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Recombinant *Streptococcus gordonii* for Mucosal Delivery of a scFv Microbicidal Antibody*

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The gram-positive bacterium *Streptococcus gordonii* was engineered to express the microbicidal molecule H6, which is an anti-idiotypic single chain antibody mimicking a yeast killer toxin. *S. gordonii* is a human commensal which we developed as a model system for mucosal delivery of heterologous proteins. The *in vivo* candidacidal activity of both H6-secreting and H6-surface-displaying streptococcal strains were assayed in a well-established rat model of vaginal candidiasis. At day 21 full clearance of *Candida albicans* infection was observed in 75% of animals treated with the H6-secreting strain, and in 37.5% of animals treated with the strain expressing H6 on the surface, while all animals treated with the control strain were still infected. The observed candidacidal effect was comparable with that observed with the antimycotic drug fluconazole. These data confirm the potential of H6 as a candidacidal agent and show how promising is the approach of using recombinant bacteria for mucosal delivery of biologically active molecules.

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INTRODUCTION

Recent advances in treatment of microbial infections determined changes with respect to disease quality and outcome, but had in some instances only minor incidence on transmission. Subclinical disease and asymptomatic carriage are thus still major problems in controlling spread of epidemics around the world. Therefore identification of antimicrobial drugs that prevent or reduce transmission is crucial for public health. This approach is not only important for life threatening diseases, but also for opportunistic infections which constitute a serious disease burden in terms of morbidity, mortality and cost of the cure. In this context it is of fundamental importance that most infectious agents use mucosal surfaces as portal of entry. Their presence at the level of the mucosa is also of prime importance for disease transmission. This fact has promoted active research both for vaccines and therapeutics with the intent to identify ways to promote local protection and treatment in order to block infectivity and transmission. A variety of approaches have been considered for local anti-infective therapy including anti-adhesion molecules [1,2] and anti-microbial peptides [3–9], but often short half lives of molecules demand for continuous administration.

A low cost solution for the problem was identified in a bacterial expression system exploiting the human commensal Gram-positive bacterium *Streptococcus gordonii* as vector of bioactive molecules. Recombinant *S. gordonii* strains were originally devised as live vaccine vectors for antigen delivery to mucosal surfaces [10–13]. Colonisation of the oral and vaginal mucosa with recombinant *S. gordonii* had been previously shown to induce local and systemic antibody responses against heterologous vaccine antigens, both in rodent and in mammal models [14–19]. It was shown that only live bacteria induced immunity when mucosal colonisation was used as route for antigen delivery [14,16]. This is underlined by the fact that recombinant strains were shown to actively produce the heterologous antigen during colonisation of the murine vagina [11,13,15,16].

Bearing in mind the potential advantage of local production of microbicidal proteins [20], the Gram-positive bacterium *S. gordonii* was engineered to express a *Candida*-cidal molecule [21]. *Candida albicans* is among the most prevalent causes of mucosal infections in humans. While oropharyngeal candidiasis is of particular concern in HIV-infected patients and patients receiving cancer therapies, vulvo-vaginal disease is estimated to occur three quarters of all otherwise healthy women of which about 5% develop difficult-to-treat recurrences of the disease with severe disturbance of the quality of their lives [22–25]. We particularly addressed the problem of treating *Candida* infection by the use of antiidiotypic antibodies mimicking the potent candidacidal action of a wide-spectrum killer toxin (KT) from *Pichia anomala* [26–28]. The H6 molecule used in our studies is a single chain fragment variable (scFv) anti-idiotypic antibody which mimics KT and was shown to be effective in the therapy of experimental vaginal infection by *C. albicans* [8,29]. Here we review the work done so far on recombinant *S. gordonii* strains secreting or displaying the H6 molecule on the surface.

EXPRESSION OF THE ANTIIDIOTIPIC MICROBICIDAL scFv IN *STREPTOCOCCUS*

Construction of Recombinant *Streptococcus gordonii*

For construction of recombinant *S. gordonii* expressing the candidacidal molecule H6 the host-vector system previously described was used [10,12,19]. This system is based on a two step procedure in which first translational fusions to the streptococcal surface protein M6 are constructed in *E. coli* vectors, than these recombinant vectors are transformed in *S. gordonii* where they integrate downstream a strong chromosomal promoter. The gene fusions include the signal sequence and parts of the N-terminal region of the M6 protein responsible for efficient export. When the C-terminal anchor sequence of M6 [30] is included in the construct, the recombinant protein will be anchored to the cell surface (Fig. 1; left panel), while when the anchoring sequence is omitted the protein will be secreted (Fig. 1; right panel) [31]. Using this system a wide variety of antigens, ranging in size from

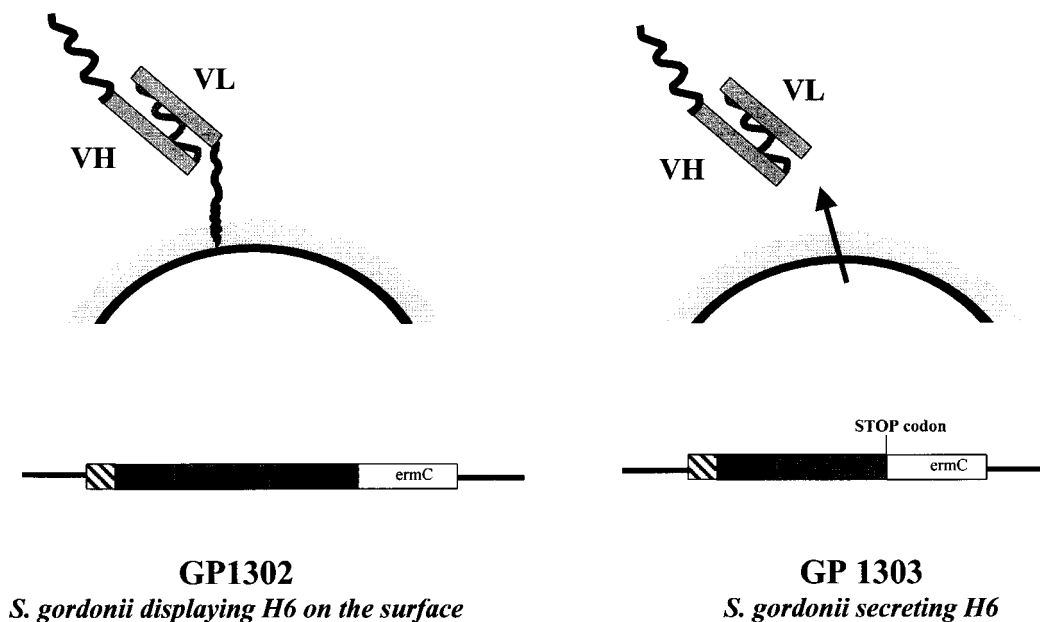


FIGURE 1 Schematic representation of the chromosomal structure of the two recombinant *S. gordonii* strains GP1302 and GP1303 (left and right of the chart respectively). In the upper part the proteins are shown schematically with their respective location towards the bacterial cell surface. The structure of the chromosomal region carrying the recombinant constructs (shown below) comprises, downstream a strong chromosomal promoter, the signal sequence of M6 protein gene (striped) (123 bas pairs/41 amino acids), portions of the mature M6 protein gene (black) (366 bp/122 AA for N-terminus; 420 bp/140 AA for C-terminal anchor part of the molecule) and the DNA coding for the H6 scFv (grey) (702 bp/234 AA). The erythromycin marker associated to the construct is shown in white.

15 to over 400 amino acids, have been displayed on the cell surface [10,14,20,21,32-35].

Expression of the Microbicidal Antibody H6

We previously described antiidiotypes which could reproduce the activity of a KT from the yeast *Pichia anomala*, including its ability to kill *C. albicans*, *Pneumocystis carinii* and multidrug-resistant strains of *Mycobacterium tuberculosis* [5,8,9,28,36]. The anti-*Candida* active polypeptide selected for expression in streptococcus was the single chain fragment antiidiotypic antibody H6 (scFv antiId), which was selected by immunisation with an anti-KT monoclonal antibody KT4. H6 was previously shown to have candidacidal activity *in vitro* and *in vivo* [8]. Two recombinant *S. gordonii* strains expressing the H6 scFv were constructed [21]: one, named GP1302, in which H6 is integrated in frame into streptococcal M6 protein leading to a surface displayed

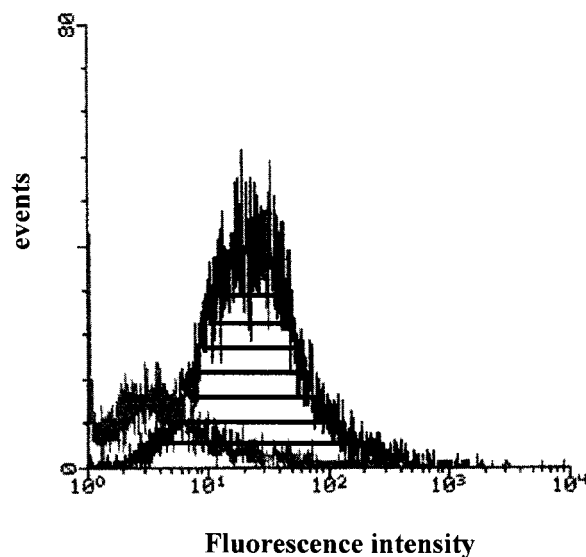


FIGURE 2 Flow cytometric analysis of recombinant *S. gordonii* GP1302 displaying the M6-H6 fusion protein on the surface. Bacterial cells were reacted with anti-M6 rabbit serum and with FITC-conjugate anti-rabbit IgG. The fluorescence peak of recombinant strain GP1302 (striped histogram) is shifted to the right when compared with the wild type control strain GP204 (grey bordered histogram).

fusion protein (Fig. 1; left panel); the second strain instead, named GP1303, was devised as to produce a secreted fusion protein by introduction of a stop codon downstream the scFv (Fig. 1; right panel), which therefore excludes the C-terminal domain of M6 necessary for surface anchoring [30,31]. Protein production and localisation was confirmed on all strains by Western blot and dot blot of bacterial cell fractions and supernatants [21]. The presence of M6-H6 fusion protein on the surface of recombinant *S. gordonii* GP1302 was also verified by flow cytometric analysis of whole cells treated with anti-M6 polyclonal serum. (Fig. 2). A shift to the right was observed in the fluorescence intensity of recombinant *S. gordonii* compared to the control wild type strain (Fig. 2). Eighty-one percent of the cells of strain GP1302 were positive for the expression of M6-H6 fusion protein.

VAGINAL COLONISATION WITH RECOMBINANT *S. GORDONII* IN RODENTS

Mucosal Colonisation in the Murine Model

We have previously exploited the approach of using recombinant streptococci as live vectors for the induction of a local immune response in the genital tract. For that purpose we have developed animal models of vaginal immunisation both in mice and in monkeys [13,15–17] using recombinant strains expressing a variety of antigens. *S. gordonii* resulted capable of colonising very efficiently the vaginal mucosa of mice for 8 weeks after a single inoculum. Recombinant and wild type *S. gordonii* were found to be equally capable of colonising the murine vagina, in agreement with what observed for oral colonisation in mice [14,18]. Vaginal colonisation was found to be independent from the dose of the inoculum in a range included between 10^6 and 10^9 CFU [16]. The concentration of *S. gordonii* in positive samples was always high (between 10^3 and 10^6 CFU/sample) indicating an efficient colonisation [15,16]. Recombinant bacteria expressing the heterologous antigen were observed by immunofluorescence in the vaginal smears collected during all the period of colonisation, indicating a stable expression of the heterologous antigen *in vivo*. Bacteria

appeared to be adhering to the superficial epithelial cells and also trapped in the mucous strands [11,13,15,16].

Rat Model for Vaginal Colonisation

We have also studied vaginal colonisation of *S. gordonii* in the rat model, which is the model of choice for vaginal candidiasis. As for the murine model also the rat model was based on induction of pseudo-estrus, a prerequisite for efficient microbial colonisation [15,16,37]. While mice received estrogen only before colonisation the rats were oophorectomized and maintained in pseudoestrus with estradiol throughout the study. Colonisation experiments were carried out inoculating 10^9 bacteria/rat at day 0, 1 and 2 [21]. Colonisation in rats by recombinant streptococci gave counts (between 10^4 and 10^6 CFU/sample) comparable to those encountered in the murine model and persistence of the bacteria was followed for 2 weeks after the last inoculum without noticing any significant decrease in bacterial load (Fig. 3), irrespective to the streptococcal strain used in the experiments.

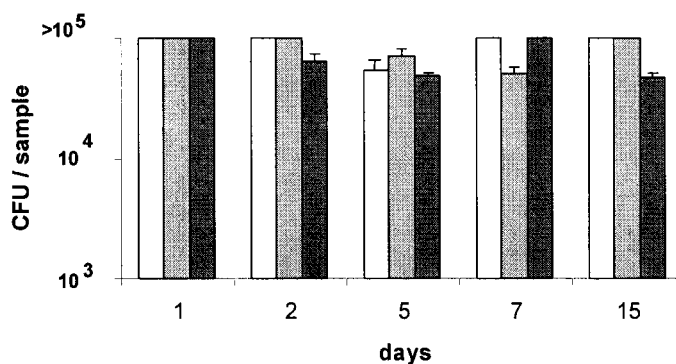


FIGURE 3 Colonisation of the rat vagina by *S. gordonii*. Streptococcal strains, grown to early stationary phase were collected and 10^9 bacterial cells were inoculated to each rat at 1, 24 and 48 hours. Bacterial colonisation was assayed for a 15 days period by swabbing the vaginal mucosa of rats and plating on blood agar (streptomycin 500 mg/l). Bacterial load was stable over the 15 day period assayed irrespective of the heterologous antigen expressed. The single columns refer to different streptococcal recombinants: GP1301 (expressing irrelevant scFv; white) [43], GP1302 (surface display of H6; light grey), and GP1303 (secretion of H6; dark grey). Two out of eight mice were negative for GP1303 at day 15 and were not included in the calculation.

FUNGICIDAL ACTIVITY OF RECOMBINANT *S. GORDONII* EXPRESSING H6

In Vitro Candidacidal Activity

To test, if H6 produced by recombinant streptococci, both on the surface and in the secreted form, maintained its candidacidal activity we used *in vitro* candidacidal assays previously described [21]. Whole streptococcal cells displaying H6 on the surface or concentrated supernatants of the secreting strain were directly incubated with *C. albicans*, to assay for their candidacidal activity. Figure 4 shows that co-incubation with *S. gordonii* GP1302, but not with the wild type strain GP204, resulted in a marked killing of *C. albicans* cells. These effects were dose-dependent over a wide range of bacteria to yeast

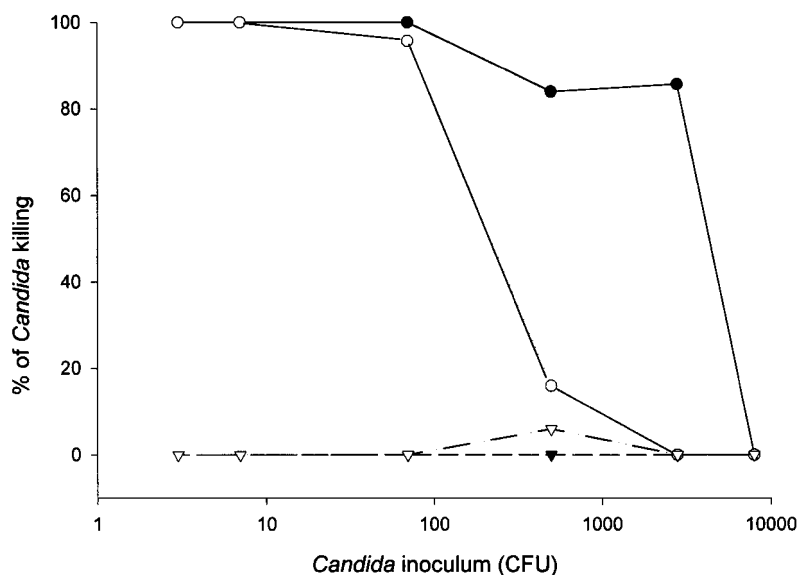


FIGURE 4 Killing of *C. albicans* *in vitro* by *S. gordonii* GP1302 displaying H6 on the surface. *Candida* cells, in which germ tube formation was induced, were co-incubated in PBS with recombinant *S. gordonii* at reciprocal dilutions. Experimental outcome was assayed by determining *C. albicans* counts at 18 hours. Two different streptococcal inocula are shown in the graph for both the recombinant strain GP1302 (solid circle 10⁸ and open circle 10⁶ cells) and for the wild type control strain GP204 (solid triangle 10⁸ and open triangle 10⁶ cells).

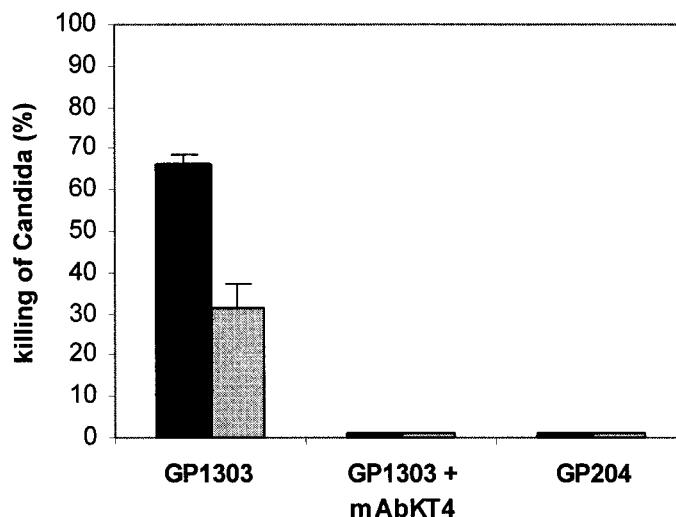


FIGURE 5 Neutralising activity of the monoclonal antibody KT4, to which the antiidiotype H6 was generated, on the killing activity of supernatants of *S. gordonii* GP1303 secreting the H6 molecule. Briefly, *C. albicans* germinating cells (3.5×10^2 in $10 \mu\text{l}$ of PBS) were added to $90 \mu\text{l}$ of the concentrated supernatants ($100 \times$ black bars; $50 \times$ grey bars) and incubated for 6 hours at 37°C . The same number of *Candida* cells was also added to $90 \mu\text{l}$ of the same concentrated supernatants preadsorbed overnight at 4°C with $100 \mu\text{g}$ of the KT-neutralising mAb KT4 [28]. Supernatants of wild type *S. gordonii* GP204 served as control. The outcome of the experiments was determined by a standard CFU assay.

ratios. But most importantly anti-candidal activity of the supernatant of the scFv H6-secreting strain (Fig. 5) was neutralised by the anti-KT mAb KT4 thus demonstrating that the recombinant bacteria indeed expressed a KT-mimicking antibody fragment and that the anti-candidal activity was mediated by this molecule.

Clearing of Mucosal Candidiasis in the Rat Model

Experiments aimed to evaluate *in vivo* the therapeutic potential of recombinant bacteria secreting the microbicidal molecule H6 were carried out in a rat model of vaginal candidiasis. This model has a strong degree of similarity to human infection due to its estrogen-dependence, type of invasive (hyphal) *Candida* growth in loco, and vaginal T lymphocytes assets [37]. As such, it has been adopted as standard to test the preclinical activity of antimycotic and

immunotherapeutic agents [38,39,40]. Moreover, experiments with the H6 molecule alone have been carried out successfully before in this model. Technically the model is based on oophorectomized female Wistar rats, maintained in pseudoestrus with estradiol, and which are inoculated intravaginally with 10^7 *C. albicans* cells (strain SA-40) [37].

The *in vivo* data are reported in Fig. 6, which shows in panel A the disease outcome while CFU counts are reported in panel B. The data clearly demonstrate that the two recombinant *S. gordonii* strains accelerated the clearance from the vagina of a high fungus burden. Importantly the therapeutic effect of colonisation by the scFv H6-secreting streptococcal recombinant did compare substantially to that of a full therapeutic course of fluconazole (100 µg at 1, 24 and 48 hours), in particular in its early (1 day) decay component (Fig. 6; panel B), a period when *C. albicans* develops invasive hyphal elements and secretes virulence enzymes [37]. This effect was not observed with the scFv H6 displayed on the bacterial cell surface whose therapeutic activity was clearly minor and occurred after the delivery of three bacterial doses only (Fig. 6). This difference was unlikely to be due to any difference in the rate and extent of vaginal colonisation, which was similar in the two strains, nor to any intrinsic difference of action, as witnessed by the similarity of their candidacidal effects *in vitro*. The activity of the secreted molecule is probably favoured *in vivo* due to a facilitated interaction with *C. albicans* cells owing to the bioavailability of the soluble product. Rats colonised with the strain expressing an unrelated scFv did, as expected, compare to control animals which did not receive any treatment (Fig. 6; panel B).

CONCLUDING REMARKS

The delivery of biological active molecules to the human host is an important problem to which a variety of solutions have been proposed. While most rely on repeated administrations (injections, tablets, capsules, etc.), only few systems have been developed for continuous release. In the field of local anti-infective therapy a variety of approaches have been considered [1,2,3,6,41,42], but as to now no continuous system for delivering these molecules to the patient has yet been devised. The therapeutic problems faced include thus still the

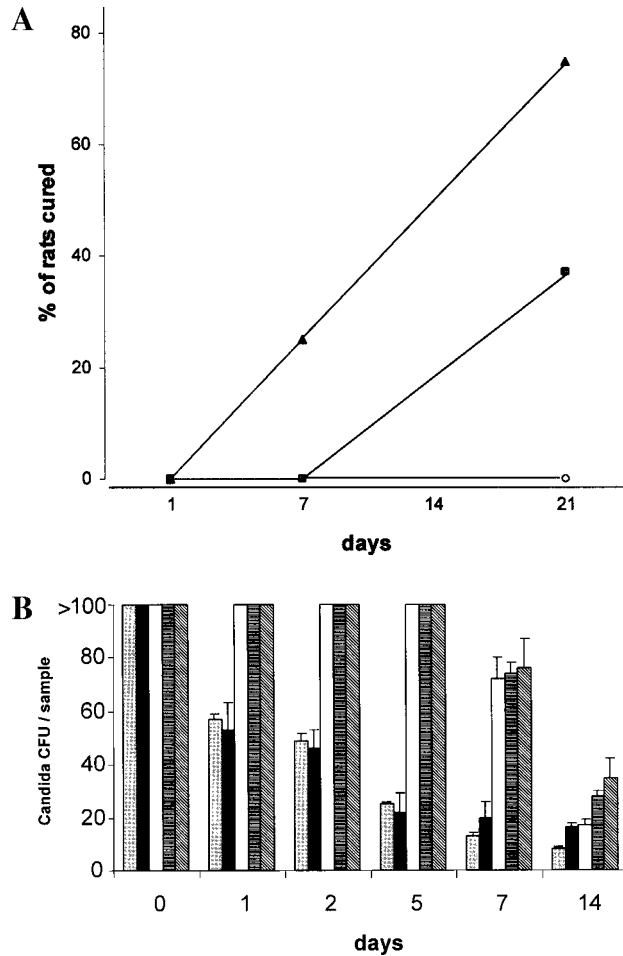


FIGURE 6 Outcome of vaginal candidiasis of rats (*Candida albicans* infection at day 0) colonised with *S. gordonii* recombinant strains (10^9 cells). Panel A shows cumulative data of two experiments. Eight mice were colonised for each group with *S. gordonii* GP1303 (secreted H6; filled triangles), GP1302 (surface displayed H6; squares), and GP1301 (expressing unrelated scFv; circles). The graph reports the outcome of the experiment where the cure of infection was determined by a drop of *Candida* counts under 10^3 CFU/ml of sample [37]. Panel B reports *C. albicans* counts per sample in an experiment considering 5 mice per group. In this experiment also a group treated with fluconazole ($100 \mu\text{g}$ at 1, 24 and 48 hours; grey bars) and untreated control group (diagonally striped bars) in addition to groups of mice colonised with the streptococcal recombinants GP1303 (secreted H6; black bars), GP1302 (surface displayed H6; white bars), and GP1301 (unrelated scFv; horizontally striped bars) [43] on days 0 (1 hour post infection), 1, and 2 post infection.

bio-availability, the precise targeting and the often short half live of the microbicidal molecules.

The work reviewed in this article describes a novel approach based on production of a microbicidal agent by live recombinant commensal bacteria [21,35]. This continuous production on the mucosal surfaces of the host of the microbicidal antibody H6 by *S. gordonii* proved to cure rats from vaginal infection by *C. albicans* as efficiently as a full course of antimycotical therapy by the drug fluconazole [21]. This approach thus demonstrated to be effective and to be a valid alternative to repeated inocula of purified molecules especially since it does circumvent the problem of the short half-life of most polypeptides. The local production of biological active molecules by recombinant bacteria may have a wide variety of applications, not necessarily linked only to anti-infective therapy.

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