

Therapy of mucosal candidiasis by expression of an anti-idiotypic in human commensal bacteria

Concetta Beninati^{1,2}, Marco Rinaldo Oggioni¹, Maria Boccanera³, Maria Rita Spinosa¹, Tiziana Maggi¹, Stefania Conti⁴, Walter Magliani⁴, Flavia De Bernardis³, Giuseppe Teti², Antonio Cassone³, Gianni Pozzi¹, Luciano Polonelli^{4*}

¹Dipartimento di Biologia Molecolare/Microbiologia, Università degli Studi di Siena, Siena, Italy. ²Istituto di Microbiologia, Università degli Studi di Messina, Messina, Italy. ³Laboratorio di Batteriologia e Micologia Medica, Istituto Superiore di Sanità, Roma, Italy. ⁴Dipartimento di Patologia e Medicina di Laboratorio, Sezione di Microbiologia, Università degli Studi di Parma, Parma, Italy. *Corresponding author: (lucpol@unipr.it).

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Two recombinant strains of *Streptococcus gordonii*, secreting or displaying a microbicidal single-chain antibody (H6), and stably colonizing rat vagina, were used to treat an experimental vaginitis caused by *Candida albicans*. A post-challenge intravaginal delivery of the H6-secreting strain was as efficacious as fluconazole in rapidly abating the fungal burden. Three weeks after challenge, 75% and 37.5% of the rats treated with the H6-secreting or displaying bacteria, respectively, were cured of the infection, which persisted in 100% of the animals treated with a *S. gordonii* strain expressing an irrelevant single-chain antibody. Thus, a human commensal bacterium can be suitably engineered to locally release a therapeutic antibody fragment.

Keywords: engineered commensal bacteria, mucosal colonization, microbicidal scFv delivery, vaginal candidiasis, therapeutic treatment

Identification of antimicrobial drugs that prevent or reduce pathogen transmission is crucial in the control of infectious diseases. Mucosal surfaces are not only portals of entry of microbial invaders but also the prime sites for their transmission. Therefore, efficient topical microbicides^{1,2} capable of clearing infectious microorganisms from the mucosa would have a fundamental impact for the control of these organisms. This approach is important not only for major life-threatening diseases but also for opportunistic infections, which presently constitute a serious and increasing burden in terms of morbidity, mortality, and cost of cure. In this context *Candida albicans* is among the most prevalent causes of mucosal infections in humans. It is of special concern not only in immunocompromised patients³ such as HIV-infected subjects, who are frequently affected by recurrent forms of oral and esophageal candidiasis, but also in noninfected subjects, as is the case of acute vaginitis in otherwise healthy women⁴.

Among novel immunotherapeutic approaches to mucosal infections, anti-idiotypic antibodies, in the single-chain format (scFv), that mimic the potent microbicidal action of a wide-spectrum killer toxin (KT) from *Pichia anomala*, have been investigated⁵⁻⁸. One of these antibodies (scFvH6) was shown to effectively kill in vitro pathogenic prokaryotic and eukaryotic microorganisms and demonstrated significant therapeutic effects in an in vivo model of rat vaginal candidiasis^{9,10}.

Bearing in mind the potential advantage of local production of microbicidal antibodies by colonization with vaginal bacterial commensals, we have now engineered the human commensal *Streptococcus gordonii* for which vaginal colonization and effective in vivo expression of heterologous antigens have been extensively described¹¹⁻¹³. ScFvH6-expressing, recombinant *S. gordonii* strains were assayed for their therapeutic activity using the KT-sensitive mucosal pathogen *C. albicans* as a model organism.

Results

Candidacidal KT-mimicking scFvH6 anti-idiotypic antibodies were produced by recombinant DNA technology from the splenic lymphocytes of mice immunized by idiotype vaccination with a KT-neutralizing monoclonal antibody (mAbKT4)^{9,14}.

ScFv were expressed as fusion proteins with the streptococcal protein M6 using the genetic system for expression of heterologous proteins in *S. gordonii*¹⁵. Three recombinant *S. gordonii* strains were constructed and used in this study: (1) strain GP1302, expressing scFvH6 on the cell surface (2) strain GP1303, expressing scFvH6 as a secreted protein, and (3) strain GP1301, expressing an irrelevant scFv (ref. 16), to be used as a control. Proteins expressed by recombinant strains were analyzed by western blot. ScFvH6 surface-displaying GP1302 strain presents two bands on blots of whole-cell lysates (Fig. 1A, lane 2) and only negligible traces in the culture supernatant (Fig. 1B, lane 2). The lower band (58 kDa) corresponds to the mature protein (arrow, Fig. 1A), whereas the upper band (63 kDa) should represent an unprocessed form. It should be pointed out that surface proteins of Gram-positive bacteria are processed at both the N and C termini¹⁷. A 47 kDa band was detected in both whole-cell lysates (Fig. 1A, lane 3) and culture supernatants (Fig. 1B, lane 3) of the H6-secreting strain GP1303. The M6-H6 fusion protein is present also in whole-cell lysates of the secreting strain GP1303, in that secreted proteins are found attached to membrane-cell wall complexes of Gram-positive bacteria. As expected from previous data, the molecular size of M6-based fusion proteins estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) appears larger than that deduced from DNA sequence¹⁸⁻²⁰.

In vitro candidacidal activity. To determine whether the bacterially expressed scFvH6 retained its biological activity, the candidacidal

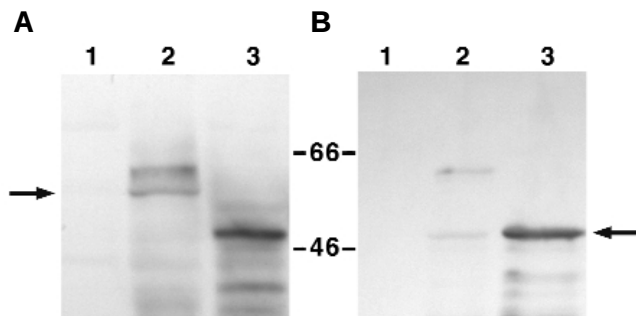


Figure 1. Western blot analysis of recombinant *S. gordonii* strains expressing M6-H6 fusion proteins. (A) Whole-cell lysates (2×10^8 cell-equivalents per lane). (B) Culture supernatants (1×10^9 cell-equivalents per lane). Lane 1, wild-type control strain *S. gordonii* GP204; lane 2, recombinant strain GP1302 expressing the M6-H6 molecule on the surface; lane 3, recombinant strain GP1303 secreting the M6-H6 fusion protein. Blots were developed with a M6-specific polyclonal rabbit serum²⁰. Molecular weight markers are shown between panels (kDa). Arrows indicate the mature form of recombinant proteins.

properties of whole bacteria and bacterial supernatants were investigated. Figure 2 shows that co-incubation with *S. gordonii* GP1302, but not with the parental strain GP204 (ref. 18), resulted in a marked killing of yeast cells. These effects were dose-dependent over a wide range of bacteria:yeast ratios. The presence of the scFvH6 molecule in the supernatant from the secreting strain GP1303 was confirmed by a dot-blot assay carried out with mAbKT4 (data not shown). The GP1303 supernatant had a clear candidacidal effect, as demonstrated by a colony-forming unit (CFU) assay, with a percentage of decrease in colony number with respect to control (GP204 supernatant) of 66.57 ± 4.84 (100-fold concentrated supernatant) and 31.68 ± 1.56 (50-fold concentrated supernatant). This killing activity was totally abolished (no decrease in colony-forming units) by pre-incubation of the supernatants with mAbKT4 (data not shown).

Therapy of experimental vaginal candidiasis. ScFvH6-expressing *S. gordonii* strains were tested for their therapeutic activity in a rat vaginal candidiasis model. In this experiment (Fig. 3), a positive control with the antifungal drug fluconazole was added. The animals receiving the scFvH6-expressing bacteria cleared the infection much more rapidly than the two negative control groups (animals untreated or treated with GP1301 strain). There were, however, significant differences between the scFvH6-secreting and surface-displaying *S. gordonii* recombinants. The animals treated with the scFvH6-secreting strain showed significantly fewer fungal colony-forming units than the controls, starting on the first day after *C. albicans* challenge, that is, after the first dose of bacterial cells. In contrast, the animals administered scFvH6 surface-displaying bacteria began to clear the infection only at one week post *C. albicans* challenge, that is, four days after being given the last of the three doses of *S. gordonii*. Nonetheless, on day 14 post-challenge, the two groups of rats had comparable yeast vaginal counts, both significantly lower than the control rats.

Figure 3 also shows that the therapeutic effect of the scFvH6-secreting recombinant was comparable to that of a full therapeutic course of fluconazole, in particular in the earliest phase of treatment. It is noteworthy that both fluconazole and GP1303 treatments caused a drop in yeast vaginal counts (from

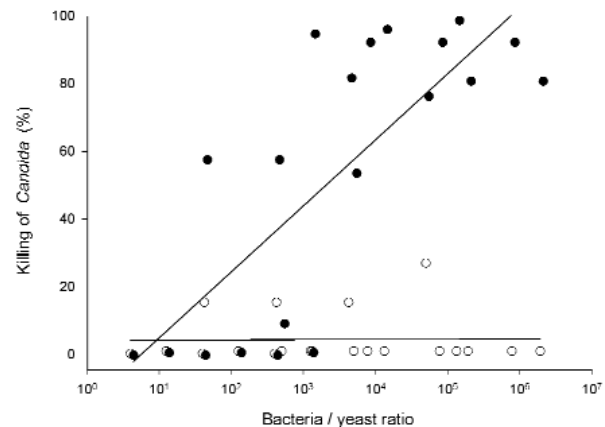


Figure 2. In vitro killing of *Candida albicans* by *Streptococcus gordonii* displaying scFvH6 on the surface. The graph indicates experiments performed with recombinant *S. gordonii* GP1302 displaying scFvH6 on the surface (●) and wild-type control strain GP204 (○). *Candida albicans*, in which germ tube formation had been induced, was co-incubated in PBS with recombinant *S. gordonii*. Bacteria:yeast ratios ranged from 4:1 to 2×10^6 :1 (reciprocal dilutions: 1/3 for yeasts; 1/10 for bacteria). The experimental outcome was assayed by determining *C. albicans* counts after 18 h of incubation. Nonlinear regression was calculated using standard parameters of Sigma Plot 4.01.

$>10^5$ to 20×10^3) in the first four days of infection, a period when *C. albicans* develops invasive hyphal elements and secretes virulence enzymes²¹.

Quite similar results were observed in a second experiment. Table 1 shows the number of infected rats versus total rats challenged with *C. albicans* on days 1, 7, and 21 post-treatment in both experiments. A cumulative assessment of all the data clearly shows that the treatment with scFvH6-expressing *S. gordonii* accelerated the elimination of the fungus from the vagina in about 21 days, whereas all the control animals were still infected. The therapeutic effect was more pronounced (and statistically significant) with the use of the scFvH6-secreting bacteria.

The vaginal fluids of rats colonized with strain GP1303 reacted with mAbKT4 in the appropriate dot-blot assay and showed killer activity against *C. albicans* cells in the CFU assay. In contrast, neither antigenic recognition nor candidacidal activity were shown by the vaginal fluids of rats treated with the control GP1301 strain (data not shown).

The extent of vaginal colonization by recombinant *S. gordonii* strains was also measured following administration of a second bacterial dose. No gross differences among the three strains were noted in their vaginal colony-forming units for as long as five days after the third inoculated bacterial dose. Bacteria were still present, in appreciable numbers (around 5×10^4 or more CFU) in the majority (strain 1303) or in all (the other two strains of *S. gordonii*) rats up to 15 days post inoculation (Table 2).

Table 1. Resolution of vaginal candidiasis of rats intravaginally administered with *S. gordonii* recombinant strains

Strain	scFv expressed	No. of rats infected ^a / total rats challenged with <i>C. albicans</i>					
		Experiment 1			Experiment 2		
		Day 1	Day 7	Day 21	Day 1	Day 7	Day 21
GP1301	Irrelevant	5/5	5/5	5/5	3/3	3/3	3/3
GP1302	H6, cell surface	5/5	5/5	3/5	3/3	3/3	2/3
GP1303	H6, secreted	5/5	4/5	2/5	3/3	2/3	0/3

^aA rat was defined as infected when at least one *C. albicans* colony-forming unit was present in 1 μ l of vaginal fluid ($>10^3$ CFU/ml). When the data from the two experiments were cumulated, there was a highly significant statistical difference ($P = 0.007$, Fisher's exact test, two-tailed) between the candidal colony-forming units of the rats treated with GP1303 and those treated with GP1301 *S. gordonii* strains.

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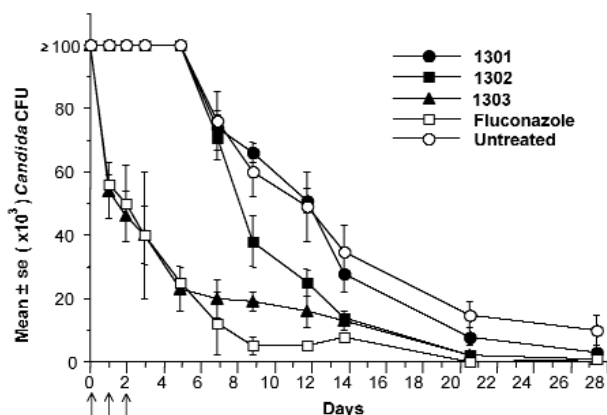


Figure 3. Outcome of vaginal infection in rats infected with *Candida albicans* (day 0) and untreated or treated with *Streptococcus gordonii* strains (10^9 cells in 0.1 ml of each strain) or fluconazole (100 μ g) as indicated, on days 0 (1 h post infection), 1, and 2 post infection (arrows). Five rats per group were used. Starting day 1 post infection there was always a statistically significant difference ($P < 0.05$, Mann-Whitney U-test, two-tailed) between the vaginal *C. albicans* colony-forming units (CFU) of the animals treated with *S. gordonii* strain 1301–treated rats. A statistically significant difference was also present between the vaginal fungal colony-forming units of the animals receiving *S. gordonii* strain 1302 and the control rats, starting on day 9 post infection. The difference between strain 1303 (or fluconazole) and strain 1302 was significant until day 9. The results are from a representative experiment out of two performed with similar results (no fluconazole-treated group in the second experiment).

Discussion

A variety of approaches have been considered for local anti-infective therapy including anti-adhesion molecules and anti-microbial peptides^{9,22–26}. However, the short half-life of these molecules demands continuous administration and raises problems of bioavailability, safety, and cost of cure. This work demonstrates that local production of a microbicide by a recombinant colonizing commensal bacterium is effective against mucosal infection by a widespread, opportunistic mucosal pathogen.

We previously described anti-idiotypic antibodies that could reproduce the activity of a KT from the yeast *P. anomala*, including its ability to kill *C. albicans*, *Pneumocystis carinii*, and multidrug-resistant strains of *Mycobacterium tuberculosis*^{10,27,28}. We also constructed a recombinant anti-idiotypic scFvH6 that, through its ability to mimic KT candidacidal activity, exerted a therapeutic activity against *C. albicans* rat vaginal infection⁹.

One of the problems connected with in vivo use of the scFvH6 antibody is the need for continuous administration of a potentially costly product. Therefore, the steady mucosal delivery of a microbicidal antibody permanently expressed by a colonizing commensal bacterium was judged of prime importance for a prospective therapeutic intervention. Toward that end we used *S. gordonii*, a naturally transformable commensal bacterium, which was engineered to express heterologous proteins. This delivery system, based on a

live recombinant microorganism, was chosen since *S. gordonii* is able to express heterologous proteins efficiently during vaginal colonization, and deliver candidate vaccines to a mammalian host, for oral, intestinal, and vaginal immunization^{11,13,20,29}.

To prove the concept of potential therapeutic benefits of the recombinant microbicidal scFvH6 antibody, we selected *C. albicans* as the target pathogen. This fungus is one of the most frequently involved microbes in the etiology of mucosal infections; in particular, it is the principal agent of vulvovaginal candidiasis in both its acute and chronic, recurrent forms³⁰. It is also the most common agent of all other forms of mucosal candidiasis, such as, the oral thrush of HIV-infected patients^{31,32}. Overall, the therapy of mucosal infections by *Candida* spp. is an area in need of progress because of the relative paucity and toxicity of truly active therapeutic agents, the increased resistance to some of the best tolerated and active antifungals, such as the triazole compounds, and the lack of valid and specific preventive measures, such as vaccines^{5,33}.

The in vitro assays in this study showed that both scFvH6 surface-displaying or secreting *S. gordonii* strains were highly efficacious in killing *C. albicans* with a clear dose response. The anti-candidal activity of the supernatant of the scFvH6-secreting strain increased at higher concentration and was neutralized by the anti-KT mAbKT4, thus demonstrating that the recombinant bacterial construct indeed expressed a KT-mimicking antibody fragment and that the anti-candidal activity was mediated by this molecule.

To test this functional activity in vivo, we selected an experimental model of rat vaginal candidiasis that among its advantages includes the requirement of persistent bacterial colonization, a prerequisite for proving our therapeutic concept. Effective *S. gordonii* vaginal colonization has been previously demonstrated in another rodent model^{11,12}. Moreover, the rat vaginal candidiasis model has, in its estrogen dependence, its characteristic invasive (hyphal) *Candida* growth in loco, and vaginal T-lymphocytes assets, a strong degree of similarity to human infection²¹. As such, it has long been adopted to test the preclinical activity of antimycotic and immunotherapeutic agents^{6,34,35}.

Our in vivo data proved that *S. gordonii* was capable of persistently colonizing the rat vagina, and also demonstrated that the two recombinant *S. gordonii* strains accelerated the clearance from the vagina of a high fungus burden. This therapeutic benefit was associated with the expression of the candidacidal scFvH6 molecule and was not observed with recombinant bacteria expressing an irrelevant scFv. Importantly, however, the scFvH6 secretory strain caused an early, rapid drop of the fungal infection after only a single therapeutic dose. This effect was not observed with the scFvH6 displayed on the bacterial cell surface, with which therapeutic activity was clearly minor and occurred after the delivery of three bacterial doses. This difference was unlikely to be due to any difference in the rate and extent of intravaginal colonization, 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. Thus the activity of the secreted molecule may be favored in vivo, perhaps because of a facilitated interaction with *C. albicans* cells resulting from the bioavailability of a soluble product. Importantly, the strain of *S. gordonii* expressing an

irrelevant scFv, though equally capable of colonizing the rat vagina, was totally ineffective as a therapeutic agent. This rules out the possibility that the observed beneficial effects were due to vaginal colonization per se.

Two aspects of our approach have not been demonstrated previously. The first is the capacity of *S. gordonii* to efficiently colonize animal mucosa and there secrete a recombinant antibody fragment in a bioactive form. The second is the nature of

Table 2. Vaginal colonization by *S. gordonii* recombinant strains

Strain	scFv expressed	<i>S. gordonii</i> (CFU $\times 10^3$) on days post delivery				
		1	2	5	7	15
GP1301	Irrelevant	>100	>100	55 $\pm$ 13	>100	>100
GP1302	H6, cell surface	>100	>100	72 $\pm$ 9	50 $\pm$ 6	>100
GP1303	H6, secreted	>100	65 $\pm$ 12	49 $\pm$ 3	>100	48 $\pm$ 5 ^a

^aTwo rats were negative for *S. gordonii* (they were not included in the enumeration of colony-forming units). In all other cases, all five rats treated were positive for *S. gordonii*, and the measured colony-forming units are the mean $\pm$ s.d. The only statistically significant differences in the CFU enumeration were between GP1303 and the other two strains on days 2 and 15, as well as between GP1302 and the other two strains on day 7 ($P < 0.05$, Student's *t*-test, two-tailed).

the scFv delivered to the mucosa: a molecule that mimics a killer toxin and that has been demonstrated to exert microbicidal activity in a wide spectrum of mucosal and nonmucosal pathogens of both prokaryotic and eukaryotic phylogeny such as *C. albicans*, *P. carinii*, and *M. tuberculosis*. The recombinant antibody, moreover, mimics the functional activities of protective microbicidal antibodies naturally produced during candidal infections in humans^{7,36}.

The development of the scFvH6 candidacidal molecule, along with other anti-infective polypeptides (protegrin, cyanovirin, and others), may open up new avenues for the prevention and therapy of mucosal diseases; however, there is still a need for effective delivery systems^{1,2,25,37}. The in vivo production by human commensal bacteria may circumvent the problem of the short half-life of polypeptide-based microbicides and thus warrants further investigation.

Experimental protocol

Construction of recombinant *S. gordonii* strains. ScFvH6, which mimics the biological activity of *P. anomala* KT, has been described⁹.

The scFvH6-encoding DNA fragment was amplified by PCR using the oligonucleotides 5'-GAAGATCTATGGCCAGGTGAAGCT-3' (upstream primer, containing a restriction site for *Bgl*II) and 5'-GGAATTCACGTTTGAATTCCAGCTTG-3' (downstream primer, containing a restriction site for *Eco*RI).

A second construct was amplified from the same plasmid using similar primers, but with the introduction of a stop codon in the downstream primer corresponding to the end of the scFvH6 coding sequence (5'-GGAATTCACGTTAGATTTCAGCTTG-3').

Recombinant *S. gordonii* strains were constructed by using a specially engineered recipient strain GP1221 as described^{15,19}. Two recombinant plasmids were obtained; one with scFvH6 inserted in a frame within M6 and one where scFvH6 sequences were preceded only by the 5' part of M6 because of the stop codon inserted by PCR. These plasmids were then transferred into *S. gordonii* GP1221 and transformants were selected as described elsewhere^{15,18}. The recombinant strain displaying scFvH6 on its surface was designated GP1302, and the strain secreting scFvH6 was designated GP1303. Both strains have been transformed for streptomycin resistance (SmR) to allow efficient recovery after intravaginal delivery^{11,29}. The same protocol was adopted to construct a recombinant *S. gordonii* strain secreting an irrelevant scFv (ref. 16), which was designated GP1301.

To verify the presence of heterologous constructs in scFv transformant *S. gordonii* strains, a western blot analysis using polyclonal rabbit anti-M6 antibodies was performed. Bacterial whole-cell lysates were obtained by lysozyme and SDS treatment, whereas culture supernatants were prepared by precipitation in eight volumes of ethanol followed by two washes in 70% ethanol. The subsequent analysis was performed as described¹⁹.

In vitro candidacidal assays. *Candida albicans* ATCC 90028 was grown in Sabouraud broth (DAKO, Milan, Italy) at 37°C to an optical density of 0.9 (540 nm, 1.6 cm). Yeast cells were washed in phosphate-buffered saline (PBS, pH 7.3; 0.15 M NaCl, 0.01 M phosphate) and incubated at 37°C for 1 h in 1 ml of heat-inactivated (56°C, 30 min) fetal calf serum to induce germ tube formation. The yeast cells were then washed again twice in PBS, and resuspended (3×10^5 CFU/ml). Bacterial suspensions were prepared by growing streptococcal cells in tryptic soy broth without dextrose (TSB w/o; Difco, Detroit, MI) to an optical density of 0.4 (540 nm, 1.6 cm), followed by washing and resuspension in PBS (1×10^{11} CFU/ml). Experiments with recombinant scFvH6 surface-displaying GP1302 and wild-type *S. gordonii* GP204 (ref. 18) were carried out in parallel. Reciprocal dilutions and successive incubation for 18 h at 37°C were carried out in 96-well microtiter plates. *Candida albicans* inocula varied from 3×10^0 to 3×10^3 cells (3.3-fold dilutions), whereas inocula of *S. gordonii* varied from 1×10^5 to 1×10^9 cells (10-fold dilutions). As control for *Candida* inocula and survival, yeast cells were incubated without streptococci.

For assaying the candidacidal activity of the scFvH6-secreting GP1303 strain, its 100- and 50-fold concentrated supernatant in TSB w/o was used in a conventional CFU assay, and a similar supernatant of strain GP204 served as the control. KT-susceptible *C. albicans* germinating cells (3.5×10^2 in 10 μ l of PBS) were added to 90 μ l of the concentrated supernatants and incubated for 6 h at 37°C. To verify the specificity of the biological activity, the same number of *Candida* cells was also added to 90 μ l of the concentrated supernatants preadsorbed overnight at 4°C with 100 μ g of the KT-neutralizing mAbKT4 (ref. 14).

For all assays, after incubation with the respective reagents, the yeast cells were seeded in Sabouraud agar plates that were then incubated at 30°C and checked after 48 h for CFU enumeration. Each experiment was done in triplicate for statistical purposes.

Immunoreactivity of supernatants of *S. gordonii* strains with mAbKT4. The reactivity of the supernatants of *S. gordonii* strains GP1303 and GP204 with mAbKT4 was ascertained by a dot-blot assay. Two-hundred microliters of the concentrated supernatants were spotted onto nitrocellulose paper. The strips were incubated with mAbKT4 (100 μ g/ml) and the reaction revealed by peroxidase-conjugate anti-mouse antibodies and 4-chloro-1-naphthol as substrate. As control, the same procedure was carried out with an irrelevant isotype-matched mAb.

In vivo anti-candidal activity of recombinant *S. gordonii* strains. For the assay of anti-candidal protection by surface-displayed or secreted scFvH6 from recombinant *S. gordonii* strains, a well-established experimental model of vaginal infection by *C. albicans* in oophorectomized, estrogen-treated rats, was used²¹. Bacteria were grown in TSB medium, at 37°C under a CO₂ atmosphere and, after reaching the stationary phase, the cultures were centrifuged (1,800 g, 15 min) and the pellet resuspended in PBS (10^{10} CFU/ml). These suspensions (0.1 ml) were inoculated intravaginally into the rats at 1, 24 and 48 h after challenge with the fungal cells (0 time). The second and third doses were administered 1 h after the sampling of the vaginal fluid for *C. albicans* CFU enumeration. As a positive control, one group of rats received 100 μ g of fluconazole (Pfizer, Rome, Italy) in PBS (0.1 ml) at 1, 24, and 48 h after the yeast challenge, as above.

Samples for assaying vaginal colonization by streptococci were taken as above and plated on blood agar plates containing streptomycin (500 μ g/ml); plates were incubated for 24–48 h at 37°C. The *S. gordonii* strains isolated from the vaginal fluid were controlled for their scFvH6 insert by PCR and sequencing.

To verify the presence of the scFvH6, the vaginal fluids of rats colonized with strain GP1303 and, as control, strain GP1301, were collected 48 h after administration of the first bacterial dose and tested for reactivity against mAbKT4 by the dot-blot assay. The same fluids were also assayed for candidacidal activity by the CFU assay.

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