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# Engineered Commensal Bacteria as Delivery Systems of Anti-infective Mucosal Protectants

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## Introduction

The mucosal surfaces of the gastrointestinal, genitourinary, oral and respiratory systems represent the principal portals of entry for most pathogenic microorganisms, as well as the first line of defence against the majority of infections. Mucus-associated lymphoid tissue represents a critical component of the mammalian immune system, and mucosal exposure to infectious agents and other foreign antigens is responsible for the production of antigen-specific secretory IgA and, frequently, serum antibodies, as well as for local cell-mediated immune responses (Conley and Delacroix, 1987; Ogra *et al.*, 1999). Based on these observations, mucosal surfaces have been seen as obvious sites for delivering protective agents against infectious diseases, mainly those mucosally acquired, thus preventing widespread colonization during the early stages of infection.

Live or live-attenuated microorganisms administered by the oral route have been associated with effective development of serum and generalized mucosal immune responses, and some of them, including typhoid, cholera, adenovirus and Sabin oral

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Abbreviations: APC, antigen-presenting cells; CT, cholera toxin; CMI, cell-mediated immunity; CTL, cytotoxic T lymphocytes; DC, dendritic cells; FimA, fimbrillin A; F, fusion protein; gp, glycoprotein; HA, haemagglutinin; HIV, human immunodeficiency virus; HPV16, human papilloma virus type 16; HSV, herpes simplex virus; Id, idiotypic; IgA, immunoglobulin A; Iscoms, immune-stimulating complexes; KT, killer toxin; KT-IdAbs, anti-Id antibodies with KT-like activity; KTR, killer toxin receptor; LT, *Escherichia coli* heat-labile enterotoxin; ML-I, mistletoe lectin I; N-9, nonoxynol 9; ODN, oligodeoxynucleotides; OPV, oral polio vaccine; *Pa*KT, *P. anomala* KT; scFv, single chain fragment variable; SDS, sodium dodecyl sulphate; SLS, sodium lauryl sulphate; STDs, sexually transmitted diseases.

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polio vaccine (OPV), are currently approved as vaccines for human use. The most remarkable success in this field has been the eradication of poliomyelitis from Europe, North America and most other parts of the world by using OPV vaccine, which is no longer recommended for routine use in the United States (Kiyono *et al.*, 1996).

Mucosal vaccines, particularly in oral formulations, are seen as safe, non-invasive, and attractive candidates for vaccination in both developed and developing countries. However, the complexity of mucosal immune regulation and the lack of the appropriate antigen delivery systems have remained substantial obstacles to the development of new efficacious mucosal vaccines, and most vaccines are still delivered by injection. Induction of a mucosal immune response starts with the recognition of an antigen by specialized cells, M cells, which are localized in the mucosal membranes of lymphoid tissues, allowing delivery to antigen-presenting cells. Cross-talk between M cells, epithelial cells and dendritic cells (DC) seems to play an important role in determining the outcome of immunity (Kaiserlian and Etchart, 1999; McGhee and Kiyono, 1999).

Mucosal delivery of soluble, non-replicating antigens usually does not stimulate efficient immune responses, requires multiple high doses, and may result in systemic unresponsiveness (McGhee and Kiyono, 1999; Nosal, 1999). Several approaches are currently under study for enhancing immune responses to mucosally-delivered vaccines. New vaccine agents, such as recombinant proteins, subunits or DNA vaccines, and transgenic edible plants, as well as new carriers to deliver the antigens into the mucosa, and new adjuvants to improve mucosal interaction with antigens have all been proposed (Ogra *et al.*, 2001).

Particular methods of antigen preparation and selective strategies of adjuvanticity have been investigated. Detoxified bacterial toxins, such as LT (*Escherichia coli* heat-labile enterotoxin) and CT (cholera toxin), have been demonstrated to be the most powerful mucosal adjuvants identified to date when co-administered with soluble antigens, dead bacteria or inactivated viruses (Freytag and Clements, 1999; Pizza *et al.*, 2001). Studies on other delivery systems, such as liposomes, microparticles, mucoadhesive microspheres, and polymeric devices (Kuntz and Saltzman, 1997; O'Hagan, 1998; Wyatt *et al.*, 1998; Baca-Estrada *et al.*, 2000; Kunisawa *et al.*, 2000), iscoms (Morein and Bengtsson, 1998), synthetic ODN (oligodeoxynucleotides) containing immunostimulatory motifs (McCluskie *et al.*, 2001), and plant lectins (Lavelle *et al.*, 2001), have highlighted their potential as mucosal vaccine carriers (Michaelek *et al.*, 1999). Finally, transgenic plants expressing vaccine antigens have been proposed as edible, low-cost vaccines, especially for use in developing countries, due to their ability to prevent or delay the onset of disease in animals, together with their safety and functionality in human clinical trials (Walmsley and Arntzen, 2000).

These new technologies for antigen delivery and knowledge of mucosal immunoregulatory mechanisms should facilitate, in the forthcoming decades, the availability of widespread, efficacious, painless, mucosally-delivered vaccines, eventually in multiple formulations, for both preventative and therapeutic use.

## Recombinant bacteria as live vaccine vectors

The use of the latest recombinant DNA technologies and availability of different vector systems, such as *Saccharomyces cerevisiae* and viruses, has resulted in the

production of large quantities of purified recombinant antigens, useful for the development of several protein-based vaccines, such as hepatitis B virus surface antigen vaccine (Jilg *et al.*, 1984; Valenzuelz *et al.*, 1985; Hammond *et al.*, 1991). To overcome the need for injection and to increase the ability to stimulate an efficient secretory immune response, live microbial vectors have been proposed for the direct delivery of heterologous antigens or DNA to mucosal sites in humans and animals (Cardenas and Clements, 1993; Babiuk and Tikoo, 2000; Pachuk *et al.*, 2000; Thole *et al.*, 2000; Medina and Guzman, 2001).

Among viral vectors, adenoviruses, vaccinia, chimeric HIV–poliovirus genomes and, particularly, poliovirus, have been employed to induce an IgA B cell immune response, in addition to specific CTL (Perkins *et al.*, 1985; Lomonossoff and Johnson, 1995; Moldoveanu *et al.*, 1995; Babiuk and Tikoo, 2000).

Initial studies on bacterial vectors focused mainly on attenuated Gram-negative pathogenic microorganisms. Among them, *Salmonella* remains the prototype. A variety of attenuated *Salmonella* strains, mostly *S. typhimurium*, have been proposed for the expression and delivery of foreign antigenic determinants to the immune system, since they are able to colonize and penetrate the intestinal mucosa following oral administration, and to be taken up by M cells (Brown *et al.*, 1987; Dougan *et al.*, 1987; Nakayama *et al.*, 1988; Poirier *et al.*, 1988; Yang *et al.*, 1990; Doggett *et al.*, 1993). Other Gram-negative attenuated bacteria, such as *Yersinia*, *Shigella*, *Vibrio* and *Brucella*, have also been proposed (Sory *et al.*, 1990; Comerci *et al.*, 1998; Killeen *et al.*, 1999; Vemulapalli *et al.*, 2000; Ogra *et al.*, 2001) as vectors for the delivery of eukaryotic antigen expression vectors (DNA vaccines) directly into APC (antigen-presenting cells), such as macrophages and DC (dendritic cells), through bacterial infection (Courvalin *et al.*, 1995; Grillot-Courvalin *et al.*, 1998, 1999; Dietrich *et al.*, 2000, 2001). Other bacterial pathogens, such as *Bordetella*, *Listeria* and *Mycobacterium*, have been also exploited for vaccine purposes (Stover *et al.*, 1991; Cirillo *et al.*, 1995; Mielcarek *et al.*, 1997; Weiskirch and Paterson, 1997; Dietrich *et al.*, 2001).

All these live antigen-delivery vectors were attenuated human or animal pathogens, which were representing a problem in terms of reduction of their pathogenicity without impairing their immunogenicity. Questions had also been raised about their safety, especially when destined for immunodeficient individuals, and, in addition, possible environmental issues inherent in their wide-scale dissemination. To circumvent some of these issues and avoid the use of engineered pathogens, commensal Gram-positive bacteria, generally recognized as safe (GRAS), have been exploited as alternative antigen-delivery vehicles. This has been achieved by developing systems that allow the expression of heterologous antigens in these host cells. The addition of mucosal targetting signals through co-display of adhesins is now under study so as to achieve targetting of the live bacteria to immunoreactive sites, thereby increasing immune responses (Fischetti *et al.*, 1996; Hansson *et al.*, 2001).

Lactic acid bacteria, and *Lactobacillus* strains in particular, show a variety of properties, such as GRAS status, low intrinsic immunogenicity, adjuvanticity, mucosal adhesive characteristics, as well as their use as food starters and probiotics, which make them an attractive new approach for oral vaccination purposes. Independent studies on recombinant lactic acid bacterial strains expressing antigens from pathogens have demonstrated that, when used as mucosal vaccines, these vectors can induce

specific protective humoral and mucosal antibodies, as well as a cellular immune response (Marteau and Rambaud, 1993; Wells *et al.*, 1996; Norton *et al.*, 1997; Pouwels *et al.*, 1998; Grangette *et al.*, 2001). *Lactococcus lactis*, a food-grade non-pathogenic and non-colonizing bacterium, has been also engineered to functionally display heterologous proteins on its surface and co-express cytokines, and has been proposed as a non-invasive mucosal vaccine delivery vehicle (Steidler *et al.*, 1998a,b, 2000).

Since some streptococcal species are part of the normal microbiote colonizing the mucosal surfaces of humans and other animals, these non-pathogenic streptococci have been proposed as potential vaccine vectors. In 1992, Pozzi and co-workers developed a host–vector system in which a foreign antigen replaces nearly all of the surface-exposed region of the fibrillar M6 protein from *Streptococcus pyogenes* and is fused to the C-terminal attachment motif of the M molecule. A segment of the *emm*-6.1 gene responsible for encoding most of the surface-exposed portion of the molecule was excised and replaced with the one encoding the foreign epitope. The fusion protein was thus expressed on the surface of *S. gordonii* Challis (formerly *S. sanguis*), a commensal bacterium of the human oral cavity, naturally competent for genetic transformation (Pozzi *et al.*, 1992a,b).

Further studies facilitated the development of a more efficient host–vector system based on the integration of plasmid insertion vectors into the chromosome of specially engineered *S. gordonii* strains, downstream from a resident promoter, and the use of the M6-protein-encoding gene (*emm*-6) as a partner for the construction of translational gene fusions. Using this strategy, upon transformation, the recipient bacterial cells acquire the capacity of displaying on the cell surface and stably expressing both *in vitro* and *in vivo* a variety of antigenic epitopes (Oggioni *et al.*, 1996, 1999a). By using such a system, different types of foreign antigens have been expressed, such as the B monomer of the *E. coli* heat-labile toxin, LT (Ricci *et al.*, 2000), the E7 protein of HPV16 (Pozzi *et al.*, 1992a), *Porphyromonas gingivalis* FimA (Sharma *et al.*, 1996, 1997, 1999), measles virus proteins HA and F (Maggi *et al.*, 2000), tetanus toxin fragment C (Medaglini *et al.*, 2001), and V3 domains of HIV-1 gp120 (Oggioni *et al.*, 1999b).

Experimental immunization by oral or vaginal colonization with such recombinant strains has been shown to induce a systemic, as well as a mucosal, immune response depending upon the effective colonization by live bacteria, since dead bacteria did not induce such a response (Medaglini *et al.*, 1995, 1997, 1998; Oggioni *et al.*, 1995; Di Fabio *et al.*, 1998; Sharma *et al.*, 2001). Live bacteria induce *de novo* synthesis of both MHC class I and II major histocompatability complex in DC and stabilize MHC class I molecules, increasing the capacity of DC to present antigens in the draining lymph nodes. Thus, recombinant streptococci appear potential useful vectors for the correct delivery of exogenous antigens to be presented efficiently on MHC class I molecules to specific CD4+ T cells (Rescigno *et al.*, 1998; Corinti *et al.*, 1999, 2000).

Recent studies demonstrating compartmentalization of the common mucosal immune system have created further challenges for the development of organ-specific vaccines. Live recombinant bacteria could possibly be engineered to co-express heterologous antigens and specific mucosal targetting signals, such as colonization factors, and used as more efficient vectors, due to their ability to colonize and persist for a longer period in a specific mucosal compartment (Ricci *et al.*, 2001).

**Mucosal drug delivery systems**

Apart from vaccines, mucosal surfaces have been used as sites for delivering many different drugs, the oral route constituting the preferred route for delivery (Shojaei, 1998), and microbicides (Stephenson, 2000). The mucosa has a rich blood supply and is relatively permeable, allowing the absorption and the bioavailability of therapeutic agents and, among them, peptide or protein drugs and antibodies. However, the bioavailability or relative potency of orally-administered peptides is usually quite low, due to several barriers encountered within the gastrointestinal tract. Hepatic first-pass metabolism, acidity and proteolytic enzymes can lead to a severe pre-systemic degradation of the drug, limiting its availability. Polypeptide drugs can be hydrolysed by brush border membrane proteolytic enzymes during their transport across the intestinal mucosa by a peptide transporter (Bai and Amidon, 1992). In order to circumvent these problems, different mucosal delivery systems have been intensively studied.

Bioadhesive drug delivery systems have been proposed to prolong and intensify the contact between the drug and the mucosal surfaces. Non-specific interactions with the mucosal surfaces, on the basis of the physico-chemical properties of the bioadhesive systems and specific interactions, by using ligands attached to the bioadhesive materials for the recognition and attachment to specific sites at the mucosal surfaces, provide an intimate contact and delivery to the mucosa, thereby reducing any possible drug degradation (Harding *et al.*, 1999: see also Lehr, 1994, 2000; Mathiowitz *et al.*, 1997; Ponchel and Irache, 1998; Lele and Hoffman, 2000). Other drug delivery systems, such as chitosan (Senel *et al.*, 2000; Janes *et al.*, 2001), cubic phase gels (Shah *et al.*, 2001), cellulose derivatives (Suzuki and Makino, 1999), as well as the use of inhibitory agents of the proteolytic enzymes (Bernkop-Schnurch, 1998), have been proposed.

Bacterial ghosts, too, have been exploited for the packaging of drugs and their site-specific delivery, taking advantage of the display on their surface of morphological and antigenic structures that permit their specific attachment to target tissues and their uptake by phagocytes and M cells (Huter *et al.*, 1999).

Controlled, prolonged topical release of specific antibodies has been proposed for long-term mucosal passive immunoprotection against infectious STDs, as well as to permit substantial systemic uptake of the antibodies (Sherwood *et al.*, 1996; Kuo *et al.*, 1998; Saltzman *et al.*, 2000).

In this field, another focus of a great deal of research activity is currently the development of new, effective topical microbicides, i.e. products to be used for protection of mucosal surfaces against bacterial and viral pathogens. The spread of STDs, the HIV pandemic in particular, has created an urgent need for new types of non-toxic, broad-spectrum microbicides, that may or may not be spermicidal, to protect the vaginal and cervical mucosa against sexually transmitted pathogens at the time of initial exposure. Detergent-based microbicides approved for use as spermicides, such as N-9, the only one tested in phase III clinical trials so far, and benzalkonium chloride, appear to damage epithelial tissues of the female reproductive tract and to reduce the population of potentially protective *Lactobacillus* species in the vagina (Patton *et al.*, 1999; VanDamme, 2000). Thus, many different products have been proposed in different formulations and are in various stages of development. Among

them, bile salts (Herold *et al.*, 1999), natural antimicrobial peptides (Lee *et al.*, 1997; Qu *et al.*, 1997; Turner *et al.*, 1998; Amiche *et al.*, 1999), dendrimers (Bourne *et al.*, 2000), monocaprin in hydrogel formulation (Neyts *et al.*, 2000), mucibodies (Fontenot *et al.*, 1998), naphthalene sulphonate derivatives (VanDamme *et al.*, 2000), pharmaceutical excipients (Neurath, 2000), plant products (Bourne *et al.*, 1999), surface active agents, such as SLS, C31G and SDS (Krebs *et al.*, 1999; Piret *et al.*, 2000), synthetic lipids (Lampe *et al.*, 1998), and thiourea compounds (D'Cruz *et al.*, 2000), have shown significant *in vitro* and *in vivo* microbicidal activity against HIV, HSV-2, *Chlamydia trachomatis* or *Neisseria gonorrhoeae*, and are seen as candidate topical microbicides.

Among the antimicrobial agents, antifungals often represent a limited therapeutic armamentarium of compounds that are frequently found to be ineffective and toxic. Cyclodextrins, cochleates, liposomes, nanoparticles, or nanospheres, have been proposed and used to modulate the pharmacokinetics of the existing antifungal compounds and to enhance their delivery to sites of infection, while reducing their toxicity (Irie and Uekama, 1997; Mandal, 2000; Santangelo *et al.*, 2000; Walsh *et al.*, 2000; Arikan and Rex, 2001). However, novel molecules and innovative delivery systems for acute and prophylactic application are required, due to the emergence of antifungal resistance among normally susceptible strains and the spreading of less susceptible species, particularly among *Candida* spp.

## Mucosal candidiasis, immunity, and KT-like fungicidal antibodies

Superficial and deep-seated candidiasis are important opportunistic diseases in immunocompromised, or otherwise modified, hosts. Yeasts belonging to species of the genus *Candida* have emerged among the most frequent pathogens in humans, representing the fourth leading cause of nosocomial bloodstream infections, accounting for 8% of all infections associated with the highest mortality of commonly encountered pathogens (Banerjee *et al.*, 1991; Beck-Sagué and Jarvis, 1993).

Epidemiologically, *C. albicans* is the prevalent pathogenic species, even though infections caused by other species, such as *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*, are reported to be significantly increasing, particularly in immunocompromised patients such as neutropenic, intensive-care, cancer, and post-surgical patients (Banerjee *et al.*, 1991; Beck-Sagué and Jarvis, 1993; Nguyen *et al.*, 1996; Abi-Said *et al.*, 1997). Oropharyngeal and oesophageal candidiasis often complicate HIV infection, and acute and chronic vulvovaginal candidiasis are relatively common in women of child-bearing ages. It has been estimated, for instance, that approximately two-thirds of fertile women experience at least one acute attack of vulvovaginal candidiasis during their life span (Morton and Rashid, 1977; Klein *et al.*, 1984; Odds, 1988; Fidel and Sobel, 1996).

T cell-dependent CMI (cell-mediated immunity) is considered to play a key role for prevention of *Candida* mucocutaneous infections (Fukazawa *et al.*, 1994; Fidel, 1998). However, recent studies indicate that protection against this pathogen requires a combination of different host resistance factors, and that CMI and antibody-mediated immunity are largely interactive immunomodulators of the host–fungus interplay (Casadevall *et al.*, 1998). The antigenic complexity of *Candida* has probably been the main reason of the past controversies concerning the role of antibodies, since

protective antibodies directed against a limited number of the epitopes displayed may not necessarily be produced at sufficient levels during natural infections.

Over the past few years, we have reported a special case of antibody-mediated immunity by fungicidal antibodies, which mimic the biological activity of a wide-spectrum KT (killer toxin) from the yeast *Pichia anomala*, showing an anti-*Candida* antibiotic activity (Cassone *et al.*, 1997; Magliani *et al.*, 1997b). In light of the theory of the Id (idiotypic) network of Jerne (1974) and the yeast killer phenomenon (Magliani *et al.*, 1997b), we used a murine mAb (mAb KT4) that neutralized the anti-*Candida* activity of KT (Polonelli and Morace, 1987) as a parenteral or mucosal Id vaccine in rodent experimental models, thereby eliciting the production of protective serum or secretory anti-Id antibodies. Parenteral Id vaccination in syngeneic mice resulted in a significant protection against lethal intravenous infection with KT-susceptible *C. albicans* cells, as well as intravaginal immunization with monoclonal antibody (mAb) KT4, which resulted in effective protection in a rat model of vaginitis. MAb KT4-affinity chromatography-purified anti-Id antibodies exerted a significant *in vitro* killer activity against KT-susceptible *C. albicans* cells, and this candidacidal activity was totally abolished by previous absorption with mAb KT4, thus attesting to the specificity of their action. KT-IdAbs were also able to confer passive immunoprotection on unvaccinated, experimentally infected animals (Polonelli *et al.*, 1993, 1994). Like KT, KT-IdAbs were able to interact with a not-yet-identified putative cell wall receptor on susceptible *Candida* cells, mainly expressed in budding cells and germ tubes (Polonelli *et al.*, 1990).

These data suggested that KT-IdAbs carry the internal image of KT, also implying some structural homology between the Id of mAb KT4 and KTR. According to this hypothesis, we demonstrated that KT-IdAbs and KT can compete for the binding site of mAb KT4, and that intravaginal or intragastric inoculations of KTR-bearing *C. albicans* cells were able to recall mucosal KT-IdAbs production in animals primarily immunized intravaginally with mAb KT4 and to elicit by themselves antibodies that functionally mimicked the KT. More interestingly, natural KT-like candidacidal antibodies, functionally similar to those previously described, were also significantly found, particularly in the vaginal fluid of women with recurrent vaginitis who had obviously never been exposed to the Id vaccine (Polonelli *et al.*, 1996). Any role of these antibodies in human disease remains to be elucitated, but they are certainly part of the antibody repertoire that follows infection or immunization with *Candida*.

Following these observations, we produced rat mAb KT-IdAbs and mouse recombinant antibodies, in the scFv format, by hybridoma and recombinant DNA technologies from the spleen lymphocytes of animals immunized with mAb KT4. MAb KT-IdAbs and scFv KT-IdAbs were, as KT-IdAbs, able to kill *C. albicans in vitro*, to bind to specific KTR on the yeast cells, and to exert a strong therapeutic effect in an experimental model of rat vaginal candidiasis (Magliani *et al.*, 1997a; Polonelli *et al.*, 1997).

## Transgenic commensal bacteria in the therapy of mucosal candidiasis

Recently, we have described a new approach to deliver a fungicidal recombinant antibody into the vaginal mucosa and demonstrated its significant therapeutic effect in a well established *in vivo* model of rat vaginal candidiasis (Beninati *et al.*, 2000).

We engineered the human commensal Gram-positive bacterium, *S. gordonii*, previously devised as live vaccine for antigen delivery to mucosal surfaces, as mentioned before, to express a candidacidal anti-idiotypic recombinant antibody in the single chain format (scFv KT-IdAbs). Construction, selection and *in vitro* and *in vivo* candidacidal activities of scFv KT-IdAbs have been previously described (Magliani *et al.*, 1997a). We used the host–vector system described by Oggioni *et al.*, based on a two-step procedure (Oggioni *et al.*, 1996). First, translational fusions to the streptococcal surface protein M6 are constructed in *E. coli* vectors, then these vectors are tranformed in *S. gordonii* where they integrate downstream a strong chromosomal promoter.

As depicted in *Figure 7.1*, after amplification by PCR and proper primers, the scFv KT-IdAbs-encoding DNA fragment has been cloned into the insertion vector pSMB55 inside the *emm-6* gene, and transformed in a specially engineered recipient strain, named GP1221 (Oggioni *et al.*, 1996, 1999a). Two different constructs were obtained, one of them having a stop codon introduced at the end of the scFv coding sequence. Integration of the heterologous DNAs into the host bacterial chromosome allowed the selection of two recombinant strains expressing the scFv molecule. In the first, named GP1302, the gene fusion included the signal sequence and parts of the N-terminal region as well as the C-terminal anchor sequence of the M6 protein, allowing the efficient expression, export, and display of the scFv molecule at the bacterial surface. In the second, named 1303, the introduction of the stop codon excluded the anchoring sequence, which determines the secretion of the scFv KT-IdAbs fusion protein. The capacity to kill *Candida albicans in vitro* was confirmed for both recombinant strains expressing the scFv KT-IdAbs. More importantly, the candidacidal activity was shown to be inhibited by the anti-KT (mAb KT4) which generated the scFv KT-IdAbs.

Based on these *in vitro* data, *in vivo* experiments have been performed with the scFv KT-IdAbs expressing *S. gordonii* strains in an oestrogen-dependent model of rat vaginal candidiasis. In two independent experiments, it was shown that streptococci secreting the scFv KT-IdAbs cleared *Candida* infection to an extent comparable to a full therapeutic course of fluconazole. The streptococcal strain displaying the scFv KT-IdAbs on the surface cleared *Candida* infection much more slowly and less efficiently (Beninati *et al.*, 2000).

The general design of the *S. gordonii* system makes it extremely useful for mucosal delivery of substances. Features include stable expression of heterologous constructs by chromosomal promoters (Pozzi *et al.*, 1992a), stability of genetic constructs obtained by chromosomal integration (Pozzi *et al.*, 1992b), efficient protein production *in vivo* (Medaglini *et al.*, 1997) and extra-cellular production of heterologous proteins (less toxicity).

All of these features contribute to the capacity for efficient colonization of mucosal surfaces by the recombinant streptococcal clones, which evidently did not lose their competitivity towards wild-type bacteria. The unaltered viability of genetically manipulated microorganisms is a central concept when using recombinant bacteria in the field, and distinguishes the streptococcal expression system from all inducible high level, plasmid-based expression systems.

Since the local production of substances is strictly related to the capacity of the recombinant bacteria to maintain colonization, efforts are presently being made to

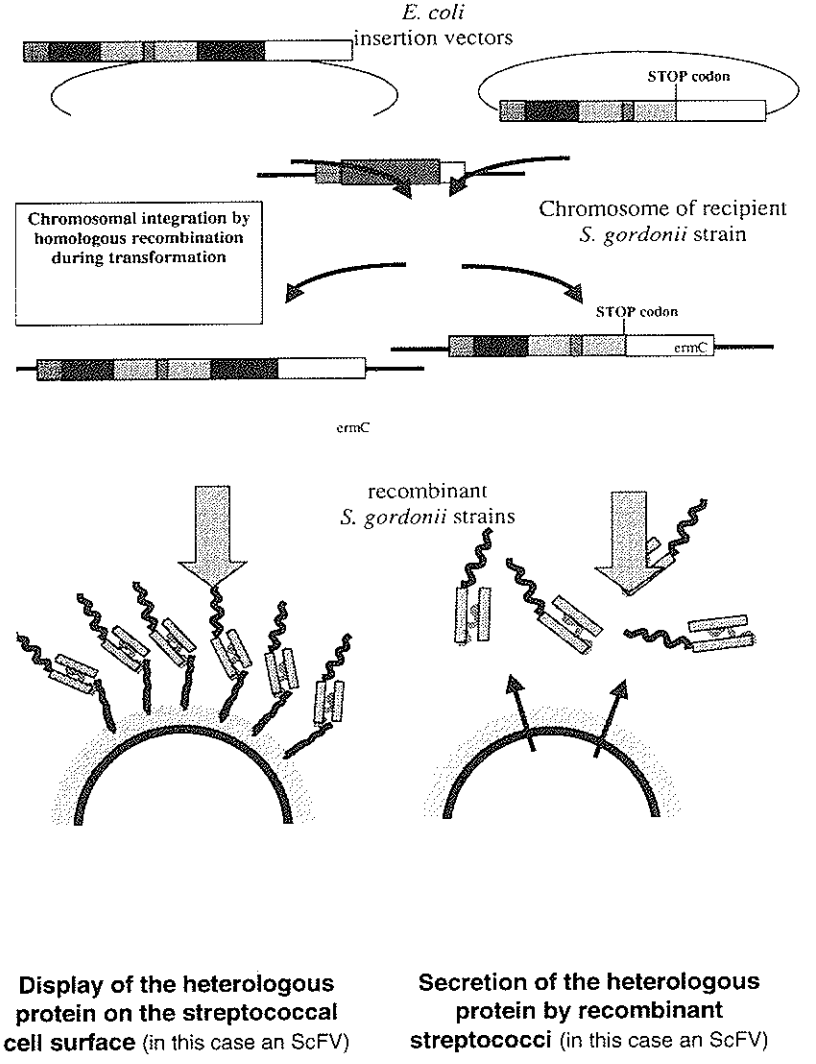


**Figure 7.1.** Schematic representation of the system for heterologous gene expression and protein production in *Streptococcus gordonii*. In the upper part, the two-step genetic system is shown. Heterologous DNA (the grey region represents the sequence of an scFv gene) is cloned in *E. coli* vectors in-frame into the gene of M6 protein (structural gene shown in black; the signal sequence represented by the striped area). When secretion of recombinant proteins is required, a stop codon is inserted downstream from the cloned DNA. Recombinant *E. coli* vectors are then transformed into specially engineered *S. gordonii* recipient strains, which permit integration by homologous recombination just downstream from a strong chromosomal promoter. Recombinant streptococci display fusion proteins on their surface or secrete the recombinant proteins, depending on the type of genetic constructs.

construct strains displaying multiple proteins, including pro-colonization proteins. A careful selection of chromosomal integration sites, which are permissive for good-level expression without altering 'visibly' the competitivity of recombinant strains, permitted the construction of bacteria expressing two heterologous genes from two independent chromosomal sites (Oggioni *et al.*, 1999a). Recently, it has been shown that the colonization potential of recombinant *S. gordonii* can be enhanced by expression of peptostreptococcal protein L (Ricci *et al.*, 2001). This capacity to modulate persistence of recombinant strains could be one of the approaches for the dosing of therapeutically active molecules produced by commensals.

An interesting point was raised by the commentary article to a paper appearing in *Nature Biotechnology* describing the microbicide secreting streptococci (Whaley and Zeitlin, 2000; see also Zeitlin *et al.*, 2002), which pointed to the fact that some of the success of the delivery of pharmaceutical substances by live bacteria could be due to the fact that commensal bacteria colonize the epithelium replicating within the mucous layer. Whaley and Zeitlin, who are working on mucosal delivery by tablet or gels (Zeitlin *et al.*, 2001, 2002), have underlined the fact that any pharmaceutical molecule that is produced *within* the mucus does not have to penetrate from outside this viscous substance, where putative pathogens (therapeutic targets) are also located.

## Conclusions

The use of transgenic commensal bacteria for the delivery of biologically active molecules as therapeutic agents opens an exciting and potential strategy, generally applicable for different purposes of therapy. The genes encoding molecules as antibodies and peptides can now be easily cloned and expressed in live vectors facilitating their mucosal delivery.

This approach re-evaluates and revitalizes interest in the well-known phenomenon of *microbial interference*, which is based on the competition amongst different microorganisms for essential nutrients or attachment sites on cell surfaces and their ability to produce metabolites, such as antibiotics, bacteriocins, and killer toxins, which allow some species to eliminate competitors (Chang, 1996). Until this recent work, the discovery of antibiotics and sulphonamides had somewhat diminished interest in the use of microbial interference as a therapeutic approach against different infectious diseases, but now it appears to have been re-established as a strategy for preventing mucosal colonization and entry of pathogenic microorganisms by this route.

Recombinant DNA technology has now made possible the engineering of different microorganisms living in symbiosis with humans and other animals, thus facilitating the local expression and delivery of recombinant bioactive products, so long as the colonization of mucosal surfaces remains. Interferon, hormones, microbicides, anti-microbial or anti-adhesive polypeptides (Kelly and Younson, 2000) and other therapeutic compounds, such as interleukins (Steidler *et al.*, 2000), could be produced by selecting the appropriate vector system and the bacterial host, depending on the surface to be colonized.

Major problems could be the amount of product that can be made locally and absorbed by the mucosa, and the persistence of the colonization. Recombinant *S.*

*gordonii* strains expressing a microbiocidal antibody in the single-chain format were demonstrated to be highly efficacious in killing *C. albicans in vitro* and in significantly accelerating the clearance of high fungus burdens in a rat vaginal candidiasis model persistently colonizing the animal vagina (Beninati *et al.*, 2000). In this case, absorption would not be a prerequisite for the recombinant product to inhibit the pathogen, so long as the colonization of the *S. gordonii* strains remained long enough so as to be therapeutic.

The mimicking of killer toxins or other natural antimicrobial molecules by the idiotypic network and the expression of the variable region of microbicidal antibodies or mimotopes on recombinant commensal bacteria could mirror the competition occurring among microorganisms in natural habitats.

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