [Lederboer et al., 2002, de Haard et al., 2005].

AIMS OF THE STUDY

GENERAL AIM

The aims of these investigations have been to evaluate the potential of lactobacilli expressing antibody fragments against various types of pathogens in order to block adherence of these pathogens to their target sites.

SPECIFIC AIMS

1. To evaluate an inducible expression of scFv against the SA I/II antigen of *Streptococcus mutans* on the surface of *Lactobacillus paracasei* and evaluate the function *in vitro* and *in vivo*.
2. Further development of the expression system by changing the promoter to allow constitutive expression of the scFv against SA I/II on the surface of *Lactobacillus paracasei* and evaluate the function *in vitro* and *in vivo*.
3. Investigate the possibility of expressing llama VHH fragments in *Lactobacillus paracasei*, both in a secreted form and on the surface, using llama "model" VHH antibody fragments directed against the *Lactococcus lactis* bacteriophage p2 and evaluate their effect *in vitro*.
4. Express llama VHH fragments against rotavirus on the surface of *Lactobacillus paracasei* and evaluate the effect *in vitro* and *in vivo*.

MATERIALS AND METHODS

Details concerning the different methods used can be found in the papers, I-IV.

THE LACTOBACILLUS STRAIN

The same *Lactobacillus* strain has been used for expression of antibody fragment in all the papers included in the thesis (paper I-IV). The strain was previously considered as a *L. casei* ATCC 393^T variant cured of plasmid pLZ15 and as been referred in the past as *L. casei* or *L. zeae* ATCC 393 ($pLZ15^-$). Recently, using molecular techniques, it has been correctly identified as a *L. paracasei* [Acedo-Félix et al., 2003]. The reason for choosing this strain was that the protocols for his transformation were available [Posno et al., 1991].

CLONING AND EXPRESSION OF scFv IN LACTOBACILLI

pLP401-anchored and pLP402-secreted constructs for inducible expression of scFv against *S. mutans*

The single-chain antibody gene fragment (VH and VL fused with a linker, $(G_4S)_3$), encoding the Guy´s 13 monoclonal antibody (scFv, 711 bp), was amplified from cDNA and cloned into the pCANTAB 5E phage vector (Amersham), fusing it to an E-tag for serological selection. A series of scFv expression vectors were constructed containing the promoter and secretion signal from the *α-amylase* gene of *L. amylovorus* as described previously [Pouwels et al., 2001] generating the expression vectors pLP401-scFv-long anchor (called pLP402-scFv-long anchor in Paper I) [Krüger et al., 2002] (Fig. 2A) and pLP402-scFv-short anchor. The pLP402 vector is an *E. coli/Lactobacillus* shuttle vector and contains a terminator region from the *ldh* gene, T*ldh*, inserted downstream of the promoter and the translation initiation region, which results in complete stability of the vector during the construction steps in *E. coli* [Pouwels et al., 1996]. Before transformation into lactobacilli, the T*ldh* was removed by restriction cutting. *L. paracasei* was transformed with the constructs by electroporation and induced by mannitol for expression of the scFv. The long anchor is approximately twice as long as the short anchor (244 aa compared to 117 aa) and was originated from the *PrtP* gene of *L. paracasei*.

E. coli JM109 (Stratagene) was used as a host strain for the construction of shuttle vectors, introduced by heat shock. The selection steps were performed on LB plates containing 100 μg/ml ampicillin for *E. coli* and MRS plates containing 3 μg/ml erythromycin for lactobacilli.

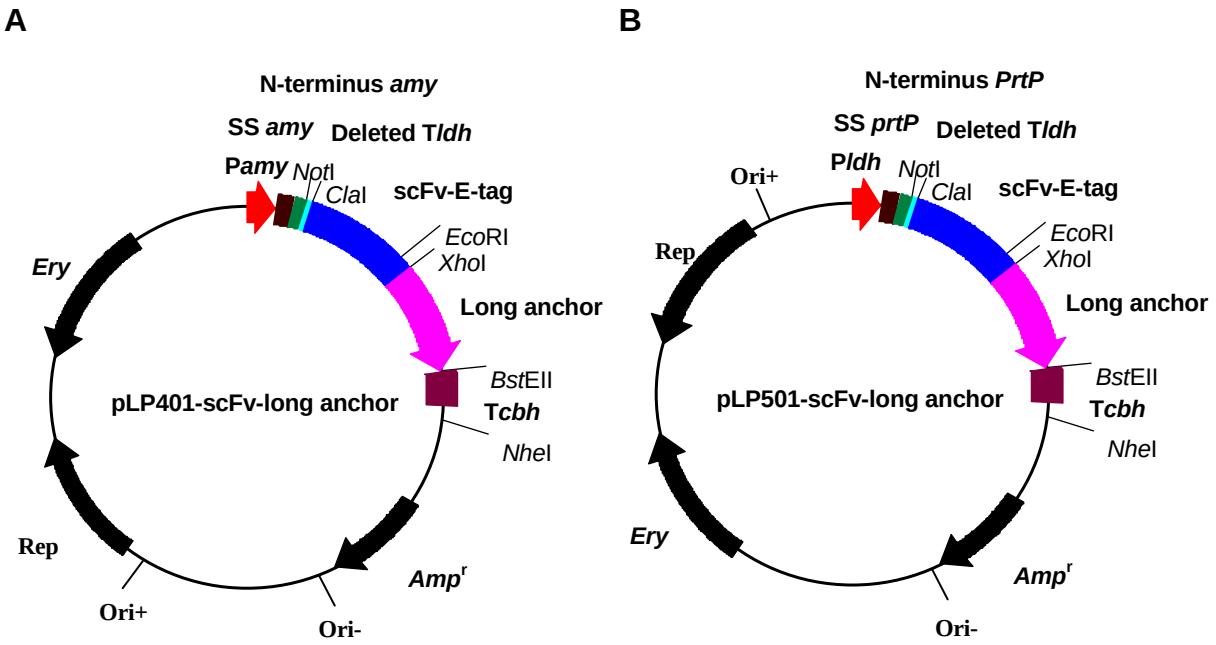

Fig. 2. *Lactobacillus* expression vectors for (A) inducible surface expression of scFv (B) for constitutive surface expression of scFv

pLP501-anchored construct for constitutive expression of scFv against *S. mutans*

The pLP502 vector contains a constitutive promoter, P*ldh*, from the lactate dehydrogenase gene of *L. plantarum* 80 [Pouwels et al., 1996]. The scFv-E-tag fused to the long anchor was excised from the pLP401-scFv-long anchor construct (previously called pLP402-scFv-long anchor) [Krüger et al., 2002] and ligated into the pLP502 vector, generating pLP501-scFv-long anchor (renamed when the anchor region was added) (Fig. 2B).

E. coli DH5α was used for the construction steps and the vector was introduced by heat shock. After removal of the terminator region, T*ldh*, the pLP501-scFv-long anchor was introduced into *L. paracasei* as described in the previous section.

CLONING AND EXPRESSION OF VHH FRAGMENTS IN LACTOBACILLI

pLP401-anchored and pLP402-secreted constructs for inducible expression of scFv against bacteriophage p2

The VHH2 encoding gene was cut out from the phagemid vector pUR3824 [de Haard et al., 2005] at the restriction sites *Sfi*I and *Not*I and ligated into pCANTAB 5E (Amersham Pharmacia Biotech, Bucks, UK) in order to fuse it with the E-tag. The VHH5-c-*myc* fragment was amplified by PCR from the pUR3825 vector [de Haard et al., 2005] adding a stop codon (TAA) after the c-*myc* gene. The VHH2-E-tag and VHH5-c-*myc* fragments were, after restriction cutting and purification, ligated into a *Lactobacillus* expression vector pLP401 (Pouwels et al., 1996, previously named pLP402, Krüger et al., 2002,) generating the vectors pLP402-VHH5-secreted (Fig. 3A) and pLP401-VHH2-anchor (Fig. 3B). *L. paracasei* was used for the transformation as described in the previous sections.

Lactobacilli containing pLP402-VHH2-secreted was also constructed, similarly to the pLP402-VHH2-anchored construct but with a stop (TAA) after the E- tag.

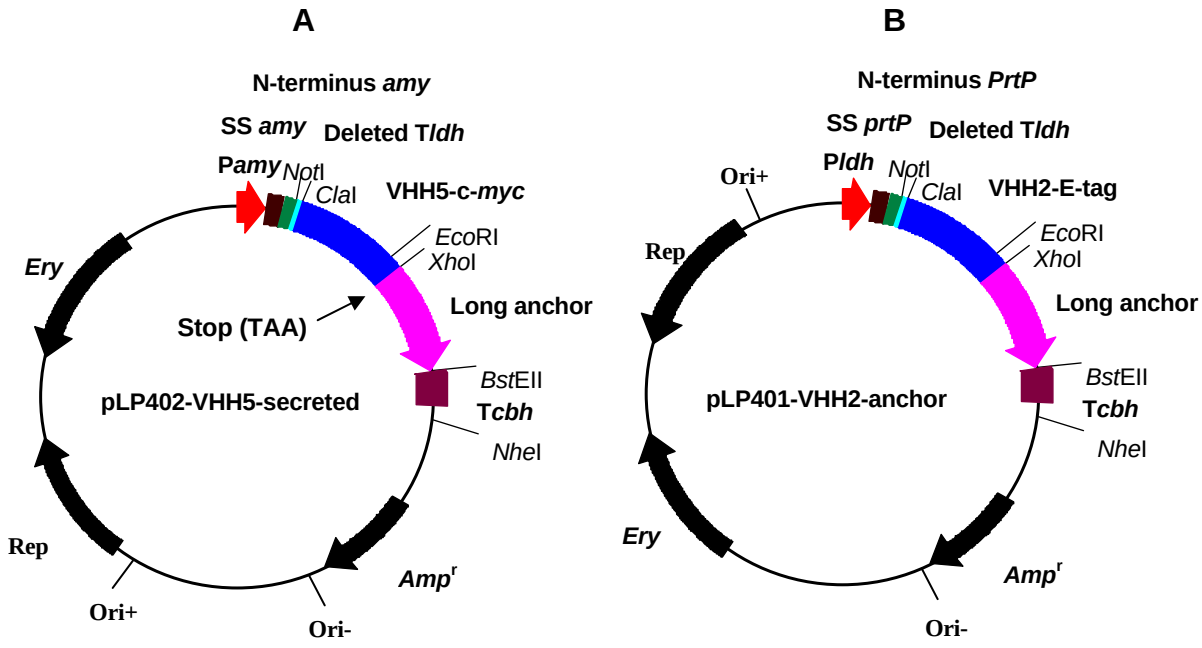

Fig. 3. *Lactobacillus* expression vectors for (A) an inducible expressed secreted llama VHH5 fragment and (B) an inducible surface expressed llama VHH2 fragment.

pLP501-anchored and pLP502-secreted constructs for constitutive expression of llama VHH against rotavirus

The immunization of llama with rhesus-monkey rotavirus serotype G3, strain RRV, and subsequent generation and selection of the VHH1 fragment has been described elsewhere [van der Vaart et al., 2006]. For generation of the surface-expressed antibody fragment, the DNA fragment coding for VHH1-E-tag gene (E-tagged for detection and purification) was fused to an anchor sequence (the last 244 amino acids of the proteinase P protein of *L. casei*) into the *Lactobacillus* expression vector pLP501 [Pouwels et al., 1996, Krüger et al., 2005]. To generate the secreted VHH1 antibody fragment, a stop codon (TAA) was included after the E-tag and introduced into pLP501, where the expression is under the control of constitutive *ldh* promoter from *L. casei* gene.

GROWTH CONDITIONS FOR EXPRESSION

Transformed lactobacilli were first grown over night in MRS broth with 3 μg/ml erythromycin at 37°C. For transformants with expression vectors containing the constitutive *ldh* promoter, a 1:50 inoculum was transferred to MRS broth or *Lactobacillus* carrying medium (LCM) [Efthymiou et al., 1962] containing 1% glucose (w/v) and 3 μg/ml erythromycin at 37°C. In order to induce expression of the heterologous proteins in transformants containing plasmids under the transcriptional control of the regulatable *α-amy* promoter (Fig. 2), cells were grown in LCM supplemented with mannitol (1% w/v) containing 3 μg/ml erythromycin. The bacteria was harvested at OD_{600} 0.8 (10^8 CFU/ml) to evaluate expression and activity of the antibody fragments.

IN VITRO EXPERIMENTS

For verification and visualization of surface expression, binding, agglutination ability and neutralization capacity, various experimental assays were used.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used in papers I-IV for verification of expression of scFv and VHH by lactobacilli and functional binding to the antigens (Carlsson et al, 1975). It was also used for estimations of the amount of antibody fragments produced by comparison to standards.

Western Blot

Western Blot was used in papers I and III to show the expression of scFv and VHH by lactobacilli detected by primary antibodies against the tags fused to the antibody fragments (E-tag and c-*myc* tag) [Laemli 1970, Towbin et al, 1979].

Scanning Electron Microscopy (SEM)

SEM was used in papers I, III and IV to visualize the binding of lactobacilli expressing antibody fragments to the pathogens [Boyde et al, 1963].

Flow cytometry

Flow cytometry was used in papers I- IV to verify surface expression of the scFv or VHH on the lactobacilli. An anti-E-tag antibody was used for detection of the E-tag and FITC conjugated or cy2-conjugated antibodies were used as secondary antibodies.

Purification of antibody fragments secreted by *Lactobacillus*

It was used in papers II and IV. Purification of secreted VHH and scFv antibody fragments was performed using the RPAS Purification module (Amersham Biosciences). The purity of antibody fragments was verified on SDS-PAGE and the concentration of total protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

Antagonism assay

It was used in paper II. The production of inhibitory substances by *L. paracasei* was measured on solid media by an antagonism test [Parrot et al., 1989]. Briefly, transformed *L. paracasei* inoculated in 2 cm lines on LCM agar plate, were allowed to grow and were then overlaid with BHI soft agar containing *S. mutans*. After incubation, the inhibition zone extending from each lactobacilli line was measured.

Agglutination assay

The agglutination assay was used in papers I and II to verify binding of the surface expressed scFv to *S. mutans*. Time and amount of agglutination was measured. The assays were performed blindly and repeated at least three times.

In vitro neutralization assay of rotavirus

This assay was used in paper IV to investigate the neutralizing capacity of lactobacilli expressing anchored or secreted and affinity purified VHH fragments against rotavirus. They were incubated with trypsin-activated RRV before subsequent inoculation of MA104 cell monolayers [Ruggeri et al., 1998, Svensson et al., 1991]. A reduction in the number of RRV-infected cells by more than 60% relative to the number in control wells suggested significant neutralization [Ruggeri et al., 1998].

Phage inhibition assay

It was used in paper III to verify inhibition of phage infection of *L. lactis* by the VHH fragments expressed by lactobacilli [Geller et al., 2005].

In situ expression of surface-anchored VHH1 on lactobacilli isolated from faeces

It was used in paper IV. Intestinal wash samples from mice daily fed with lactobacilli expressing anchored VHH were smeared on glass slides, fixed and incubated with mouse anti-E-tag antibody and a cy2-labeled donkey anti-mouse antibody before being examined in a fluorescence microscope.

Histology

Histology was used in paper IV. Sections of small intestines were stained with hematoxylin and eosin according to standard protocols and individual slides were evaluated blindly for typical signs of rotavirus infection [Boshuizen et al., 2003].

Quantification of RNA

It was used in paper IV. Total cellular RNA was isolated from small intestinal tissue, treated with Rnase-free DNase® (QIAGEN) and analyzed by real-time PCR using the EZ RT-PCR® core reagent kit (PE Applied Biosystems). Rotavirus *vp7* mRNA or viral genomic RNA was amplified. A standard curve was generated using a RRV *vp7* gene containing plasmid and the lowest level of detection of the PCR was 10 viral RNA copies. The RNA samples from each animal were normalized against the GAPDH gene [Overbergh et al., 1999]. The presence of less than 10 copies of *vp7* RNA was defined as clearance of infection.

Statistical analysis

In paper I, the Mann-Whitney test was used for the *S. mutans* counts and χ^2 was used for the caries score. Significance was considered when the *P* value was < 0.05.

In paper II, one-way analysis of variance in conjunction with the Tukey multiple comparison test was used for statistical analysis of *S. mutans* counts and caries activity data. Differences between groups were considered significant when a P value of < 0.05 was obtained.

In paper IV, diarrhea in the pups was assessed on the basis of consistency of faeces. Watery diarrhea was given a score of 2 and loose stool was given a score of 1. No stool or normal stool was given a score of 0. Presence or absence of diarrhea was compared among treatment groups in a day-wise manner by Fischer's exact test and was presented as percentage diarrhea in the graphs. Severity was defined as the sum of diarrhea scores for each pup during the course of the experiment (severity = Σ diarrhea score (day 1 + day 2 + day 3 + day 4)) and duration was defined as the sum total of days with diarrhea. Both severity and duration were analyzed by Kruskal-Wallis and Dunn´s comparison tests. Differences in the intestinal virus load as assessed by real-time PCR were tested using the Mann-Whitney test.

The analyses were performed using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA).

IN VIVO EXPERIMENTS

To investigate the *in vivo* capacity of the different lactobacilli expressing antibody fragments against *S. mutans* and rotavirus, two animal models were used.

The desalivated rat model

An established rat model was used in paper I and II to investigate the *in vivo* capacity of lactobacilli expressing scFv against *S. mutans* [Bowen et al., 1988, Krüger et al., 2002, 2005]. The rats used in this model have a decreased salivary secretion which creates a dry mouth situation in the oral cavity of the rats and in combination with a high sucrose diet and infection with *S. mutans*, they rapidly develop caries. The Keyes index was used for evaluation of dental caries in the desalivated rats [Keyes, 1958].

The rotavirus mouse pup model

The mouse pup model for rotavirus induced diarrhea is a well established model [Boshuizen et al., 2003]. Occurrence of diarrhea was recorded daily until the day of termination [Ruggeri et al., 1998]. By the time of termination, sections of small intestine were stabilized in

RNAlater® (QIAGEN) for RNA isolation and fixed in neutral buffered formalin for histopathological analysis.

All animal experiments were approved by the local Animal ethics committee of the Karolinska Institutet at Karolinska University Hospital, Huddinge.

RESULTS

PAPER I

Lactobacilli have previously been used to deliver vaccine components for active immunization *in vivo*. In this paper we describe a novel method where single-chain Fv (scFv) are produced *in situ* for delivery of passive immunity. Vectors encoding a scFv antibody fragment, which recognizes the streptococcal antigen I/II (SA I/II) adhesion molecule of *S. mutans*, were constructed and expressed in *Lactobacillus paracasei*. The scFv antibody fragments secreted into the supernatant or expressed on the surface of the bacteria, showed binding activity against SA I/II in enzyme-linked immunosorbent assay (ELISA). Surface scFv-expressing lactobacilli agglutinated SA I/II-expressing *Streptococcus mutans* without affecting the corresponding SA I/II knockout strain. Binding of lactobacilli expressing the scFv fragment fused to an E-tag were visualized by scanning electron microscopy (SEM) using beads coated with a monoclonal anti-E-tag antibody. Oral administration of scFv-expressing bacteria in desalivated rats resulted in less *S. mutans* bacteria and lower incidence of dental caries.

PAPER II

In this paper we describe a further development of the first series of lactobacilli constructs presented in paper I. The previous constructs contained an inducible promoter region and therefore was dependent upon the presence of mannitol for expression of scFvs. In this set of new constructs, the inducible promoter has been replaced and the new constructs have a continuous expression of antibody fragments directed against the SA I/II antigen. The expression of scFv on the surface of *L. paracasei* binding to SA I/II was verified by ELISA and immunoblotting methods. The amount of surface expression of scFv was similar to the previous construct. Lactobacilli transformed with the pLP501-long anchor construct were able to agglutinate *S. mutans* to the same extent as the previous pLP401-long anchor construct. The protective effect against dental caries was evaluated in a desalivated rat model. We found that rats treated daily (swabbing and in drinking water) with *L. paracasei* pLP501-long anchor had a lower development of smooth surface caries in comparison to controls. The protection was slightly better to that found in the pLP401-long anchor construct group suggesting that continuous *in situ* production of scFv in the oral cavity may be therapeutically superior to intermittent production.

PAPER III

Selected llama IgG subclass antibodies are composed of elongated heavy chains but no light chains. The variable regions may be expressed as single domains (VHH), which are suitable for introduction into the lactobacillus expression systems without loosing their antigen binding properties. These antibody fragments are more stable than scFv and have been produced at high level in *Saccharomyces cerevisiae*. Two VHH fragments, VHH2 (directed against the capsid of the phage) and VHH5 (directed against a receptor-binding protein located at the tip of the phage tail) against a lactic acid bacteriophage p2, have previously been expressed by *S. cerevisiae* and prevents the bacteriophage p2 from causing lysis of lactococci used in cheese production. The VHH2 and VHH5 fragments have been introduced into the *Lactobacillus* inducible vector system, pLP402. The VHH5 was expressed as a secreted fragment and the VHH2 as an anchored product. Both VHH fragments were well expressed and by ELISA, the amounts could be calculated to approximately 0.5 μg/ml for the VHH5 and about 10^3-10^4 fragments/bacteria for the anchored VHH2. Flow cytometry was used to confirm the surface expression of the VHH2. Scanning Electron Microscope (SEM) showed binding of the lactobacilli expressing VHH2 on the surface to the phage p2. A neutralization assay showed an inhibition of the phage infection on lactococci both when secreted (VHH5) and when expressed on the surface of the lactobacilli (VHH2). Lactobacilli expressing VHH fragments against a bacteriophage may be used for prevention of lysis of the starters and destruction of cheese batches.

PAPER IV

Rotavirus induced diarrhea poses a worldwide medical problem with substantial morbidity and mortality among children in developing countries. We have therefore developed a system for passive immunotherapy where recombinant lactobacilli constitutively express neutralizing llama VHH antibody fragments against rotavirus. A VHH directed against rotavirus (VHH1) has been selected at Unilever and been expressed by *Saccharomyces cerevisiae*. The VHH1 was expressed in *Lactobacillus paracasei,* both in secreted and in cell surface anchored forms, in the pLP502 vector system for continuous expression. Electron Microscopy (Scanning and Transmission) demonstrated efficient binding of the VHH antibody fragments to rotavirus. Expression of VHH fragments in lactobacilli conferred significant reduction of infection in cell cultures in a neutralization assay. When administered orally, lactobacilli producing surface expressed VHH markedly shortened disease duration, severity and viral load in a mouse model of rotavirus-induced diarrhea both when given fresh or in a freeze-dried form. Such

“lactobodies” may thus form the basis of a novel form of therapy against rotavirus infections and other diarrheal diseases.

DISCUSSION AND FUTURE PERSPECTIVES

The "perfect" vaccine is safe, cheap, stable, easy to administer and should give protection against a variety of diseases. Antibodies, used for passive immunization provides an immediate protection by inhibiting the binding of the pathogen to its host. They have been produced in bovine colostrums or egg yolk and used to reduce dental caries caused by *S. mutans* [Weiner et al., 1999, Krüger et al., 2004], or gastrointestinal infections caused by *H. pylori* [Casswall et al., 1998, 2002] or rotavirus [Sarker et al., 1998, 2001]. IgY antibodies from eggs and antibodies from colostrum have the disadvantage that they do not give as high titres as monoclonal antibodies. The production and purification of the antibodies is also very costly, making it necessary to find other approaches. The use of selected monoclonal antibodies or antibody fragments is therefore increasing.

Since the majority of the pathogens enter and give rise to infections on the mucosal sites, it is an advantage to use a type of vaccine that can be delivered and confer protection at the mucosal surface. GRAS (Generally Regarded As Safe) organisms with probiotic properties such as *Lactobacillus* are suitable candidates as vectors for delivery of foreign antigens or antibody fragments in especially the human gastrointestinal tract. They are gram-positive bacteria that are normal constituents of the human oro-gastrointestinal tract and have been suggested to have health promoting (probiotic) effects. Lactobacilli have several advantages as delivery vehicles in the oro-gastrointestinal tract, they are a natural ingredient in many of our food products and our microbiota, many lactobacilli are acid resistant [Kociubinski et al., 1999] and they can adhere to the mucosal epithelium [Morita et al., 2002].

This thesis involves the production of antibody fragments against mucosal pathogens using lactobacilli as delivery vehicles. We have expressed antibody fragments, scFv or VHH anchored or secreted using an inducible promoter (paper I and III) or a constitutive promoter (paper II and IV).

In the first study, *L. paracasei* was expressing a scFv anchored on the surface under the control of an inducible promoter (paper I). The scFv was derived from a monoclonal antibody (Guy´s 13) recognizing the SA I/II adhesion of *S. mutans,* a major mucosal pathogen involved in the development of dental caries. When administered to rats, the scFv expressing lactobacilli decreased the number of *S. mutans* and the development of dental caries. The amylase promoter present in this vector, pLP401-long anchor, is inducible by sugars such as mannitol. In order to make the production of scFv against *S. mutans* more efficient, the inducible promoter was

exchanged with a constitutive one and the vector, pLP501-long anchor, was introduced into lactobacilli (paper II). The expression of the scFv was shown to be slightly lower for the constitutive promoter, but the protection against smooth surface caries was slightly better to that found in the pLP401-long anchor construct group, suggesting that continuous *in situ* production of scFv in the oral cavity may be therapeutically superior to intermittent production. *Lactobacillus* transformants were detectable in the oral cavity throughout the study suggesting a possible *in situ* production of scFv against *S. mutans.* We observed a low, non-significant anti-cariogenic effect of the pLP402-empty vector control bacteria on the development of caries as well as the ability of these control lactobacilli to inhibit growth of *S. mutans in vitro*. This effect could be due to competition for receptors, production of inhibitory substances such as lactic acid, hydrogen peroxide or bacteriocins. Long-term administration of non-transformed *Lactobacillus rhamnosus* GG in children has indeed been shown previously to protect against development of dental caries [Näse et al., 2001].

The mechanisms by which the transformed *Lactobacillus* prevented caries could be blocking of the SA I/II adhesion, aggregation of *S. mutans* and the effect of antimicrobial substances produced by the lactobacilli. The *S. mutans* bacteria bound to the surface of the lactobacilli might thus be effectively killed by the high local concentration of bactericidal and bacteriostatic proteins produced by the latter (e. g. bacteriocins) [Eijsink et al., 2002, Maldonado et al., 2004]. Another mechanism could be that binding or crosslinking of the SA I/II induces a transduction signal upregulating or downregulating gene expression, thereby influencing the metabolism of *S. mutans* and/or adhesion to the tooth pellicle. However, the nature of the antimicrobial substances involved and the mode of inhibition remain to be determined. The delivery of antibody fragments by transformed lactobacilli could provide a future prophylactic strategy against dental caries. Expression of antibody fragments directed against several epitopes of interest, such as the Gtfs and/or the SA I/II as well as development of new systems for more stabile integration, constitute future developments that would be highly interesting.

Selected llama IgG subclass antibodies are composed of an elongated heavy chain but no light chain. They may be expressed as single domains only (VHH), which are suitable for introduction into the lactobacillus expression systems without loosing antigen binding properties. Llama VHH have been shown to have advantages over scFv, such as being smaller, more acid and heat resistant and being formed by a single polypeptide making them easier to express [Frenken et al., 2000, Vu et al., 1997, van der Linden et al., 1999]. We expressed two characterized VHH fragments against a lactococcal bacteriophage, p2 [Lederboer et al., 2002,

de Haard et al., 2005, Spinelli et al., 2006] under the control of the inducible promoter (pLP402) in lactobacilli (paper III). They were both found to prevent the bacteriophage from infecting the lactococci and cause lysis. Even though VHH2 is non-neutralizing as a monovalent fragment, VHH2 expressed on the surface on lactobacilli, may bind and neutralize the phages. However, the secreted VHH5 appears more effective in neutralization than the anchored VHH2. This was the first time that we could demonstrate an ability of *Lactobacillus* to express functional VHH fragments. Our results thus suggest a potential of lactobacilli expressing anti-phage VHH fragments as a powerful tool for prevention for phage attacks in the production of dairy products.

In subsequent studies, we expressed a VHH fragment directed against rotavirus, produced by *S. cereviseae* and which were previously shown to reduce diarrhea in a mouse pup model [van der Vaart et al., 2006]. The VHH was well expressed by lactobacilli and reduced the duration and severity of rotavirus induced diarrhea in a mouse pup model (paper IV). These results show the potential of lactobacilli to deliver therapeutic VHH fragments in the intestinal tract. The reconstituted freeze-dried VHH1-anchored lactobacilli were shown to be as protective against rotavirus-induced diarrhea as their fresh counterparts, suggesting that the antiviral activity is retained after freeze-drying. This is useful if it should be used in developing countries, where the genetically modified lactobacilli would have to be freeze-dried and administered in oral rehydration solutions or soft drinks.

VHH1-secreted was effective in neutralizing rotavirus *in vitro*. Nevertheless, only lactobacilli expressing VHH1-anchored successfully reduced viral load, normalised pathology and mitigated diarrhea in our animal model. In line with this, we have recently observed that purified monovalent VHH1 fragments produced in yeast mitigate rotavirus-induced diarrhea in the mouse pup model, but only when administered at very high doses (10 μg daily) [van der Vaart et al., 2006]. Although we used a strong constitutive promoter, VHH1 secreting lactobacilli cannot produce this amount of VHH1. Using the anchored construct, however, the numerous antibody fragments expressed on the bacterial surface result in the formation of "biological beads" which allow high-avidity binding due to multivalency, thus promoting strong agglutination and subsequent clearance of the virus. This is of particular importance in the gastrointestinal tract where microbial attachment is often based on low-affinity carbohydrate–protein interactions for tight adhesion to mucosal surfaces and biofilms.

We made several attempts to express scFv against rotavirus and *H. pylori* but were unsuccessful [unpublished data]. Some were expressed on the surface as detected by the E-tag, but were non-functional and some were not expressed at all. This points to the fact that scFv

expression and functionality is probably dependent on the folding of the scFv and display on the surface. The VHH fragments seem to be more stable and were all expressed well and functional in binding to their antigens, probably due to the fact that they are single domain antibody fragments and thereby more easy to express with the correct folding compared to the scFv, formed by a VH and a VH held together with a linker. This and the fact that they have other advantages over scFv, make them attractive for expression in lactobacilli against other pathogens of the mucosa. VHH fragments, so called nanobodies®, are now being selected against a variety of diseases such as rheumatoid arthritis, Chron´s disease and tumours, to be used as therapeutics [Gibbs, 2005].

The vectors used in these studies have previously been used for expression of antigens and are well characterized [Pouwels et al., 1996, 2001]. These vectors contain antibiotic resistance genes for selection of the transformed lactobacilli but to avoid this problem, new, safe biologically contained expression systems where the antibody gene is integrated into the chromosome of lactic acid bacteria and devoid of antibiotic selection markers are necessary [Steidler et al., 2003, Hanniffy et al., 2004]. By selecting lactobacilli with different tropism, the effect of the lactobacilli expression system could be even further improved. The choice of strain would depend on the ability to colonize (at least transiently) at the site where the protection is needed.

The advantages of using genetically modified lactobacilli include exceedingly cost-efficient production, long shelf life when lyophilized, simple logistics for distribution, and ease of administration. *In situ* production of antibody fragments locally on the mucosal surfaces of the host not only reduces the cost of purification but also circumvents the practical problem of degradation of orally administered antibodies in the stomach. If increased avidity or multispecificity is the key to efficient neutralization of pathogens, it should theoretically be possible to improve these results by concomitant expression of anti-pathogen fragments of varying specificities on the surface of lactobacilli or by using a mixture of recombinant clones. The use of lactobacilli expressing antibody fragments might be the prophylaxis of choice for children suffering from malnutrition and immunodeficiency. Tailor-made “lactobodies” may thus represent a new and versatile system for passive immunization at mucosal surfaces, and may be of major medical importance – especially in developing countries.

GENERAL SUMMARY AND CONCLUSION

Lactobacilli are non-pathogenic, probiotic bacteria, believed to have a health promoting effect. They have been used for delivery of antigens for induction of a protective immune response. Antibodies are the major protection against pathogens of the oro-gastrointestinal tract by their blocking of adhesion of the pathogen to the mucosal surface. They have been used for passive immunization against various pathogens, but are expensive to produce and purify. The antigen binding part can be isolated and expressed as a single-chain fragment (scFv) or VHH, if derived from llama. We have evaluated the potential of lactobacilli expressing antibody fragments against various types of pathogens in order to block adherence of these pathogens to their target sites.

The following conclusions can be drawn from the results herein presented work:

- Lactobacilli can successfully express functional scFv against SA I/II on *S. mutans*, under the control of an inducible promoter. They decrease the amount of smooth surface caries in a desalivated rat model when applied orally and in the drinking water.
- Lactobacilli can also successfully express functional scFv against SA I/II on *S. mutans*, under the control of a constitutive promoter. They were able to decrease the amount of total smooth caries in a desalivated rat model and were slightly better than the lactobacilli expressing scFv under the control of an inducible promoter.
- Llama VHH fragments against bacteriophage p2 can be functionally expressed in lactobacilli and inhibits the phage infection of lactococci in a neutralization assay, both when expressed on the surface of the lactobacilli and in a secreted form.
- When lactobacilli expressing functional llama VHH fragments against rotavirus were administered orally, the recombinant lactobacilli markedly shortened disease duration, severity and viral load in a mouse model of rotavirus-induced diarrhea, both when given fresh or in a freeze-dried form.

We have demonstrated the potential of lactobacilli expressing antibody fragments against various types of pathogens in order to block adherence of these pathogens to their target sites. This may provide a new strategy for cost efficient production of antibody fragments for *in situ* delivery of passive immunity.

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