Microorganisms as Carriers of Nucleotide Sequences Coding for Antigens and Protein Toxins, Process of Manufacturing and Uses thereof

Description

Technical field

The invention relates to microorganisms as carriers of heterogeneous nucleotide sequences coding for antigens and protein toxins, a process of manufacturing thereof as well as corresponding plasmids or expression vectors. These microorganisms can be used as medicaments, in particular as tumor vaccines for the treatment of various tumors.

Prior art

Immunotherapy of cancer represents a promising option of tumor-treatment. A multiplicity of clinical trials using different approaches concentrates on its efficiency in patients. In principle, a distinction is drawn between passive and active immunotherapy.

Active immunotherapy aims at the induction of a vaccine-related tumor-specific immune response. The latter is currently being clinically probed using several different approaches. For instance there are so called whole – cell vaccines, whose feed stock are tumor cells that are either directly obtained from the patient (autologous) or derived from appropriate cell lines (heterologous). These cells are then usually inactivated, differentially manipulated and (re)applied to the patient.

In contrast, antigen-specific vaccines contain one (or more) tumor-specific antigens, parts of the antigen or the specific antigen-coding DNA as well as so called anti-idiotype vaccines. Normally these vaccines are not isolated, but injected in combination with an appropriate carrier. Hence, on one hand different classic adjuvants are utilized, but likewise combinations with biological immuno-stimulants such as cytokines.

For the purpose of immuno-stimulation, approaches are being applied, that contain antigen associated with immuno-stimulants, such as tetanus toxin. Furthermore, there exist attempts, applying antigens in combination with dendritic cells. And finally there are several attempts with recombinant live-vaccines with viral or bacterial carriers.

Fusion proteins of bacterial toxins, such as tetanus toxin, shiga toxin, lethal toxin or cholera toxin, as adjuvants with an antigen are utilized as vaccines especially against infections for quite some time (Freytag and Clements, 1999). Additionally, native toxins, often merged with a target-cell specific molecule, such as a cell surface molecule of tumor cells, are also used in order to destroy target cells.

At this, fusion proteins with the native toxin, that generally comprise an enzymatic unit and a protein-binding domain, develop their optimum effect in use as adjuvants (Freytag and Clements, 1999). By means of these vaccines a satisfying immune response is obtained even after mucosal and particularly after oral immunization. The trouble with these fusion proteins is, that native toxins are highly toxic and therefore cannot be established in humans (Holmgren et al., 2005).

A whole string of research is thus occupied with detoxification of toxins that at the same time preserve the adjuvant-effect. However, since in most cases the adjuvant-effect coincides with enzymatic-activity, which is responsible for the toxic effect (Lycke et al., 1992), detoxification cannot be performed in a straightforward way, even if it appears to be possible for some toxins that do not lose their enzymatic, adjuvant activity (Hormozi et al., 1999; Lycke et al., 1992).

In the case of cholera toxin (CT) several attempts of detoxification are pursued (Agren et al., 1999; Byun et al., 2001; Eriksson et al., 2004; Kweon et al., 2002; Sanchez et al., 2002), for which, however, the use as mucosal adjuvant prevails (Freytag and Clements). Therefore, above all, efficient induction of an antibody response (primarily mucosal IgA), which receive an increased toxin-related MHC class II restricted T-cell support, is the main prerequisite for a mucosal adjuvant for a vaccine that consists of protein-antigen and a fused or co-applied toxin (Freytag and Clements).

As for cholera toxin, especially its B-subunit (CtxB) is tested as adjuvant since it is responsible for binding to the GM-1 receptor and does not show toxic effects when isolated (Holmgren et al.). Protein fusions with CtxB are characterized by primary induction of so called Th2 immune responses. These are T-cell responses, which are mainly characterized by cytokines, such as IL-4 or IL-6, and which primarily cause induction of antibodies, but which do not at all or at most restrictedly initiate a cellular immune response, particularly of cytotoxic T cells (CTL) (Holmgren et al.).

In addition, CtxB as mucosal adjuvant induces protein-antigen systemic tolerance. Systemic tolerance describes the depletion or inactivation of antigen-specific lymphocytes, in particular T-cells or B-cells. This kind of approach is therefore inapplicable for the induction of a systemic immune response (Holmgren et al.).

In contrast to mucosal application intraperitoneal or subcutaneous application of a toxin-antigen fusion protein is able to induce systemic as well as low cytotoxic responses. This has indeed been utilized for tumor-vaccination in model systems (refer to e.g. (Becerra et al., 2003)). However such response is also obtained with the purified antigen itself and is primarily depending on the adjuvant used. Apart from the fact that the measured CTL responses in the model are rather low, there was no evidence that the protection was even depending on these effects. Moreover, the antigen was not applied orally, but only by direct injection (s.c., i.d., i.m., i.p.) of the antigen.

Antigen proteins fused to a detoxified toxin are generally ineffective if applied as oral tumor vaccine. The main reasons are, if present at all, only low induction of a systemic immune response or even, in the case of CtxB, induction of systemic tolerance as well as induction of mucosally restricted antibody and Th2 type immune responses.

McSorley et al., for instance, showed that nasal immunization with a CtxB-antigen fusion protein (which for a tumor vaccine represents the preferred way of inducing a systemic response) preferably tolerates and therefore inactivates Th1 cells, whereas Th2 cells are not influenced (McSorley et al., 1998). Th2 responses are characterized by T-helper cells that predominantly produce IL-4 or IL-6. These cytokines are particularly responsible for the initiation of antibody production by B-cells, which provide protection in the case of most conventional vaccines. In contrast, Th1 T-cells mostly secrete IL-2 and IFN-gamma, thus cytokines, which play a role in cellular immune response. Depending on the objective of an immunization strategy it is of crucial importance, whether an antibody dominated Th2 immune response (so-called Th2 bias) or a cellular dominated Th1 response (so called Th1 bias) is initiated.

By contrast, systemic induction of a Th1 dominated cellular immune response with IFN-gamma producing T-helper cells and induction of cytotoxic T-cells (CTL) is indispensable for tumor immunotherapy.

The prior art shows that toxins can function as adjuvants. Especially cholera toxin (CT) shows a strong adjuvant effect. However, this effect is significantly attenuated as soon as the toxin is detoxified. Regarding its subunit CtxB, oral application even induces systemic tolerance. This problem can be partially avoided by nasal application. However, if nasal administration is applied, other problems arise, in particular such as the ones associated with CtxB subunit concerning the expression of GM-1 receptor in the brain (van Ginkel et al., 2000), which are reflected in an increasing risk of particular severe side effects (Mutsch et al., 2004).

To overcome these problems, it may be possible to generate recombinant live-vaccines that co-express toxins and (heterologous) antigens. This option has already been pursued with regard to infection vaccines, i.e. vaccines that are directed against a specific pathogen, for instance the tuberculosis pathogen, and are meant to induce immunity against that pathogen.

In the course of developing such infection vaccines, recombinant toxins fused to appropriate (heterologous) antigens have already been generated, using several recombinant bacterial strains that are administered orally, either as a live or inactivated vaccine. In most cases, the generated strains only expressed a recombinant toxin. Hence, these approaches primarily aimed at solely immunizing against the toxin itself (induction of an antibody response) and, consequently, the toxin expressing strain (pathogen). These kinds of vaccines are not suitable for use as potential tumor vaccines in tumor therapy and, not surprisingly, corresponding trials do not mention any such possible application (Reveneau et al., 2002) (Vertiev et al., 2001) (Freytag and Clements, 1999; Jackson et al., 1996).

With respect to such infection vaccines, in the course of induction of an antibody response the toxin does not function as adjuvant, it is the induction of a mucosal antibody immune response which prevails. These infection vaccine trials include no or only a very weak systemic immune response. In contrast to tumor vaccines, such vaccines do not pursue an induction of cellular immune response, especially of cytotoxic T-cells. The toxin expressing bacterial pathogens normally show an extracellular live pattern and therefore do not contribute to an activation of CTL, i.e. the protection conferred by such infection vaccines is CTL independent.

The following bacterial strains have been used in the mentioned infection vaccine trials: recombinant Lactobacilli (Reveneau et al., 2002), Listeria (Vertiev et al., 2001), Bacillus anthracis (Mendelson et al., 2005; Mesnage et al., 1999), Shigellae (Anderson

et al., 2000; Tzschaschel et al., 1996b), E. coli (Walker et al., 1992), Vibrio (Butterton et al., 1995; Chen et al., 1998; Thungapathra et al., 1999) und Salmonella (Jackson et al., 1996).

In addition, it was tried to enhance mucosal immune responses by surface presentation (Konieczny et al., 2000) or secretion of the toxin as heterologous antigen (Salmonella, Shigellae: (Garmory et al., 2003; Orr et al., 1999; Su et al., 1992; Tzschaschel et al., 1996a; Tzschaschel et al., 1996b), Yersinia: (Sory and Cornelis, 1990), Vibrio (Ryan et al., 1997a), E. coli: (Zhu et al., 2006)). However, in these additional cases the toxins themselves represent the antigens to which the immune response is directed to.

Thus, it has to be noted that the toxins neither function as adjuvants nor did the describing studies intend to express toxins as fusion proteins with an additional separate (heterologous) antigen. Moreover, fusions proteins with CT or CtxB were neither generated with these systems nor were they suggested.

Consequently, fusion proteins mentioned in above cited documents are fusion proteins of a peptidic secretion signal, such as HlyA, and a toxin protein, but not fusion proteins, consisting of a toxin and a (heterologous) antigen.

The primary objective of these studies was to merely obtain an optimum mucosal immune response (Tzschaschel et al., 1996a). Induction of a systemic immune response was not pursued. Yet if measured at all, analyses of systemic immune responses were restricted to antibodies (particularly systemic IgA) only, since protection in these kind of models is mainly mediated by antibodies (compare e.g. [31]). Induction of a systemic cellular immune response, in particular a cytotoxic T-cell response, is not described. The fusion with a secretion signal was primarily used in order to increase the solubility of the toxins and enhance their stability, respectively, since a strong, mere cytoplasmatic expression often results in the production of insoluble aggregates (Gentschev et al., 2002a).

In this respect, some authors observe that fusion proteins with a secretion signal cause a fast cytoplasmatic degradation (Tzschaschel et al., 1996a), whereas others observe a stabilization (Orr et al., 1999). The prior art, hence, is contradictory here. So far, previous experiments with secreted toxins (toxin + secretion signal) are above all aimed at increasing stability, which obviously was not achieved in all cases.

One reason certainly is found in variable expression intensity and plasmid stability. Tzschaschel et al. describe, that the plasmid system used, is highly instable and above all, without selection, is merely found in a few bacteria. As a possible solution the authors use chromosomal integration via a mini-transposon (Tzschaschel et al., 1996a; Tzschaschel et al., 1996b).

However, such a system possesses several disadvantages. On one hand the point of integration is not defined, which can lead to an undesired phenotype alteration of the host strain (e.g. increased / decreased expression of flanking genes). On the other hand the expected expression level using a single genomic copy is only low, which has a negative effect on immunogenicity. After all, chromosomal transposon integration is relatively instable, for it leads quite frequently to spontaneous excisions at the sides of the repetitive elements.

As mentioned above, the prior art is contradictory concerning the stability of a secreted heterologous toxin.

Garmory et al. even assume, that secretion of a heterologous antigen does not have any special benefit with regard to immunogenicity (Garmory et al., 2002; Roland et al., 2005). In other cases increased systemic antibody response to a secreted toxin after intravenous administration has indeed been observed, but not after oral application. All the contrary, oral application is even indirectly put into question (Roland et al., 2005).

Eventually, studies with a gram-positive strain (Lactobacillus plantarum) which produces tetanus toxin showed no significant difference in induction of a systemic antibody response compared to strains that secrete the toxin, present it bound to their membrane or contain the toxin cytoplasmatically (Reveneau et al., 2002).

Systemic cellular immune responses, in particular cytotoxic T-Cell responses, and the resulting protection, were not studied nor described.

The above cited prior art of toxin-based infection vaccines thus cannot declare if a secretion of toxin used as adjuvant represents an advantage, concerning the induction of a systemic (cellular) immune response. All the contrary, above cited studies rather point the stability problem out and mention the missing benefit of a secreted heterolo-

gous toxin. Fusion proteins, consisting of secretion signal, toxin and heterologous antigen, are not at all described or suggested.

In above passages the prior art was presented, that describes bacterial carriers which express toxins heterologously and can be used as infection vaccines. In the cited examples mainly modifications regarding the expression or stability of toxins and their solubility were carried out, for instance insertion of strong expression promoter or fusion of a toxin to a secretion signal.

Other authors have also studied genetic fusions of toxins to heterologous antigens in live-vaccines. In these cases the toxin was mostly used as adjuvant. In some cases (e. g. (Brossier et al., 2000)) the heterologous antigen functioned as adjuvant and the toxin as proper antigen.

However, it is important to note, that in the mentioned cases, expression of the toxin-antigen gene fusion construct exclusively took place cytoplasmatically or periplasmatically. The toxin-antigen construct was neither fused to an additional secretion signal (which would lead to its complete secretion) nor was it directly secreted.

In the course of these toxin-antigen gene fusion constructs, recombinant E. coli (Clemens et al., 2004), Bacillus anthracis (Brossier et al., 2000), Shigella (Koprowski et al., 2000; Ranallo et al., 2005; Zheng et al., 2005) and Vibrio strains (Silva et al., 2003) were used. For Salmonella (summarized in (Garmory et al., 2002)), too, fusions of CT variants with antigens (Hajishengallis et al., 1996; Huang et al., 2000) or other toxins with antigens (Barry et al., 1996; Cardenas and Clements, 1993; Chabalgoity et al., 1997; Chabalgoity et al., 1996; Chabalgoity et al., 2000; Chabalgoity et al., 1995; Chacon et al., 1996; Jagusztyn-Krynicka et al., 1993; Khan et al., 1994a; Khan et al., 1994b; Lee et al., 2000; Pogonka et al., 2003; Schodel et al., 1990; Smerdou et al., 1996; Ward et al., 1999; Wu et al., 2000) have been described.

In the majority of these cases the main focus was on induction of a mucosal (antibody) immune response and for the induction of a systemic immune response, only subcutaneous, but not oral application was chosen [36].

Some work with Salmonella as supporter strain are limited to a mere characterization of the strain (Gomez-Duarte et al., 1995; Jagusztyn-Krynicka et al., 1993), others only analyze the mucosal and/or systemic antibody response and/or protection (Barry et al., 1996; Cardenas and Clements, 1993; Dunstan et al., 2003; Hajishengallis et al.,

1996; Harokopakis et al., 1997; Khan et al., 1994a; Khan et al., 1994b; Pogonka et al., 2003; Smerdou et al., 1996; Somner et al., 1999). In all these cases, in which incidentally a fusion between antigen and tetanus toxin was used and which are exclusively used as infection vaccines, systemic cellular immune responses, particularly cytotoxic T-Cell responses, have not been studied.

Therefore, from an immunological point of view, no conclusions about a potential use as a tumor vaccine can be drawn from those studies, since their focuses were set on antibody mediated effects as infection vaccines.

Investigations that include an isotype analysis of the immune response studied appear to be more relevant. In fact, cellular immune responses are not directly measured in these cases, but the isotype profile of the antibody response allows a conclusion on the Th1/Th2 bias of the immune response. Antibody isotypes like IgG1 are associated with Th2 responses and isotypes like IgG2a are associated with Th1 responses. As already mentioned, Th1 responses are cellular dominated immune responses, whereas Th2 responses mainly represent humoral antibody-driven responses. Still, those studies do neither describe tumor vaccines nor do they suggest any use as anti-tumor agent.

One infection vaccine study based on Salmonella as carrier, which expresses a tetanus-toxin antigen fusion protein has been realized in dogs. The low antibody responses that were induced in dogs, show a Th1 bias, regarding the antibody profile, hence, a response that rather correlates with a cellular type immune response (Chabalgoity et al., 2000). Admittedly, dog immunology is only scarcely researched, and therefore it is not clear to which extent a dog's antibody profile can give information about a Th1 bias.

Studies in mice, performed by the same group, using comparable constructs, however showed an antibody profile with the same level for IgG1 as for IgG2a, which would indicate a mixed Th1/Th2 response.

Interestingly, an existing immunity against tetanus toxin, as it is found in most humans due to previous immunizations, causes another relatively strong induction of IgG1, whereas IgG2a is hardly induced. This clearly indicates a distinct Th2 bias (Chabalgoity et al., 1995). For this reason, a tetanus toxin based live-vaccine as tumor vaccine is rather harmful for human use. For it can be expected, that a strong Th2 anti-

body-dominated response is induced in the majority of these patients, who show a tetanus toxin specific response.

Only few studies also analyze cellular immune response and compare genetic constructs with and without toxin fusion. In one case, for instance, a fusion of an antigen with and without tetanus toxin has been compared in Salmonella (Lee et al.). There, the tetanus toxin antigen fusion construct mainly increased the total antibody level, whereas the Th1/Th2 profile was hardly altered. Even the antigen specific $CD4^+$ T cell secretion of typical Th1 cytokines, such as IFN-gamma and IL-2, respectively, only showed weak divergence. In an earlier study by the same group, cellular IFN-gamma levels had been measured, too. However, no comparison drawn with constructs without tetanus toxin (Chabalgoity et al.). Other studies with different gram-negative bacterial carriers, like Shigella (Koprowski et al., ; Ranallo et al., ; Zheng et al.) or Vibrio (Campos et al., ; Ryan et al.), did not analyze isotypes and cellular immune responses, respectively, either.

In summary, it can be stated, that the studies mentioned clearly focus on the induction of an antibody-driven humoral immune response. Indeed, genetic toxin antigen constructs are used, but these are not provided with a secretion signal nor are they directly secreted. On no account, however, systemic cellular immune responses, in particular cytotoxic T-cell responses, were analyzed. What is more, such cellular cytotoxic T-cell responses cannot be concluded from humoral antibody response and cannot be detected if the antibody response is Th1/Th2 – composed.

However, it is exactly these cellular cytotoxic T cell immune responses which are crucial for use in tumor vaccination therapy.

Hence, in terms of toxin-antigen fusions, which are restricted to mucosal infection vaccines only, the state-of–the-art does not allow any statement about possible use of any such constructs as tumor vaccines.

As already mentioned, expression of the genetic fusion constructs is realized without assistance of a secretion system. Toxins and toxin antigen constructs, respectively, are usually located cytoplasmatically as well as periplasmatically, i.e. between the two membranes. In order to induce an efficient cellular immune response the toxin must be freely accessible for the antigen-presenting cell (APC). Usually, native toxin is pro-

duced in the periplasm of gram-negative bacteria. This is sufficient for mere mucosal immune responses, because periplasmatic toxins can escape from the periplasm in the colon and consequently are accessible, too (Hunt and Hardy, 1991). This does not count, however, if the carrier targets antigen-presenting cells outside the colon, such as e.g. Peyer's Patches or lymphatic organs like lymph nodes or spleen.

In principle, two factors are crucial for the efficiency of a tumor vaccine: induction of a cellular immune response of the Th1-type and participation of components of the innate immune system, such as NK cells, NKT-cells and gamma-delta T cells, which play an important role for the efficiency of tumor therapy (Dunn et al., 2004).

The importance of these components of the innate immune systems lies on multiple levels. Properly activated NK- and gamma-delta T cells are able to locally produce large amounts of IFN-gamma. This interferon, which is also produced by specific Th1 polarized T-cells, has multiple functions, relevant to tumor therapy. One of its central functions is the inhibition of angiogenesis, which cuts off the tumor's oxygen and nutrients supply and de facto starves the tumor. Furthermore, NK cells possess receptors, which recognize MHC class I molecules. If these molecules are present on a cell, NK cells are inhibited.

As for a vaccine, which induces specific cytotoxic T-cells, tumor cells can be killed by these CTLs. If the tumor cell loses its capability to express MHC class I molecules, which occurs quite frequently in tumors, specific cytotoxic T-cells are ineffective. Hence, in this case, inhibition of NK cells is stopped, and they are able to eliminate tumor cells directly.

Consequently, it would be ideal if a tumor vaccine induces both components efficiently. There are contradictory data concerning the Th1-Th2 bias of a fused toxin adjuvant. As discussed earlier, toxin antigen fusion constructs applied in an isolated fashion obviously induce a strong Th2 polarized immune response. Some authors still describe a low Th2 bias for live-carriers; other authors see a slight Th1 bias.

However, these data again are exclusively based on non-secreted constructs. Induction of innate immunity by means of such kinds of infection vaccines has never been compared nor contemplated.

As already mentioned, the main reason is that the existing vaccines are mucosal infection vaccines, not tumor vaccines. Therefore, induction of Th1 immune responses, CTL immune responses and responses of the innate immune system were not in the focus. In contrast, regarding tumor vaccines, induction of these immune responses is indispensable.

Interestingly, in cell biology native toxins are commonly used as inhibitors of signaling pathways. So among others it has been shown, that native pertussis toxin, but not cholera toxin, is able to inhibit a particular apoptosis pattern of NK cells (Ramirez et al., 1994). A different research study was able to show that cholera toxin, but not its B-subunit, blocks specific NK cell functions (Poggi et al., 1996). Native pertussis toxin is used in order to inhibit chemotaxis of lymphocytes (Spangrude et al., 1985). Even if these studies do not deal with tumor vaccination, a person skilled in the art would conclude, that the use of toxins as tumor vaccines would be harmful, because the response of the innate immune system, crucial for tumor therapy, is rather inhibited than induced.

Other research studies were able to show that toxins like native pertussis toxin (but not inactive pertussis toxin) efficiently induce components of the innate immune system. Regarding immunotherapy against tumors, this would mean that, if at all, native toxins would need to be employed. However, due to toxicity reasons such kind of administration is infeasible. Further, such induction would inevitably lead to a Th2 directed secondary immune response, which in turn would be deleterious for tumor therapy (Boyd et al., 2005). Consequently, concerning the induction of an innate immune response, the prior art does not describe nor contemplates tumor vaccination. All the contrary, critical analysis of the literature even militates against the use of toxins in tumor therapy.

Interesting, though, is an analysis of the synergistic effect of toxins or their subunits with other stimulants, such as immune stimulating DNA oligonucleotides with hypomethylated CpG motives (CpG ODN) (Holmgren et al., 2005) or liposaccharides (LPS). In the case of LPS primarily the induction of monocytes appears to be increased mainly through the B-subunit of the toxins, whereas it is inhibited by the toxin as a

whole (holotoxin) (Hajishengallis et al., 2004). However, these studies exclusively rely on the use of purified toxin-antigen-fusion constructs, to which substances like LPS or CpG are added as adjuvants. Furthermore, analysis there is only carried out on macrophages, which induce an adaptive immune response, but do not attack tumours directly. Hence, these studies have no significance regarding the induction of components of the innate immune system, in particular NK cells, which can attack tumors directly.

Other studies, however, show that NK cells can be activated and chemotacticly attracted by toxins like Pseudomonas aeruginosa Exotoxin A (Muhlen et al., 2004). Depending on the experimental system, Th1 responses can also be induced, although a suppression of NK cells and Th1 responses thereby mostly occurs (Michalkiewicz et al., 1999). However, these assays primarily aim at the analysis of the hepatotoxicity of Enterotoxin A and do not refer to tumor vaccination. Interestingly, the effects are highly dose-dependent, and only a slight change of dose can invert the effects. Yet, the authors could not reveal which response effectively occurs in vivo. As a consequence, the data do not provide a prediction which effects may occur if a toxin or even a detoxified toxin is used as adjuvant.

All in all, the immune response strongly depends on the particular system applied. In most cases, a mucosal anti-infection vaccine aims at the local manipulation of the immune system at the mucosa, in order to induce an efficient mucosal immune response (Lycke, 2005). However, these studies do not include the development of a tumor vaccine. In addition, those studies lack information about the induction of systemic cellular immune responses, particularly responses of cytotoxic T-cells, which are essential for tumor vaccination.

It has already been shown, that secretion of a heterologous antigen confers advantages for a systemic immune response (Hess et al., 1996). However, the secreted antigens described were not secreted toxin-antigen constructs; toxins or subunits thereof were not used. The thereby attainable immune responses in a transgenic tumor model were highly limited, too (Gentschev et al., 2005). Indeed, weak antibody and cytotoxic T-cell responses could be induced in this case, that partially protected from a tumor progression. However, not only the immune responses themselves, but the protection itself was limited. Likewise, these tumor vaccination studies lack a comparison with

non-secreted constructs. Furthermore, the comparative studies were not carried out in the context of tumor vaccination (Hess et al., 1996) and are in contrast to further studies, that do not see any advantage with regard to secretion (Garmory et al., 2002; Roland et al., 2005).

In summary and as previously mentioned, the prior art is highly contradictory regarding secretion and, all in all, does not give any hint towards potential advantages of secreted toxin-antigen constructs in tumor therapy. All the contrary, critical analysis of the existing literature rather disagrees with such kind of use.

Bacterial toxins (Todar, 2002): At a chemical level, there are two types of bacterial toxins, lipopolysaccharides, which are associated with the cell walls of gram-negative bacteria, and proteins, which are released from bacterial cells and may act at tissue sites remote from the site of bacterial growth. The cell-associated lipopolysaccharide (LPS) toxins are referred to as endotoxins and the extracellular diffusible toxins are referred to as exotoxins.

Exotoxins are typically soluble proteins secreted by living bacteria during exponential growth but in some cases they are released by lysis of the bacterial cell. The production of the toxin is generally specific to a particular bacterial species that produces the disease associated with the toxin (e.g. only *Clostridium tetani* produces tetanus toxin; only *Corynebacterium diphtheriae* produces the diphtheria toxin). Both gram-positive and gram-negative bacteria produce soluble protein toxins.

In general there exist three classes of protein (exo-) toxins: (i) type I toxins (superantigens), that bind to the host cell surface and modulate the immune response but are not translocated into the cell, (ii) type II toxins (pore-forming toxins), which act on the host cell membrane and make the host cell leak and die, and (iii) type III toxins (A-B toxins), which bind to the host cell via one specific receptor, are translocated into the cell, become active therein an modify proteins or other components of the host cell.

As indicated above, type III toxins, acting intracellularly with regard to host cells, consist of two components: one component (subunit A) is responsible for the enzymatic activity of the toxin; the other component (subunit B) is concerned with binding to a specific receptor on the host cell membrane and transferring the enzyme across the membrane. The enzymatic component is not active until it is released from the native

(A+B) toxin. Isolated A subunits are enzymatically active but lack binding and cell entry capability. Isolated B subunits may bind to target cells (and even block the binding of the native toxin), but they are non-toxic.

There are a variety of ways that toxin subunits may be synthesized and arranged: A + B indicates that the toxin is synthesized and secreted as two separate protein subunits that interact at the target cell surface; A-B or A-5B or AB5 indicates that the A and B subunits are synthesized separately, but associated by noncovalent bonds during secretion and binding to their target; 5B or B5 indicates that the binding domain of the protein is composed of 5 identical subunits. AB or A/B denotes a toxin synthesized as a single polypeptide, divided into A and B domains, which may be separated by proteolytic cleavage. Examples of AB or A/B toxins are Diphtheria Toxin, Exotoxin A, Botulinum toxin and Tetanus Toxin. Examples of A-5B or AB5 toxins are Cholera Toxin and Shiga Toxin, whereas Anthrax Toxin LF and Anthrax Toxin EF are examples of A-B toxins.

Further relevant documents of the prior art comprise the following:

Michl et al. describe the use of bacteria and bacterial toxins as therapeutic agents for solid tumors. Toxin-Antigen fusion constructs are disclosed as well as bacterial targeting of such construct. Use of diphteria toxin (DT), pseudomonas exotoxin A (PE) and clostridium perfringens enterotoxin (CPE) is studied. However, the authors do neither mention the use of cholera toxin nor do they show or render obvious secretion of bacteria-delivered toxin-antigen fusion constructs (Michl and Gress, 2004).

Lahiri gives an overview about different bacterial toxins and discuss their manifold uses. Although the author mentions toxin-antigen fusion proteins he is silent about cholera toxin and bacterial targeting of secreted toxin-antigen fusion constructs (Lahiri, 2000).

Lavelle et al. disclose molecules of infectious agents as immunomodulatory drugs. The authors also mention cholera toxin-antigen fusion proteins, but such constructs are only applied directly as proteins and not by means of genetically modified live vaccines (Lavelle et al., 2004).

WO 01/74383 is directed to chimeric antigen-enterotoxin mucosal immunogens and also mentions the use of cholera toxin subunits A2 and B. Such chimeric immuno-

gens, however, comprises always A2 and B subunits at the same time and are intended for use in mucosal immunization but not in tumor therapy.

WO 02/077249 describes non-virulent Yersinia enterocolitica mutant strains for the delivery of heterologous proteins to specific mutated target cells. Use of cholera toxin subunit A1 is also mentioned but the patent document is silent about secretion and refers to the treatment of infections and infectious states only.

WO 2004/018630 discloses recombinant double stranded RNA phages encoding a double stranded eukaryotic expression cassette. Although cholera toxin subunit A is mentioned, the document is not of further relevance.

Holmgren et al. give a brief overview about the field of mucosal immunization and adjuvants. They discuss among others the effects of cholera toxin as mucosal adjuvants but do not disclose information about genetic expression systems or live vaccines and are also silent about tumor therapy (Holmgren et al., 2003).

Holmgren and Czerkinsky also give an overview about mucosal immunity and vaccines. However, this article is restricted to anti-infectives only and does not discuss or render obvious possible uses in the field of tumor therapy (Holmgren and Czerkinsky, 2005).

Another review by Freytag and Clements discusses mucosal adjuvants for application in anti-infective immunotherapy. Although cholera toxin is mentioned as mucosal adjuvants the authors are silent about secreted toxin-antigen constructs and tumor therapy as a possible field of administration (Freytag and Clements, 2005).

Shaw and Starnbach describe the use of modified bacterial toxins to deliver vaccine antigens. However, the article does not mention cholera toxin and further is limited to direct application of toxin-antigen fusion proteins for vaccination reasons (Shaw and Starnbach, 2003).

WO 03/072789 is directed to microorganisms as carriers of nucleotide sequences coding for cell antigens used for the treatment of tumors. Although the patent document mentions secretion and use in the field of tumor therapy it is silent about bacterial toxins and fusion proteins at all.

Gentschev, Dietrich and Goebel as well as Gentschev et al. describe bacterial targeting and its use in tumor vaccine development. However, these two documents do not mention the use of bacterial toxins and fusion proteins in tumor therapy (Gentschev et al., 2002a; Gentschev et al., 2002b).

WO 98/23763 discloses Vibrio cholerae cells expressing E.coli hemolysin B and D subunits along with a fusion polypeptide that includes a heterologous antigen fused to hylA. Further described is a Vibrio cholerae vaccine strain that expresses cholera toxin subunit B and a fusion polypeptide of a secretory signal sequence, heterologous antigen and choleratoxin A2 subunit. Last, a fusion polypeptide that includes cholera toxin B subunit fused to an antigenic portion of C. difficile toxin A or toxin B subunit is disclosed. However, the patent application does not mention use of a fusion protein of protein toxin plus a heterologous non-protein toxin antigen in tumor therapy.

Dietrich and co-workers discuss two vaccine delivery tools – haemolysin A and listeriolysin – that can be used for cell-mediated immunity. However, no protein toxin – heterologous antigen fusion proteins or co-expression is mentioned (Dietrich et al., 2003).

Gentschev et al. describe the use of the alpha-hemolysin secretion system of Escherichia coli for antigen delivery in the Salmonella typhi Ty21a vaccine strain. However, the authors do not mention the use of bacterial toxins and fusion proteins in tumor therapy (Gentschev et al., 2004).

WO 02/47727 is directed to therapeutic agents comprising a B-subunit of a protein toxin. The document only discloses fusion proteins of CtxB and EtxB with viral antigen. No bacterial vaccine or bacterial vaccine delivery is mentioned.

Cheng-hua S and co-workers describe a gene fusion of cholera toxin B subunit and HBV PreS2 epitope and the antigenicity of the fusion protein in direct immunization studies. However, no bacterial vaccine or bacterial vaccine delivery is mentioned (Cheng-hua et al., 1995).

Sanchez et al. disclose that cholera toxin B-subunit gene enhances mucosal immunoglobulin A, Th1-type and $CD8^+$ cytotoxic responses when co-administered intradermally with a DNA vaccine (Sanchez et al., 2004). However, in this approach the authors use DNA as a carrier which acts as a Th1 promoting adjuvant on its own. In addition, the proteins are produced by host cells and not delivered directly or via a bacterial carrier and are therefore directly available for eukaryotic cells.

WO 01/29233 is directed to chimeric immunogenic compositions and nucleic acids encoding them. However, no bacterial vaccine or bacterial vaccine delivery is mentioned.

WO 2007/044406 relates to methods for stimulating an immune response using a bacterial antigen delivery system that is based on a SopE bearing type III secretion signal. However, the patent application does not mention the use of bacterial toxins and fusion proteins in tumor therapy.

In summary, it can be concluded from the prior art that native toxins cannot be established for use in humans due to their strong toxicity. Further, their application in tumor therapy would be harmful because the response of the innate immune system, in particular that of NK cells, would be inhibited. However, it is this immune response which is a crucial component for a successful tumor therapy since tumor cells very frequently lose their capability to express MHC class I molecules and therefore are resistant to CTL recognition and attack.

Use of the detoxified toxin subunits, on the other hand, alone or fused to (heterologous) antigen proteins results in a strongly attenuated adjuvant effect and/or even an induced systemic tolerance of the immune system as well as mucosally restricted antibody and Th2 type immune responses.

Furthermore, secretion of (heterologous) antigen-toxin fusion proteins, which is only described in the course of anti-infection vaccines (i.e. targeting the antigen or even the toxin itself), is said to not only display no advantages over cytoplasmatic expression, but to be generally rather unsuitable.

All in all, neither a tumor therapeutic approach is presented nor were a systemic induction of a Th1 dominated cellular immune response with IFN-gamma producing T-helper cells, an induction of CTL nor the activation of the innate immune system described or achieved, all of which are indispensable for tumor therapy.

Description of the invention

The present invention has the object to provide novel tumor vaccines by means of which a strong systemic cellular immune system response is induced and an efficient tumor therapy can be achieved.

The object of the present invention has been surprisingly solved in one aspect by providing a microorganism as a carrier of nucleotide sequences coding for antigens and protein toxins comprising the following components:

(I) at least one nucleotide sequence coding for at least one complete or partial antigen of at least one wild-type or mutated protein; and

(II) at least one nucleotide sequence coding for at least one protein toxin and/or at least one protein toxin subunit; and

(III) **a)** at least one nucleotide sequence coding for at least one transport system which enables the expression of the expression products of component (I) and component (II) on the outer surface of the microorganism and/or enables the secretion of the expression products of component (I) and component (II); and/or coding for at least one signal sequence which enables the secretion of the expression products of component (I) and component (II); and/or

b) optionally, at least one nucleotide sequence coding for at least one protein for lysing the microorganism in the cytosol of mammalian cells and for intracellularly releasing plasmids or expression vectors, which are contained in the lysed microorganism; and

(IV) at least one nucleotide sequence for at least one activation sequence for the expression of one or more of components (I) to (III), wherein said activation sequence can be activated in the microorganism and/or is tissue cell-specific, tumor cell-specific, macrophage-specific, dendrite-specific, lymphocyte-specific, function-specific or non-cell-specific";

wherein any of components (I) to (IV) can be present either once or several times and if a component of components (I) to (IV) is present several times it can independently from each other be either identical or different.

In a preferred embodiment, a microorganism comprising above components (I) to (IV) is provided, wherein the component (I) and component (II) are <u>not</u> identical, that is component (I) does <u>not</u> code for at least one nucleotide sequence coding for at least one protein toxin and/or at least one protein toxin subunit.

The term "tissue cell-specific" in connection with component (IV) in the course of the present invention refers to activation sequence(s) which are specifically activated in target tissue cells, such as hormone dependent promoters in prostatic tissues for instance.

The term “tumor cell-specific” in connection with component (IV) in the course of the present invention refers to activation sequence(s) which are specifically activated in tumor cells, such as promoter elements activated by the action of tumor specific oncogenes.

The term “macrophage-specific” in connection with component (IV) in the course of the present invention refers to activation sequence(s) which are specifically activated in macrophages, such as promoter elements which encode for macrophage specific genes, for instance the gene encoding for F4/80.

The term “dendrite-specific” in connection with component (IV) in the course of the present invention refers to activation sequence(s) which are specifically activated in dendritic cells, such as promoter elements controlling the expression of B7.1. The terms “dendrite-specific” and “dendritic-cell-specific” are equivalent, i.e. they have the same meaning and both refer to dendritic cells.

The term “lymphocyte-specific” in connection with component (IV) in the course of the present invention refers to elements which are specifically activated in cells of the lymphocytic lineage like promoter elements regulating the expression of CD3 molecules in T-cells or promoter elements regulating the expression of CD20 in mature B-cells.

The term “function-specific” in connection with component (IV) in the course of the present invention refers to activation sequence(s) which are specifically activated in cellular context, e.g. in tumor cells which have lost p53 expression, or activation sequence(s) which are activated within bacteria depending on the context, e.g. cellular localization or oxygen pressure.

The term “non-cell-specific” in connection with component (IV) in the course of the present invention refers to activation sequence(s) which are ubiquitously active, such as constitutively active bacterial promoters.

The term “nucleotide sequence” in the course of the present invention refers to dsDNA, ssDNA, dsRNA, ssRNA or dsDNA/RNA hybrids. Preferred is dsDNA.

The term “antigen” in the course of the present invention refers to molecules that react with antibodies, i.e. that are able to generate antibodies. Some antigens do not, by themselves, elicit antibody production; only those that can induce antibody production are called immunogens. For the purpose of the present invention, all kinds of known antigens are intended to be comprised. It is within the knowledge of a person

skilled in the art to retrieve the necessary information about potential antigens by means of databases and/or experimental screening without undue burden. Examples of antigens are among others cell antigens, tissue-cell specific antigens (e.g. tissue cells from which the tumor derives), cell protein antigens, viral antigens, viral protein antigens and the like. Preferred are protein antigens. Further preferred are heterologous antigens or foreign antigens, i.e. antigens which are not endogenous to the respective microorganism of the invention or antigens which are not expressed by the respective microorganism of the invention by nature, but are introduced into it by means of standard molecular biotechnological methods.

The term “complete antigen” in the course of the present invention refers to complete molecules that react with antibodies according to the definition above. Examples of complete antigens are for instance full-length proteins, which are also preferred.

The term “partial antigen” in the course of the present invention refers to specific parts of molecules that react with antibodies according to the definition above. Partial antigens can be for instance protein motives such as amino acid loops within proteins, protein kinase domains, epitopes and the like. Preferred are protein kinase domains and epitopes, the latter of which are specific sites of an antigen recognized by an antibody (also referred to as antigenic determinants).

The terms “wild-type” and “mutated” in connection with “protein” in the course of the present invention refer to proteins consisting of either their “natural” dominating amino acid sequence (encoded by the respective nucleotide sequence) and proteins that have one or more mutations in their amino acid sequence (encoded by the respective nucleotide sequence) compared to the wild-type sequence, respectively. Preferably wild-type and/or mutated proteins are derived from tumor cells. As for partial antigens it is further preferred that the sequence encompasses mutations, i.e. an epitope is chosen that preferably contains one or more mutations, for instance the B-Raf V600E epitope.

Microorganisms in the meaning of the invention are bacteria, gram-positive bacteria, gram-negative bacteria and eukaryotic cells, the latter of which comprise unicellular parasites, yeasts, tumor cells and cell line cells, such as Sacharomyces cerevisiae, Leishmania spp., autologous patient derived tumor cells and tumor cell lines. Such microorganisms are usually used as carriers for the transfer of nucleotide sequences being foreign (heterologous or heterogeneous) for the microorganism. Preferably, bacte-

ria are used, which are attenuated in their virulence, for instance bacteria that carry a deleted or inactivated aroA, aro, asd, gal, pur, cya, crp, phoP/Q, omp gene or are temperature-sensitive mutants or antibiotic-dependent mutants (Cardenas and Clements, 1992). Further preferred as a microorganism comprising above components (I) to (IV) is a gram-negative, attenuated, facultative intracellular bacterium as a carrier, which is able to overcome the intestinal mucosa (e.g. Salmonella spp. or Shigella spp.).

In a preferred embodiment, a microorganism comprising above components (I) to (IV) is provided, wherein the microorganism is selected from the group consisting of "bacterium, gram-positive bacterium, gram-negative bacterium, eukaryotic cell" and preferably is selected from the group consisting of "Escherichia spp., Escherichia coli, Salmonella spp., Salmonella typhi, Salmonella typhimurium, Yersinia spp., Yersinia enterocolitica, Vibrio spp., Vibrio cholerae, Listeria spp., Listeria monocytogenes, Shigella spp., Shigella flexneri", wherein preferably the virulence of the microorganism is attenuated. Further preferred, Vibrio cholerae is excluded from above defined microganisms.

The term "spp." in connection with any microorganism is intended to comprise for the purpose of the present invention all members of a given genus, including species, subspecies and others. The term "Salmonella spp." for instance is intended to comprise all members of the genus Salmonella, such as Salmonella typhi and Salmonella typhimurium.

In another preferred embodiment, a microorganism according to above definitions is provided, wherein the at least one complete or partial antigen of at least one wild-type or mutated protein according to component (I) is selected from the group consisting of the following wild-type proteins and their known mutants: "receptor; extracellular, transmembranic or intracellular part of a receptor; adhesion molecule; extracellular, transmembranic or intracellular part of an adhesion molecule; signal-transducing protein; cell-cycle protein; transcription factor; differentiation protein; embryonic protein; viral protein; allergen; protein of microbial pathogen; protein of eukaryotic pathogen; cancer testis antigen protein; tumor antigen protein; and/or tissue-cell specific protein", wherein the tissue cell is selected from the group consisting of "glandula thyroidea,

glandula mammaria, glandula salivaria, nodus lymphoideus, glandula mammaria, tunica mucosa gastris, kidney, ovarium, prostate, cervix, tunica serosa vesicae urinariae and nevus".

As for the mutated protein, the mutation may have been oncogenic and may have caused a loss or a gain of its original cellular functions.

Such antigens perform in the cell the control of the cell growth and of the cell division and are presented on the cell membrane of normal cells, for instance by the MHC class I molecule. In tumor cells, these antigens are frequently over-expressed or specifically mutated. Such mutations can have function limitations of oncogene suppressors or the activation of proto-oncogenes to oncogenes as a consequence and can be involved alone or commonly with over-expressions in the tumor growth. Such cell antigens are presented on the membrane of tumor cells and thus represent antigens on tumor cells, without however causing an immune reaction affecting the tumor disease of the patient. Rapp (US 5,156,841) has already described the use of oncoproteins, i.e. expression products of the oncogenes, as an immunogen for tumor vaccines.

Examples for antigens and their (oncogenic) mutations according to the invention are i) receptors, such as Her-2/neu, androgen receptor, estrogen receptor, lactoferrin receptor, midkine receptor, EGF receptor, ERBB2, ERBB4, TRAIL receptor, FAS, TNFalpha receptor, TGF-beta receptor; ii) signal-transducing proteins, such as c-Raf (Raf-1), A-Raf, B-Raf, B-Raf V599E, B-Raf V600E, B-Raf KD, B-Raf V600E kinase domain, B-Raf V600E KD, B-Raf V600E kinase domain KD, B-Raf kinase domain, B-Raf kinase domain KD, Ras, Bcl-2, Bcl-X, Bcl-W, Bfl-1, Brag-1, Mcl-1, A1, Bax, BAD, Bak, Bcl-Xs, Bid, Bik, Hrk, Bcr/abl, Myb, C-Met, IAP1, IAO2, XIAP, ML-IAP LIVIN, survivin, APAF-1; iii) proteins of the cell cycle control, such as cyclin D(1-3), cyclin E, cyclin A, cyclin B, cyclin H, Cdk-1, Cdk-2, Cdk-4, Cdk-6, Cdk-7, Cdc25C, p16, p15, p21, p27, p18, pRb, p107, p130, E2F(1-5), GAAD45, MDM2, PCNA, ARF, PTEN, APC, BRCA, p53 and homologues; iv) transcription factors, such as C-Myc, NFkB, c-Jun, ATF-2, Spl; v) embryonic proteins, such as carcinoembryonic antigen, alpha-fetoprotein, MAGE, MAGE-1, MAGE-3, NY-ESO-1, PSCA; vi) differentiation antigens, such as MART, Gp100, tyrosinase, GRP, TCF-4, basic myelin, alpha-lactalbumin, GFAP, prostate specific antigen (PSA), fibrillary acid protein, tyrosinase, EGR-1, MUC1; vii) viral antigens, such as of the following viruses: HIV, HPV, HCV, HPV, EBV, CMV, HSV, influenza virus, influenza virus type A, influenza virus type A (H5N1) and (H3N2), influenza virus type B, influenza virus type C; hemagglutinins, hemagglutinin

H1, hemagglutinin H5, hemagglutinin H7, hemagglutinin HA1 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12C (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), neuramidase, microbial antigens: p60, LLO, urease etc. Antigens of eukaryotic pathogens: CSP (malaria), calflagin (trypanosoma), CPB (Leishmania major) etc.

In yet another preferred embodiment, a microorganism according to above definitions is provided, wherein the at least one complete or partial antigen of at least one wild-type or mutated protein according to component (I) is selected from the group consisting of the following wild-type proteins and their known mutants: “Her-2/neu, androgen receptor, estrogen receptor, midkine receptor, EGF receptor, ERBB2, ERBB4, TRAIL receptor, FAS, TNFalpha receptor, TGF-beta receptor, lactoferrin receptor, basic myelin, alpha-lactalbumin, GFAP, fibrillary acid protein, tyrosinase, EGR-1, MUC1, c-Raf (Raf-1), A-Raf, B-Raf, B-Raf V599E, B-Raf V600E, B-Raf KD, B-Raf V600E kinase domain, B-Raf V600E KD, B-Raf V600E kinase domain KD, B-Raf kinase domain, B-Raf kinase domain KD, N-Ras, K-Ras, H-Ras, Bcl-2, Bcl-X, Bcl-W, Bfl-1, Brag-1, Mcl-1, A1, Bax, BAD, Bak, Bcl-Xs, Bid, Bik, Hrk, Bcr/abl, Myb, C-Met, IAP1, IAO2, XIAP, ML-IAP LIVIN, survivin, APAF-1, cyclin D(1-3), cyclin E, cyclin A, cyclin B, cyclin H, Cdk-1, Cdk-2, Cdk-4, Cdk-6, Cdk-7, Cdc25C, p16, p15, p21, p27, p18, pRb, p107, p130, E2F(1-5), GAAD45, MDM2, PCNA, ARF, PTEN, APC, BRCA, Akt, PI3K, mTOR, p53 and homologues, C-Myc, NFkB, c-Jun, ATF-2, Sp1, prostate specific antigen (PSA), carcinoembryonic antigen, alpha-fetoprotein, PAP; PSMA; STEAP; MAGE, MAGE-1, MAGE-3, NY-ESO-1, PSCA, MART, Gp100, tyrosinase, GRP, TCF-4, viral antigens of the viruses HIV, HPV, HCV, HPV, EBV, CMV, HSV, influenza virus, influenza virus type A, influenza virus type A (H5N1) and (H3N2), influenza virus type B, influenza virus type C; hemagglutinins, hemagglutinin H1, hemagglutinin H5, hemagglutinin H7, hemagglutinin HA1 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12C (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), neuramidase, p60, LLO, urease, CSP, calflagin and/or CPB”.

In yet another preferred embodiment, a microorganism according to above definitions is provided, wherein the at least one complete or partial antigen of at least one

wild-type or mutated protein according to component (I) is selected from the group of kinases consisting of the following wild-type proteins and their known mutants (accession numbers in parantheses): AAK1 (NM 014911), AATK (NM 004920), ABL1 (NM 005157), ABL2 (NM 005158), ACK1 (NM 005781), ACVR1 (NM 001105), ACVR1B (NM 020328), ACVR2 (NM 001616), ACVR2B (NM 001106), ACVRL1 (NM 000020), ADCK1 (NM 020421), ADCK2 (NM 052853), ADCK4 (NM 024876), ADCK5 (NM 174922), ADRBK1 (NM 001619), ADRBK2 (NM 005160), AKT1 (NM 005163), AKT2 (NM 001626), AKT3 (NM 005465), ALK (NM 004304), ALK7 (NM 145259), ALS2CR2 (NM 018571), ALS2CR7 (NM 139158), AMHR2 (NM 020547), ANKK1 (NM 178510), ANKRD3 (NM 020639), APEG1 (NM 005876), ARAF (NM 001654), ARK5 (NM 014840), ATM (NM 000051), ATR (NM 001184), AURKA (NM 003600), AURKB (NM 004217), AURKC (NM 003160), AXL (NM 001699), BCKDK (NM 005881), BCR (NM 004327), BIKE (NM 017593), BLK (NM 001715), BMPR1A (NM 004329), BMPR1B (NM 001203), BMPR2 (NM 001204), BMX (NM 001721), BRAF (NM 004333), BRD2 (NM 005104), BRD3 (NM 007371), BRD4 (NM 014299), BRDT (NM 001726), BRSK1 (NM 032430), BRSK2 (NM 003957), BTK (NM 000061), BUB1 (NM 004336), BUB1B (NM 001211), CABC1 (NM 020247), CAMK1 (NM 003656), CaMK1b (NM 198452), CAMK1D (NM 020397), CAMK1G (NM 020439), CAMK2A (NM 015981), CAMK2B (NM 001220), CAMK2D (NM 001221), CAMK2G (NM 001222), CAMK4 (NM 001744), CAMKK1 (NM 032294), CAMKK2 (NM 006549), CASK (NM 003688), CCRK (NM 012119), CDC2 (NM 001786), CDC2L1 (NM 001787), CDC2L5 (NM 003718), CDC42BPA (NM 014826), CDC42BPB (NM 006035), CDC7L1 (NM 003503), CDK10 (NM 003674), CDK11 (NM 015076), CDK2 (NM 001798), CDK3 (NM 001258), CDK4 (NM 000075), CDK5 (NM 004935), CDK6 (NM 001259), CDK7 (NM 001799), CDK8 (NM 001260), CDK9 (NM 001261), CDKL1 (NM 004196), CDKL2 (NM 003948), CDKL3 (NM 016508), CDKL4 (NM 001009565), CDKL5 (NM 003159), CHEK1 (NM 001274), CHUK (NM 001278), CIT (NM 007174), CLK1 (NM 004071), CLK2 (NM 003993), CLK3 (NM 003992), CLK4 (NM 020666), CRK7 (NM 016507), CSF1R (NM 005211), CSK (NM 004383), CSNK1A1 (NM 001892), CSNK1D (NM 001893), CSNK1E (NM 001894), CSNK1G1 (NM 022048), CSNK1G2 (NM 001319), CSNK1G3 (NM 004384), CSNK2A1 (NM 001895), CSNK2A2 (NM 001896), DAPK1 (NM 004938), DAPK2 (NM 014326), DAPK3 (NM 001348), DCAMKL1 (NM 004734), DCAMKL2 (NM 152619), DCAMKL3 (XM 047355), DDR1 (NM 013993), DDR2 (NM 006182), DMPK (NM 004409), DMPK2 (NM 017525.1), DYRK1A (NM 001396), DYRK1B (NM 006484), DYRK2 (NM 006482), DYRK3 (NM 003582), DYRK4 (NM 003845), EEF2K

(NM 013302), EGFR (NM 005228), EIF2AK3 (NM 004836), EIF2AK4 (NM_001013703), EPHA1 (NM 005232), EPHA10 (NM 001004338), EPHA2 (NM 004431), EPHA3 (NM 005233), EPHA4 (NM 004438), EPHA5 (NM 004439), EPHA6 (XM 114973), EPHA7 (NM 004440), EPHA8 (NM 020526), EPHB1 (NM 004441), EPHB2 (NM 017449), EPHB3 (NM 004443), EPHB4 (NM 004444), EPHB6 (NM 004445), ERBB2 (NM 004448), ERBB3 (NM 001982), ERBB4 (NM 005235), ERK8 (NM 139021), ERN1 (NM 001433), ERN2 (NM 033266), FASTK (NM 025096), FER (NM 005246), FES (NM 002005), FGFR1 (NM 000604), FGFR2 (NM 022970), FGFR3 (NM 000142), FGFR4 (NM 022963), FGR (NM 005248), FLJ23074 (NM 025052), FLJ23119 (NM 024652), FLJ23356 (NM 032237), FLT1 (NM 002019), FLT3 (NM 004119), FLT4 (NM 002020), FRAP1 (NM 004958), FRK (NM 002031), FYN (NM 002037), GAK (NM 005255), GPRK5 (NM 005308), GPRK6 (NM 002082), GPRK7 (NM 139209), GRK4 (NM 005307), GSG2 (NM 031965), GSK3A (NM 019884), GSK3B (NM 002093), GUCY2C (NM 004963), GUCY2D (NM 000180), GUCY2F (NM 001522), H11 (NM 014365), HAK (NM 052947), HCK (NM 002110), HIPK1 (NM 152696), HIPK2 (NM 022740), HIPK3 (NM 005734), HIPK4 (NM 144685), HRI (NM 014413), HUNK (NM 014586), ICK (NM 016513), IGF1R (NM 000875), IKBKB (NM 001556), IKBKE (NM 014002), ILK (NM 004517), INSR (NM 000208), INSRR (NM 014215), IRAK1 (NM 001569), IRAK2 (NM 001570), IRAK3 (NM 007199), IRAK4 (NM 016123), ITK (NM 005546), JAK1 (NM 002227), JAK2 (NM 004972), JAK3 (NM 000215), KDR (NM 002253), KIS (NM 144624), KIT (NM 000222), KSR (XM 290793), KSR2 (NM 173598), LAK (NM 025144), LATS1 (NM 004690), LATS2 (NM 014572), LCK (NM 005356), LIMK1 (NM 016735), LIMK2 (NM 005569), LMR3 (XM 055866), LMTK2 (NM 014916), LOC149420 (NM 152835), LOC51086 (NM 015978), LRRK2 (XM 058513), LTK (NM 002344), LYN (NM 002350), MAK (NM 005906), MAP2K1 (NM 002755), MAP2K2 (NM 030662), MAP2K3 (NM 002756), MAP2K4 (NM 003010), MAP2K5 (NM 002757), MAP2K6 (NM 002758), MAP2K7 (NM 005043), MAP3K1 (XM 042066), MAP3K10 (NM 002446), MAP3K11 (NM 002419), MAP3K12 (NM 006301), MAP3K13 (NM 004721), MAP3K14 (NM 003954), MAP3K2 (NM 006609), MAP3K3 (NM 002401), MAP3K4 (NM 005922), MAP3K5 (NM 005923), MAP3K6 (NM 004672), MAP3K7 (NM 003188), MAP3K8 (NM 005204), MAP3K9 (NM 033141), MAP4K1 (NM 007181), MAP4K2 (NM 004579), MAP4K3 (NM 003618), MAP4K4 (NM 145686), MAP4K5 (NM 006575), MAPK1 (NM 002745), MAPK10 (NM 002753), MAPK11 (NM 002751), MAPK12 (NM 002969), MAPK13 (NM 002754), MAPK14 (NM 001315), MAPK3 (NM 002746), MAPK4 (NM 002747), MAPK6 (NM 002748), MAPK7 (NM

002749), MAPK8 (NM 002750), MAPK9 (NM 002752), MAPKAPK2 (NM 032960), MAPKAPK3 (NM 004635), MAPKAPK5 (NM 003668), MARK (NM 018650), MARK2 (NM 017490), MARK3 (NM 002376), MARK4 (NM 031417), MAST1 (NM 014975), MAST205 (NM 015112), MAST3 (XM 038150), MAST4 (XM 291141), MASTL (NM 032844), MATK (NM 139355), MELK (NM 014791), MERTK (NM 006343), MET (NM 000245), MGC33182 (NM 145203), MGC42105 (NM 153361), MGC43306 (C9orf96), MGC8407 (NM 024046), MIDORI (NM 020778), MINK (NM 015716), MKNK1 (NM 003684), MKNK2 (NM 017572), MLCK (NM 182493), MLK4 (NM 032435), MLKL (NM 152649), MOS (NM 005372), MST1R (NM 002447), MST4 (NM 016542), MUSK (NM 005592), MYLK (NM 053025), MYLK2 (NM 033118), MYO3A (NM 017433), MYO3B (NM 138995), NEK1 (NM 012224), NEK10 (NM 152534), NEK11 (NM 024800), NEK2 (NM 002497), NEK3 (NM 002498), NEK4 (NM 003157), NEK5 (MGC75495), NEK6 (NM 014397), NEK7 (NM 133494), NEK8 (NM 178170), NEK9 (NM 033116), NLK (NM 016231), NPR1 (NM 000906), NPR2 (NM 003995), NRBP (NM 013392), NRBP2 (NM 178564), NRK (NM 198465), NTRK1 (NM 002529), NTRK2 (NM 006180), NTRK3 (NM 002530), OBSCN (NM 052843), OSR1 (NM 005109), PACE-1 (NM 020423), PAK1 (NM 002576), PAK2 (NM 002577), PAK3 (NM 002578), PAK4 (NM 005884), PAK6 (NM 020168), PAK7 (NM 020341), PASK (NM 015148), PCTK1 (NM 006201), PCTK2 (NM 002595), PCTK3 (NM 212503), PDGFRA (NM 006206), PDGFRB (NM 002609), PDK1 (NM 002610), PDK2 (NM 002611), PDK3 (NM 005391), PDK4 (NM 002612), PDPK1 (NM 002613), PFTK1 (NM 012395), PHKG1 (NM 006213), PHKG2 (NM 000294), PIK3R4 (NM 014602), PIM1 (NM 002648), PIM2 (NM 006875), PIM3 (NM 001001852), PINK1 (NM 032409), PKE (NM 173575), PKMYT1 (NM 004203), pknbeta (NM 013355), PLK (NM 005030), PLK3 (NM 004073), PRKAA1 (NM 006251), PRKAA2 (NM 006252), PRKACA (NM 002730), PRKACB (NM 002731), PRKACG (NM 002732), PRKCA (NM 002737), PRKCB1 (NM 002738), PRKCD (NM 006254), PRKCE (NM 005400), PRKCG (NM 002739), PRKCH (NM 006255), PRKCI (NM 002740), PRKCL1 (NM 002741), PRKCL2 (NM 006256), PRKCM (NM 002742), PRKCN (NM 005813), PRKCQ (NM 006257), PRKCZ (NM 002744), PRKD2 (NM 016457), PRKDC (NM 006904), PRKG1 (NM 006258), PRKG2 (NM 006259), PRKR (NM 002759), PRKWNK1 (NM 018979), PRKWNK2 (NM 006648), PRKWNK3 (NM 020922), PRKWNK4 (NM 032387), PRKX (NM 005044), PRKY (NM 002760), PRPF4B (NM 003913), PSKH1 (NM 006742), PSKH2 (NM 033126), PTK2 (NM 005607), PTK2B (NM 004103), PTK6 (NM 005975), PTK7 (NM 002821), PTK9 (NM 002822), PTK9L (NM 007284), PXK (NM 017771), QSK (NM 025164), RAD53 (NM

007194), RAF1 (NM 002880), RAGE (NM 014226), RET (NM 020975), RHOK (NM 002929), RIOK1 (NM 031480), RIOK2 (NM 018343), RIPK1 (NM 003804), RIPK2 (NM 003821), RIPK3 (NM 006871), RIPK5 (NM 015375), RNASEL (NM 021133), ROCK1 (NM 005406), ROCK2 (NM 004850), ROR1 (NM 005012), ROR2 (NM 004560), ROS1 (NM 002944), RPS6KA1 (NM 002953), RPS6KA2 (NM 021135), RPS6KA3 (NM 004586), RPS6KA4 (NM 003942), RPS6KA5 (NM 004755), RPS6KA6 (NM 014496), RPS6KB1 (NM 003161), RPS6KB2 (NM 003952), RPS6KC1 (NM 012424), RPS6KL1 (NM 031464), RYK (NM 002958), SBK (XM 370948), SCYL1 (NM 020680), SCYL2 (NM 017988), SGK (NM 005627), SgK069 (SU SgK069), SgK085 (XM 373109), SgK110 (SU SgK110), SGK2 (NM 016276), SgK223 (XM 291277), SgK269 (XM 370878), SgK424 (CGP SgK424), SgK493 (SU_SgK493), SgK494 (NM 144610), SgK495 (NM 032017), SGKL (NM 013257), SK681 (NM 001001671), SLK (NM 014720), SMG1 (NM 015092), SNARK (NM 030952), SNF1LK (NM 173354), SNF1LK2 (NM 015191), SNK (NM 006622), SNRK (NM 017719), SRC (NM 005417), SRMS (NM 080823), SRPK1 (NM 003137), SRPK2 (NM 003138), SSTK (NM 032037), STK10 (NM 005990), STK11 (NM 000455), STK16 (NM 003691), STK17A (NM 004760), STK17B (NM 004226), STK18 (NM 014264), STK19 (NM 032454), STK22B (NM 053006), STK22C (NM 052841), STK22D (NM 032028), STK23 (NM 014370), STK24 (NM 003576), STK25 (NM 006374), STK3 (NM 006281), STK31 (NM 031414), STK32B (NM 018401), STK33 (NM 030906), STK35 (NM 080836), STK36 (NM 015690), STK38 (NM 007271), STK38L (NM 015000), STK39 (NM 013233), STK4 (NM 006282), STLK5 (NM 001003787), STYK1 (NM 018423), SUDD (NM 003831), SYK (NM 003177), TAF1 (NM 138923), TAF1L (NM 153809), TAO1 (NM 004783), TAOK1 (NM 020791), TAOK3 (NM 016281), TBCK (NM 033115), TBK1 (NM 013254), TEC (NM 003215), TEK (NM 000459), TESK1 (NM 006285), TESK2 (NM 007170), TEX14 (NM 031272), TGFBR1 (NM 004612), TGFBR2 (NM 003242), TIE (NM 005424), TIF1 (NM 003852), TLK1 (NM 012290), TLK2 (NM 006852), TNIK (NM 015028), TNK1 (NM 003985), TOPK (NM 018492), TP53RK (NM 033550), TRAD (NM 007064), TRIB1 (NM 025195), TRIB2 (NM 021643), TRIB3 (NM 021158), TRIM28 (NM 005762), TRIM33 (NM 015906), TRIO (NM 007118), TRPM6 (NM 017662), TRPM7 (NM 017672), TRRAP (NM 003496), TSSK4 (NM 174944), TTBK1 (NM 032538), TTBK2 (NM 173500), TTK (NM 003318), TTN (NM 003319), TXK (NM 003328), TYK2 (NM 003331), TYRO3 (NM 006293), ULK1 (NM 003565), ULK2 (NM 014683), ULK3 (NM 015518), ULK4 (NM 017886), VRK1 (NM 003384), VRK2 (NM

006296), VRK3 (NM 016440), WEE1 (NM 003390), Wee1B (NM 173677), YANK1 (NM 145001), YES1 (NM 005433), ZAK (NM 016653), and/or ZAP70 (NM 001079)".

The term "allergen" in the course of the present invention refers to complete or partial antigens as defined herein that elicit hypersensitivity and/or allergic reactions. Examples are Der p 5 (mite), Bet v 1 (birch pollen), Phl p 1 (grass pollen), Asp f I/a (Aspergillus), PLA 2 (bee), Hev b (latex) (Schmid-Grendelmeier and Crameri, 2001).

Antigens of microbial and eukaryotic pathogens and of cancer testis antigens are enclosed in the list above.

In the course of the present invention, protein toxins and/or their subunits according to component (II) are preferably bacterial protein toxins, more preferably exotoxins. Examples of bacterial exotoxins are type I toxins (superantigens), type II toxins (pore-forming toxins) and (iii) type III toxins (A-B toxins).

In a preferred embodiment, a microorganism according to above definitions is provided, wherein component (II) is selected from the group consisting of "bacterial toxin, enterotoxin, exotoxin, type I toxin, type II toxin, type III toxin, type IV toxin, type V toxin, RTX toxin, AB toxin, A-B toxin, A/B toxin, A+B toxin, A-5B toxin and/or AB5 toxin.

In yet a further preferred embodiment, a microorganism according to above definitions is provided, wherein component (II) is selected from the group consisting of "Adenylate cyclase toxin, Anthrax toxin, Anthrax toxin (EF), Anthrax toxin (LF), Botulinum toxin, Cholera toxin (CT, Ctx), Cholera toxin subunit B (CTB, CtxB), Diphtheria toxin (DT, Dtx), E. coli LT toxin, E. coli heat labile enterotoxin (LT), E. coli heat labile enterotoxin subunit B (LTB), E. coli ST toxin, E. coli heat stabile enterotoxin (ST), Erythrogenic toxin, Exfoliatin toxin, Exotoxin A, Perfringens enterotoxin, Pertussis toxin (PT, Ptx), Shiga toxin (ST, Stx), Shiga toxin subunit B (STB, StxB), Shiga-like toxin, Staphylococcus enterotoxins, Tetanus toxin (TT), Toxic shock syndrome toxin (TSST-1), Vero toxin (VT), Toxin A (TA) and Toxin B (TB) of Clostridium difficile, Lethal Toxin (LT) and Hemorrhagic Toxin (HT) of Clostridium sordellii, alpha Toxin (AT) of Clostridium novyi".

However, if Cholera Toxin or its subunit CtxB is used as toxins according to component (II) of the invention, it is preferred not to employ Vibrio cholerae as bacterial carrier (microorganism).

In a preferred embodiment, a microorganism according to above definitions is provided, wherein component (I) and component (II) are linked together to enable the expression and/or secretion of a fusion protein encoded by both components. More preferably, this fusion protein is selected from the group consisting of "CtxB–PSA, CtxB–B-Raf V600E KD (kinase dead), CtxB–B-Raf V600E kinase domain, CtxB–B-Raf V600E kinase domain KD (kinase dead), CtxB-B-Raf, CtxB-B-Raf KD (kinase dead), CtxB B-Raf kinase domain KD (kinase dead), CtxB-HA1 (subunit 1 of a hemagglutinin of an influenca virus), CtxB-HA12C".

Secretion is the process of segregating, elaborating, and releasing chemicals from a cell, or a secreted chemical substance or amount of substance. Secretion is not unique to eukaryotes alone; it is present in bacteria and archaea as well. ATP binding cassette (ABC) type transporters are common to all the three domains of life. The Sec system is also another conserved secretion system which is homologous to the translocon in the eukaryotic endoplasmic reticulum consisting of Sec 61 translocon complex in yeast and Sec Y-E-G complex in bacteria. Gram-negative bacteria have two membranes, thus making secretion topologically more complex. So there are at least five specialized secretion system in Gram negative bacteria:

(1) Type I secretion system: It is same as the ATP binding cassette transporters mentioned above.

(2) Type II secretion system: It depends on the Sec system for a protein to cross the inner membrane and another special system to cross the outer membrane. Bacterial pili use modifications of the sec system, but are different from type I system.

(3) Type III secretion system (T3SS): It is homologous to bacterial flagellar basal body. It is like a molecular syringe through which a bacterium (e.g. Shigella or Yersinia) can inject proteins into eukaryotic cells. The low Ca^{2+} concentration in the cytosol opens the gate that regulates T3SS. The Hrp system in plant pathogens injects hairpins through similar mechanisms into plants.

(4) Type IV secretion system: It is homologous to conjugation machinery of bacteria (and archaeal flagella). It is capable of transporting both DNA and proteins. It was discovered in *Agrobacterium tumefaciens*, which uses this system to introduce the Ti plasmid and proteins into the host which develops the crown gall (tumor). *Helicobactor pylori* uses a type IV secretion system to inject Cag A into gastic epithelial cells. *Borde-*

tella pertussis, the causative agent of whooping cough, secretes the pertussis toxin partly through the type IV system.

(5) Type V secretion system, also called autotransporter system: This use the sec system for crossing the inner membrane. The proteins which use this path have the capability to form a beta barrel in their C terminus and insert into the outer membrane to transport the rest of the peptide out. Finally the beta barrel may be cleaved and left back in the outer membrane. Some people believe these remnants of the autotransporters gave rise to the porins which are similar beta barrels.

Bacteria as well as mitochondria and chloroplasts also use many other special transport systems such as the twin-arginine translocation (Tat) pathway which, in contrast to Sec-dependent export, transports fully folded proteins across the membrane. The name of the system comes from the requirement for two consecutive arginines in the signal sequence required for targeting to this system. Secretion in gram-negative bacteria involves overcoming the inner and outer membrane by the way of a suitable secretion system, like e.g. the Hly type I or type III secretion system or AIDA autotransporter. In gram-positive bacteria the secretion system has to overcome the inner membrane and the cell wall, which, in most strains, can be achieved by fusion with a suitable secretion signal.

Component (III) a) is at least one nucleotide sequence coding for at least one transport system which enables the expression of the expression products of component (I) and component (II) on the outer surface of the microorganism and/or enables the secretion of the expression products of component (I) and component (II). The respective component can as an option be either secreted or expressed on the membrane of the microorganism, i.e. is membrane-bound. Such transport systems are for instance i) type I secretion system, type II secretion system, type III secretion system, type IV secretion system, type V secretion system, ii) the hemolysin transport system (signal) of E. coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter); the following transport signals are to be used: for the secretion - the C-terminal HlyA transport signal, in presence of HlyB and HlyD proteins; for the membrane-bound expression - the C-terminal HlyA transport signal, in presence of HlyB protein, iii) the hemolysin transport system (signal) of E. coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of a not hly-specific bacterial promoter), iv) the transport signal for the S-layer protein (Rsa A) of Caulobac-

ter crescentus; the following transport signals are to be used: for the secretion and the membrane-bound expression - the C-terminal RsaA transport signal, v) the transport signal for the TolC protein Escherichia coli; the following transport signals are to be used: for the membrane-bound expression - the N-terminal transport signal of TolC (the integral membrane protein TolC of E. coli is a multi-functional pore-forming protein of the outer membrane of E. coli, which serves – in addition to functions such as the reception of colicin E1 (Morona et al., 1983) and the secretion of colicin V (Fath et al., 1991) also as a receptor for the U3 phage (Austin et al., 1990); this protein is not only found in E. coli, but also in a multitude of gram-negative bacteria (Wiener, 2000); the localization in the outer membrane and the wide occurrence make TolC to an ideal candidate to present heterologous antigens, in order e.g. to cause an immune reaction.

Gram positive bacteria do not encompass an outer membrane. Secretion in these cases is simpler and usually does not require dedicated secretion machinery for transport through the cell membrane and cell wall. In the case of gram positive bacteria, secretion signals fused N-terminally to the heterologous protein are usually necessary and sufficient for secretion. Proteins, for which these signal sequences have been described, comprise principally all secreted bacterial proteins. Examples for listeria are the secretion signals derived from Listeriolysin, p60 or ActA. In general, a similar approach applies to eukaryotic cells, where a signal sequence (for instance the ubiquitous secretion signal Vtgss) targeting the protein to the endoplasmatic reticulum in the absence of a retention signal is necessary and sufficient for secretion.

Optional component (III) b) is at least one nucleotide sequence coding for at least one protein for lysing the microorganism in the cytosol of mammalian cells and for intracellularly releasing plasmids or expression vectors, which are contained in the lysed microorganism. Such lytic proteins (endolysins) are for instance Listeria-specific lysis proteins, such as PLY551 (Loessner et al., 1995) and/or the Listeria-specific holin under the control of a listerial promoter. A preferred embodiment of this invention is the combination of different components (III) b), for instance the combination of a lysis protein and the holin.

Both components (III) a) and/or (III) b) may be independently from each other constitutively active.

In a preferred embodiment, a microorganism according to above definitions is provided, wherein component (III) a) is selected from the group consisting of "type I secretion system, type II secretion system, type III secretion system, type IV secretion system, type V secretion system, hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter), hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of a not hly-specific bacterial promoter), transport signal for the S-layer (Rsa A) protein of Caulobacter crescentus, transport signal for the TolC protein of Escherichia coli, secretion signal Vtgss and/or secretion signals derived from listeriolysin, p60 and/or ActA" and wherein component (III) b) is selected from the group consisting of "endolysins, lytic protein of gram-positive bacteria, lytic protein of Listeria monocytogenes, PLY551 of Listeria monocytogenes and/or holin of Listeria monocytogenes".

In a further preferred embodiment, the same component (III) a) enables the expression of the expression products of component (I) and component (II) on the outer surface of the microorganism and/or enables the secretion of the expression products of component (I) and component (II). That is in this preferred embodiment component (III) a) is at least one nucleotide sequence coding for only one transport system, which enables the concomitant expression of the expression products of component (I) and component (II) on the outer surface of the microorganism and/or enables the concomitant secretion of the expression products of component (I) and component (II), wherein such preferred component (III) a) is at least one nucleotide sequence coding for the hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter) or the hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of a not hly-specific bacterial promoter).

In yet another preferred embodiment, a microorganism according to above definitions is provided, wherein according to component (III) a) the expression products of components (I) and component (II) are secreted. More preferably, component (I) and component (II) are linked together, expressed together and secreted as a fusion protein encoded by both components. Most preferably, this fusion protein is selected from the group consisting of "CtxB–PSA, CtxB–B-Raf V600E KD (kinase dead), CtxB–B-Raf V600E kinase domain, CtxB–B-Raf V600E kinase domain KD (kinase dead), CtxB-B-Raf, CtxB-B-Raf KD (kinase dead), CtxB B-Raf kinase domain KD (kinase dead), CtxB-HA1 (subunit 1 of a hemagglutinin of an influenca virus), CtxB-HA12C".

In the course of the present invention, the term “secretion” refers to the secretion of a protein antigen, protein toxin and/or toxin-antigen fusion protein through both membranes of a gram-negative bacterium or through the inner membrane and cell-wall of a gram-positive bacterium into the neighboring environment, by the means of a proper secretion system, such as the ones illustrated above.

As it is known that using a secretion system, such as the hemolysin type I secretion system from E. coli, secretion product is usually found in all cellular fractions: cytoplasmatically, membrane-associated, located in auto-membrane vesicles and fully secreted into the neighboring environment (Balsalobre et al., 2006), secretion in the course of the present invention does not need to be complete. However, a complete or an almost complete secretion, i.e. a majority quantity of fully secreted secretion product, is desired and preferred.

Component (IV) represents at least one nucleotide sequence for at least one activation sequence for the expression of one or more of components (I) to (III), wherein said activation sequence can be activated in the microorganism and/or is tissue cell-specific, tumor cell-specific, macrophage-specific, dendrite-specific, lymphocyte-specific, function-specific or non-cell-specific.

If the expression is membrane-bound on the outer surface of the microorganism, the activation sequence has preferably to be selected such that it is capable of being activated in the microorganism. Such activation sequences are for instance: i) constitutively active promoter regions, such as the promoter region with "ribosomal binding site" (RBS) of the beta-lactamase gene of E. coli or of the tetA gene (Busby and Ebright, 1994), endogenous promoter of the E. coli hly locus; ii) promoters, which are capable of being induced, preferably promoters, which become active after reception in the cell. To these belong the actA promoter of L. monocytogenes (Dietrich et al., 1998) or the pagC promoter of S. typhimurium (Bumann, 2001).

If the plasmids are released from the microorganism after its lysis into the cytosol of the mammalian cell, the activation sequence is not cell-specific, but tissue cell-specific, cell cycle-specific or function-specific. Preferably, such activation sequences are selected, which are particularly activated in macrophages, dendritic cells and lymphocytes.

In a further preferred embodiment, a microorganism according to above definitions is provided, wherein

component **(I)** is selected from the group consisting of B-Raf V600E, B-Raf V600E kinase domain, B-Raf V600E KD (kinase dead), B-Raf V600E kinase domain KD (kinase dead), B-Raf KD (kinase dead), B-Raf kinase domain, B-Raf kinase domain KD (kinase dead), prostate specific antigen (PSA), hemagglutinin HA1 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12C (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1)";

component **(II)** is selected from the group consisting of "Cholera toxin subunit B (CTB, CtxB), E. coli heat-labile enterotoxin subunit B (LTB), tetanus toxin (TT)";

component **(III) a)** is selected from the group consisting of "HlyA hemolysin transport signal of Escherichia coli together with components of the Hly secretion system (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter)";

component **(IV)** is selected from the group consisting of "endogenous promoter of the E. coli hly locus";

wherein component (I) and component (II) are linked together to enable the expression of a fusion protein encoded by both components and wherein the fusion protein is secreted.

The above illustrated microorganisms according to components (I) to (IV) as well as the preferred embodiments are hereinafter referred to as microorganisms of the invention.

The microorganisms of the invention are advantageously suited for use in tumor therapy, as live vaccines in the course of tumor-targeting. That is by means of microorganisms of the invention, which function as carriers of genetic information, heterologous antigens together with protein toxins are transported to the tumor site, expressed as fusion proteins and secreted in situ.

The microorganisms of the invention are surprisingly and advantageously characterized through an efficient and superior expression and secretion of the transported,

encoded and expressed toxin-antigen fusion proteins, i.e. no cytoplasmatic and/or periplasmatic aggregates occur.

Moreover, by administration of the microorganisms of the invention a strong systemic cellular immune system response is surprisingly elicited compared to orally administered protein vaccines which in contrast induce a systemic tolerance of the immune system.

Most notably, beside the induction of a systemic cellular immune system response of Th1 type, the microorganisms of the invention induce the activation of MHC class I restricted $CD8^+$ cytotoxic T-cells (CTL) and strongly enhance this CTL immune response.

Further, in addition to the induction and/or enhancement of strong cellular systemic Th1 and CTL immune system responses, the innate immune system, e.g. NK cells, NKT cells and/or gamma-delta T-cells, is also surprisingly activated by the microorganisms of the invention in a synergistic manner.

If Cholera Toxin is used as toxin component, the microorganisms of the invention possess the advantage that in humans no preexisting immunity against the toxin normally exists (in contrast to tetanus toxin for instance due to anti-tetanus vaccinations during childhood). Use of Cholera-Toxin and/or its subunits, in particular CtxB, is therefore preferred.

The microorganisms of the invention are particularly suitable for oral administration in live-vaccine based targeted tumor (immune) therapy. Thereby an improved patient compliance can be accomplished.

However, the microorganisms of the invention are not limited to the use in tumor therapy only. Principally, the microorganisms of the invention are also suitable for the treatment and/or prophylaxis of all these diseases that require a therapy in which induction of a systemic cellular Th1 immune system response is mandatory. Examples of such infectious diseases include, but are not limited to, HIV, influenza, HCV and other viral diseases, Mycobacterium tuberculosis, Listeria monocytogenes and other bacterial diseases.

The microorganisms of the invention are perfectly suited for the treatment of diseases like influenza, which require an optimum protection by the combination of mucosal immune system response and systemic cellular immune response. In addition, they might be useful for specific induction of Th1 like immunity in allergic diseases, e.g. al-

lergic rhinitis. In such a case, the antigen would be an allergen that is fused to the protein toxin component and the principle of action of the respective vaccine would be the shift of the immune response from a Th2 dominated immune response against the allergen in allergic reactions (diseases) towards a Th1 immune response.

A preferred mode of application is oral application. In a preferred embodiment, a Salmonella strain according to this invention is fermented in appropriate medium, harvested and washed by centrifugation and subsequently formulated and stabilized using appropriate substances and lyophylized. The lyophylized substance is filled into stomach resistant capsules containing life cell numbers preferably between 10^9 to 10^{10} bacteria. The capsules are orally uptaken with liquid.

Alternatively, lyophylized bacteria as described above are distributed together with sachets containing buffer which is able to neutralize the stomach acid (pharmaceutical kit). In a preferred embodiment, this buffer is a carbonate buffer. Immediately prior to use, the buffer is prepared with water and taken up, immediately afterwards the lyophylized bacteria are uptaken mixed with water.

Yet another alternative is the use of frozen bacteria. In this case, after washing bacteria are stabilized via a stabilizer, preferably succhrose or glycerine, and subsequently frozen and stored at -80°C preferably in concentrations between 10^9 to 10^{10} bacteria per dose. This preparation is preferably used in a pharmaceutical kit as described above in conjunction with carbonate buffer.

In a preferred embodiment, a pharmaceutical composition is provided comprising at least one microorganism of the invention, preferably at least one lyophilized microorganism of the invention, and a pharmaceutically acceptable carrier, preferably capsules.

The components (I) to (IV) according to the present invention are introduced into the microorganisms of the invention by methods well known to the man skilled in the art. If the microorganisms represent bacteria, the components are inserted into plasmids or expression vectors, and the plasmids or expression vectors are transferred into the bacteria. The molecular biologic cloning and transformation techniques suitable for the manufacturing of the plasmids, expression vectors and microorganisms of the in-

vention are well-known to the person skilled in the art and represent routine experimental work.

Another subject matter of the invention is the administration of a medicament preparation containing the microorganisms of the invention. The administration is made locally or systemically, for instance orally, perorally, rectally, into the epidermis, into the subcutis, into the musculature, into a body cavity, into an organ, into the tumor or into the blood circulation.

Such medicament preparations are for instance suspensions of the microorganisms of the invention in solutions familiar to the pharmacist, suitable for injection.

A particular subject matter of this invention is the peroral or rectal administration of a medicament according to the invention for the treatment and/or prophylaxis of diseases. The administration can be made once or several times. In each administration, approximately 10 to 10^{11} microorganisms of the invention are administered. If the administration of this number of microorganisms of the invention does not cause a sufficient immune reaction, the number to be injected has to be increased.

After administration of the microorganisms of the invention, the tolerance for a cell presenting component (I), for instance a tumor cell, or a tissue cell, from which the tumor originates, is broken, and a strong systemic immune response directed against the tumor and/or its tissue cells is triggered. Depending on the selection of component (I), this cellular immune reaction is directed either exclusively against the tumor or also against tumor cells including the tissue cells, from which the tumor cells originate.

In another aspect, the object of the present invention has been solved by providing a medicament comprising at least one microorganism of the invention comprising above illustrated genetic components (I) to (IV) or at least one pharmaceutical composition as defined herein.

In a preferred embodiment, the microorganisms of the invention can be used for the production of a medicament for the treatment and/or prophylaxis of physiological and/or pathophysiological conditions selected from the group consisting of “uncontrolled cell division, malignant tumors, benign tumors, solid tumors, sarcomas, carcinomas, hyperproliferative disorders, carcinoids, Ewing sarcomas, Kaposi sarcomas, brain tumors, tumors originating from the brain and/or the nervous system and/or the meninges, glio-

mas, neuroblastomas, stomach cancer, kidney cancer, kidney cell carcinomas, prostate cancer, prostate carcinomas, connective tissue tumors, soft tissue sarcomas, pancreas tumors, liver tumors, head tumors, neck tumors, oesophageal cancer, thyroid cancer, osteosarcomas, retinoblastomas, thymoma, testicular cancer, lung cancer, bronchial carcinomas, breast cancer, mamma carcinomas, intestinal cancer, colorectal tumors, colon carcinomas, rectum carcinomas, gynecological tumors, ovary tumors/ovarian tumors, uterine cancer, cervical cancer, cervix carcinomas, cancer of body of uterus, corpus carcinomas, endometrial carcinomas, urinary bladder cancer, bladder cancer, skin cancer, basaliomas, spinaliomas, melanomas, intraocular melanomas, leukemia, chronic leukemia, acute leukemia, lymphomas, infection, viral or bacterial infection, influenza, chronic inflammation, organ rejection and/or autoimmune diseases".

Bacterial infections comprise, but are not limited to, anthrax, bacterial meningitis, botulism, brucellosis, campylobacteriosis, cat scratch disease, cholera, diphtheria, epidemic typhus, impetigo, legionellosis, leprosy (Hansen's disease), leptospirosis, listeriosis, lyme disease, melioidosis, MRSA infection, nocardiosis, pertussis (whooping cough), plague, pneumococcal pneumonia, psittacosis, Q fever, Rocky Mountain Spotted Fever (RMSF), salmonellosis, scarlet fever, shigellosis, syphilis, tetanus, trachoma, tuberculosis, tularemia, typhoid fever, typhus, urinary tract infections, bacterially caused heart diseases.

Viral infections comprise, but are not limited to, AIDS, AIDS related complex (ARC), chickenpox (varicella), common cold, cytomegalovirus infection, Colorado tick fever, Dengue fever, Ebola haemorrhagic fever, hand, foot and mouth disease, hepatitis, Herpes simplex, Herpes zoster, HPV, influenza (flu), Lassa fever, measles, Marburg haemorrhagic fever, infectious mononucleosis, mumps, poliomyelitis, progressive multifocal leukencephalopathy, rabies, rubella, SARS, smallpox (variola), viral encephalitis, viral gastroenteritis, viral meningitis, viral pneumonia, West Nile disease, Yellow fever.

Chronic inflammations or chronic inflammatory diseases comprise, but are not limited to, chronic cholecystitis, bronchiectasis, rheumatoid arthritis, Hashimoto's thyroiditis, inflammatory bowel disease (ulcerative colitis and Crohn's disease), silicosis and other pneumoconiosis.

Autoimmune diseases comprise, but are not limited to, systemic syndromes, such as SLE, Sjögren's syndrome, scleroderma, rheumatoid arthritis and polymyositis as well as local syndromes, such as IDDM, Hashimoto's thyroiditis, Addison's disease,

pemphigus vulgaris, psoriasis, atopic dermatitis, atopic syndrome, asthma, autoimmune haemolytic anaemia, multiple sclerosis.

Corresponding medicaments comprising at least one one microorganism as defined herein or at least one pharmaceutical composition as defined herein according to all herein described embodiments for use in the treatment and/or prophylaxis of physiological and/or pathophysiological conditions as described and defined herein are also comprised by the present invention.

In another aspect, the object of the present invention has been solved by providing plasmid or expression vector comprising components (I) to (IV) as illustrated herein. Preferred are microorganism of the invention that carry at least one plasmid or expression vector comprising components (I) to (IV) as illustrated herein.

In another aspect, the object of the present invention has been solved by providing a process for the production of a microorganism of the invention, wherein a plasmid or expression vector as illustrated herein is produced, and a microorganism is transformed with this plasmid or expression vector.

In another aspect, the object of the present invention has been solved by providing a pharmaceutical kit comprising at least one microorganism of the invention or a pharmaceutical composition as described above or a medicament as described above and a pharmacologically acceptable buffer, preferably a carbonate buffer.

Brief description of the drawings

Figure 1 depicts the nucleotide sequence (5'→3') of E. coli hemolysin A (hlyA) secretion signal. The NsiI site is underlined.

Figure 2 depicts the nucleotide sequence (5'→3') of E. coli hemolysin B (hlyB) of the E. coli hemolysin type I transport system.

Figure 3 depicts the nucleotide sequence (5'→3') of E. coli hemolysin D (hlyD) of the E. coli hemolysin type I transport system.

Figure 4 depicts the nucleotide sequence (5´-3´) of human prostate specific antigen (PSA) without signal peptide, accession number M26663 (Homo sapiens prostate-specific antigen mRNA, complete cds), region 100-807 (corresponding peptide: AA 26-261).

Figure 5 depicts the nucleotide sequence (5'→3') of Cholera Toxin subunit B (CtxB) without signal peptide encompassing region 204-494, accession number K01170 (Vibrio cholerae toxA and toxB genes for cholera enterotoxin subunits A2 (gamma).

Figure 6 depicts the nucleotide sequence (5´-3´) of B-Raf kinase domain (B-Raf KD) of human B-raf protein (BRAF), accession number M95712 (Homo sapiens B-raf protein (BRAF) mRNA, complete cds), region 1403-2359 without region 1448-1477 (corresponding peptide: AA 448-766 without AA 463-472) containing the V600E-mutation.

Figure 7 depicts the nucleotide sequence (5'→3') of the human HLA B27 restricted B-Raf V600E epitope.

Figure 8 depicts the nucleotide sequence (5'→3') of the genetic fusion construct of CtxB – PSA – HlyA.

Figure 9 depicts the nucleotide sequence (5'→3') of the genetic fusion construct of Ctx – B-Raf V600E – HlyA.

Figure 10 depicts the nucleotide sequence (5'→3') of the genetic fusion construct of CtxB – B-Raf V600E kinase domain KD – HlyA

Figure 11 depicts the functional expression and secretion of PSA-CtxB fusion proteins in different bacterial species recombinant for pMKhly-PSA-CtxB

Figure 12 depicts IFN-gamma secreting splenocytes as a measure for $CD8^+$ T-cell and innate immune system (NK cell) responses in mice immunized with different live vaccine carriers.

Figure 13 depicts the reduction of tumor volume in response to immunization with different live vaccine carriers.

Figure 14 depicts IFN-gamma secreting splenocytes as a measure for $CD8^+$ T-cell and innate immune system (NK cell) responses in mice immunized with different live vaccines carrying different immunological adjuvants with CtxB-OVA fusion constructs.

Figure 15 depicts the efficient expression and functional secretion of chicken influenza hemagglutinin H1 – CtxB fusion proteins in a veterinary vaccine strain encompassing the full sequence of H5N1 haemaglutinin (HA1+HA2, depicted, HA12), without the transmembrane region (HA12c). Live attenuated *Salmonella enterica* serovar Typhimurium vacT (*gyrA* D87G) and live attenuated *Salmonella enterica* serovar Enteritidis vacE, (strain Sm24/Rif12/Ssq), both LOHMANN ANIMAL HEALTH GMBH & CO KG

Figure 16 depicts the nucleotide sequence encoding for the CtxB-HA12c-HlyA fusion protein.

Figure 17 depicts the expression and secretion of the fusion protein using the vector pMBKDC electroporated into the strain *Salmonella typhi* Ty21a and other bacterial strains:

A) lanes A1-A3: Analysis of supernatant according to TP StMoBKDC using anti-B-Raf antibody for detection. Lane A1: S. typhi Ty21a pMoBKDC, A2: S. typhi Ty21a, A3: S. typhimurium aroA. The BRaf-V600E KD-CtxB fusion protein is marked with the upper arrow and has the expected size of approx. 48 kDa. Protein Marker: Invitrogen BenchMark Pre-Stained protein ladder, #10748-010

B) equivalent analysis as in A, using the newly produced anti-HlyA antibody as detection antibody. Lane 1, 2: StMoPC constructs, lane 3: S. typhi Ty21a MoBKDC; lane 4: E. coli MoBKDC, lane 5: S. typhi Ty21a, lane 6: S. typhimurium aroA. Protein Marker: Invitrogen BenchMark Pre-Stained protein ladder, #10748-010, Lot. 1315592

Figure 18 depicts the full nucleotide sequence (5'→3') of the empty vector pMKhly1.

The contents of all cited references and patents are hereby incorporated by reference in their entirety. The invention is explained in more detail by means of the following examples without, however, being restricted thereto.

Examples

Example 1: Construction, expression and secretion of PSA–CtxB fusion proteins in different bacterial carrier strains

To prove the feasibility and demonstrate the efficacy of the E. coli type I hemolysin secretion system to secrete fusion proteins of tumor antigens, here prostate specific antigen (PSA), and protein toxin components, here CtxB, as adjuvant, a PSA–CtxB fusion protein was constructed as described herein. Expression and secretion was tested in different gram-negative bacterial strains, which are potentially useful as live vaccine strains in tumor therapy. The molecular biologic cloning is based on the plasmid/expression vector pMOhly1, which has been previously described (Gentschev et al., 2005; Gentschev et al., 1996, WO 03/072789).

1A Construction of kanamycin–resistant pMOhly1 – expression vector derivative

The replacement of the ampicillin resistance cassette of pMOhly1 was performed as described in (Datsenko and Wanner, 2000).

In brief, a sense primer P1 (5'-GAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGGTGTAGGCTGGAGCTGCTTC-3') and an anti-sense primer P2 (5'-GCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCATATGAATATCCTCCTTA-3') and plasmid pKD4 as template were used for PCR to produce a fragment carrying the kanamycin resistance gene (Kan^R) flanked by regions homologous to the ampicillin resistance gene (underlined).

E. coli strain BW25114, harboring the pKD46 plasmid and the target plasmid pMOhly1 was grown at 37°C in LB medium (Difco) supplemented with 0,2% L-(+)-arabinose for 3-4 hours before transformation of the PCR fragment.

After transformation the bacterial cells were spread on LB agar plates containing 25µg/mL kanamycin and incubated at 37°C overnight.

The next day, Kan^R clones were picked and incubated for additional 48h in LB medium containing 50µg/mL kanamycin to get rid of all ampicillin resistance conferring plasmids.

Finally, clones were selected with a Kan^R and ampicillin-sensitive phenotype. The replacement of the Ap^R gene by the Kan^R cassette was confirmed by PCR and sequencing. The resulting plasmid was called pMKhly1.

1B Cloning of pMKhly-PSA plasmid

Sense primer PSA-Nsi1 (5´-GATTGGTGATGCATCCCTCAT-3´; *NsiI* restriction sites are underlined) and anti-sense primer PSA-Nsi2 (5`-GGTGCTCATGCATTGGCCACG-3`) were used to amplify a DNA fragment encoding PSA by PCR. PCRs were performed in a Thermal Cycler 60 (Biometra, Göttingen, Germany) for 30 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min.

After digestion with the *NsiI* restriction enzyme, the DNA fragment, carrying the *psa* gene, was inserted into the single *NsiI* site of the export vector pMKhly1. The resulting plasmid pMKhly-PSA was isolated from *E. coli* DH5alpha (Invitrogene, Germany), analyzed by restriction analysis and sequenced.

1C Cloning of pMKhly-PSA/CtxB plasmid

Sense primer Ptac-SalI (5´-AAAAAAGTCGACGGCTGTGCAGGTCGTAAATCACTGC-3´) and anti-sense Primer Ptac-NotI (5´-AAAAAAGCGGCCGCGAAATTGTTATCCGCT CACAATTCC-3´) were used to amplify by PCR a 201 bp DNA-Fragment encoding Ptac-Promoter from pGEX-6p-1-Plasmid (Amersham Bioscience, Germany). The PCR was performed in T3 thermocycler (Biometra, Germany): for 30 cycles at 95°C for 30 s, 55°C for 30s, and 72°C for 90s. Sense primer Rbs-NotI-forward (5´-AAAAAAGCGGCC GCTAAGGATGAATTATGATTAAATTAAAATTTGG-3´) and antisense-primer ctb-SalI-reverse (5´-TTTATAGTCGACTTAATTTGCCATACTAATTGCGGCAATCGC-3´) were used to amplify by PCR the 413 bp DNA-Fragment encoding the ribosome binding site and the whole coding sequence of CtxB from *V. cholerae* El tor. The PCR was performed in a T3 Thermocycler (Biometra, Germany): step1: 30 cycles at 95°C for 30 s, 50°C for 30s, and 72°C for 2 min. After purification with the Qiaquick PCR Purification Kit (Qiagen, Germany) and the digestion of both fragments with the *NotI* restriction enzyme, the two fragments were ligated resulting in the 594 bp Ptac-*ctx*B-fragment. The resulting fragment was also purified and after digestion with the *SalI* restriction enzyme, the Ptac-*ctx*B-Fragment was inserted into the single *SalI* site of the export vector

pMKhly-PSA. The resulting plasmid pMKhly-PSA/CtxB was isolated from *E. coli* DH5alpha (Invitrogene, Germany), analyzed and sequenced.

1D Cloning of PSA-CtxB-HlyAs fusion construct

Sense primer 5'*ctxB* NsiI (5´-GCATATGCACATGCATCACCTCAAAATATTACTGAT-3´) and anti-sense primer 3' *ctxB* SrfI NsiI (5´-GGCTTTTTTATATCTTATGCAT**GCCC GGGC**ATTGCGGCAATCGC-3´) (*SrfI* site is bold) were used to amplify by PCR a ~300bp DNA fragment representing the *ctxB* gene from *V. cholerae* El tor. After purification with the QIAquick PCR Purification Kit (Qiagen, Germany) and digestion with the *NsiI* restriction enzyme, the DNA fragment, carrying the whole *ctxB* gene without the N-terminal signal sequence, was inserted into the single *NsiI* site of the export vector pMKhly1. The resulting plasmid pMKhly-CtxB was isolated from *E. coli* DH5alpha (Life Technologies), analyzed and sequenced. The human *psa* gene was amplified from plasmid pCDNA3PSA by PCR with primers 5-PSA-Blunt (5´-GTGGGAGGC TGGGAGTGC-3´) and 3-PSA-Blunt (5´-GGGGTTGGCCACGATGGT-3´). PCR was carried out in a Thermal Cycler 60 (Biometra, Göttingen, Germany) for 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. The 0.7 kb DNA product was subsequently cloned into the *SrfI* site of pMKhly-CtxB. The resulting plasmid pMKhly-CtxB-PSA was verified by restriction analysis and sequencing.

1E Expression and secretion of fusion proteins in different bacterial species

Using standard electroporation procedures, plasmid pMKhly-CtxB-PSA was transformed into different electro-competent bacterial strains. Kanamycin resistant single colonies were picked and grown in BHI medium (Beckton Dickinson, USA: Calf Brains, Infusion from 200g, 6.0g/L; Beef Heart, Infusion from 250g, 9.8g/L; Proteose Peptone 10.0g/L ; Sodium Chloride 5.0g/L; Dextrose 2.0g/L; Disodium Phosphate 2.5g/L) to a density of $1x10^9$ cells per ml. Following growth 20 ml of culture were centrifuged for 30 min at 4000 rpm (3000 g) in a Heraeus centrifuge at 4°C. 18 ml of the supernatant were transferred into a fresh tube. Subsequently, 1.8 ml TCA (trichloroacetic acid, Applichem, Germany) were added, the liquid was mixed and incubated for at least 1 hour on ice. After incubation, the suspension was centrifuged for 30 min at 4000 rpm (3000 g) in a Heraeus centrifuge at 4°C. The supernatant was decanted and the pellet was washed with 1 ml acetone p.a. (Applichem, Germany). The precipitate was centrifuged

for 10 min at 4000 rpm (3000 g) in a Heraeus centrifuge at 4°C. The pellet was air-dried and taken up in 150 µl 5x Laemmli buffer (70 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 3% (v/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue) with or without beta-mercaptoethanol (Laemmli, 1970). 20 µl of solution were used for each lane in SDS PAGE. The separated proteins were electrophoretically transferred (Western blot) to Hybond ECL nitrocellulose membrane (Amersham-Pharmacia, Little Chalfont, UK) and blocked overnight with PBS (Potassium chloride 0.20 g/l, Potassium dihydrogen phosphate 0.20 g/l, Sodium chloride 8.00 g/l, di-Sodium dihydrogen phosphate anhydrous 1.15g/l) containing 1% BSA. The membrane was washed in PBS-Tween 0.05%, incubated with polyclonal rabbit anti PSA antibody (1:750, DAKO, Denmark), CtxB antibody (1:1000, Zytomed, Berlin, Germany) or HlyAs antibody (Gentschev et al., 1996)) and subsequently incubated with HRP-coupled anti rabbit IgG (1/2,000; Dianova, Hamburg, Germany) for 1 h. The Western blot was developed with the enhanced chemiluminescence kit (GE Healthcare Life Science, Germany).

Figure 11 depicts the functional expression and secretion of PSA-CtxB fusion proteins in a variety of bacterial strains, including Escherichia coli spp., Salmonella dublin, Citrobacter spp, Salmonella typhi Ty21a, Salmonella typhimurium spp, Erwinia spp and Shigella flexneri spp.

The Salmonella typhi Ty21a strain expressing and secreting PSA-CtxB fusion protein was deposited at German Collection of Microorganisms and Cell Cultures (DSMZ) under DSM 19244.

Example 2: Immune response and protection against tumor cell challenge in mice immunized with Salmonella strains secreting fusion proteins of tumor antigens and CtxB or CtxB alone

By the following experiments the superior protective efficacy of secreted fusion proteins of tumor antigens and protein toxins was demonstrated in an animal tumor model. For this model, the strain Salmonella typhimurium aroA (SL7207) pMKhly-CtxB-PSA, expressing and secreting the CtxB-PSA fusion protein was compared with other control strains.

2A Immunization procedures

DBA/2 mice were immunized three times with an interval of 3 weeks. For the immunization with bacteria, animals were pre-treated by applying 50 µl 7% $NaHCO_3$ intragastrically to increase the intragastric pH. 5 to 10 minutes after pre-treatment, $5x10^8$ live, Kanamycin-insensitive bacteria were applied in a volume of 100 µl PBS intragastrically. As a control, mice were immunized intramuscularly with naked plasmid DNA encoding PSA (pcDNA-PSA) as described in (Fensterle et al., 2005).

2B T-cell responses

Seven days after the last immunization, splenocytes of immunized mice were prepared as published previously (Fensterle et al., 1999) by passaging the spleen through a mesh followed by lysis of erythrocytes. ELISPOT analysis for the detection of PSA specific $CD8^+$ T-cells was performed according to a protocol published in (Fensterle et al., 1999).

In brief, for the ex-vivo analysis of PSA specific $CD8^+$ T-cells 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with 5 µg/ml of the anti-mouse IFN-gamma monoclonal antibody (mAb) R4 (PharMingen) in 100 µl of carbonate buffer (Fensterle et al., 1999), pH 9.6. After overnight incubation at 4°C and blocking with 1% BSA in PBS, $1x10^5$ splenocytes from vaccinated mice were added in 100 µl RP10 (Fensterle et al., 1999) per well.

For analysis of the $CD8^+$ T-cell response the PSA expressing P815 cell clone PPSA 24 was used; in brief, this clone expresses the full length PSA via a CMV promotor en-

coded by the plasmid pCDNA3 (Fensterle et al., 2005). After incubation for 20-22 h at 37°C, 5% CO_2 in the presence of 30 U/ml IL-2, plates were washed and incubated for additional 2h with 100 µl biotinylated anti-mouse IFN-gamma mAb XMB1.2 (0.25 µg/ml, PharMingen). Plates were washed and incubated for 1 h at 37°C in the presence of 100 µl of a 1/20,000 dilution of alkaline phosphatase-coupled streptavidin (PharMingen). Spots were visualized by adding 50 µl of the ready-to-use substrate BCIP/NBT (Sigma, St. Louis, MO) dissolved in water. Spots were counted under a dissecting microscope at 3-fold magnification.

The frequency of peptide-specific T cells (CTL) is expressed as the number of IFN-gamma-secreting cells per 10^5 splenocytes. For the analysis of T-cell responses after restimulation, 2.5 x 10^7 splenocytes were restimulated with $2x10^6$ irradiated PSA expressing P815 cells (Fensterle et al., 2005) in RP10 medium (Fensterle et al., 1999)in the presence of 60 U/ml recombinant IL-2 for 5 days. ELISPOT analysis was performed as described above using various amounts of restimulated cells (10^5, $3x10^4$, 10^4 or $3x10^3$ per well) mixed with $4x10^5$ feeder cells (= freshly prepared splenocytes from naïve DBA/2 mice) and 10^5 PPSA24 cells.

The induction of a cellular immune response, especially of $CD8^+$ cytotoxic T cells, plays an important role for the efficiency of tumor therapy (Boon et al., 2006; Rosenberg, 2001). Therefore, the efficacy of the recombinant bacterial strain Salmonella typhimurium (SL7207) carrying a pMKhly-CtxB-PSA expression vector to induce a PSA-specific $CD8^+$ T-cell immune response was first tested.

For this purpose 64 female DBA-2 mice at the age of 10-14 weeks were immunized p.o. with recombinant SL7207/pMKhly-CtxB (n=10), SL7207/pMKhly-CtxB-PSA (n=10), SL7207/pMKhly-PSA (n=10), SL7207/pMKhly-PSA/CxtB (n=10), SL7207/pMKhly1 (n=7) and naked pcDNA3-PSA (n=10) as a positive control as described herein.

Figure 12 shows the ELISPOT data revealing that DNA immunized mice showed profound $CD8^+$ T-cell responses. After restimulation, marked immune responses were detected in animals vaccinated with *Salmonella* secreting PSA protein fused to CtxB, but not in strains secreting PSA alone or PSA and CtxB separately. Interestingly, animals vaccinated with *Salmonella* secreting CtxB toxin alone also showed significant immune responses after restimulation. These responses are most likely due to NK cells or other cells of the innate immune system, which non-specifically recognize the target cell line. Hence, from the data for secreted CtxB alone it can be concluded that the

immune response observed for secreted CtxB-PSA fusion protein (IFN-gamma secreting splenocytes) is composed of a CD8+ T-cell response as well as a profound response of the innate immune system (most likely NK cells).

2C Protection against tumor cell challenge

To analyze the protective capacity of the immunization, 6-7 mice per group were immunized according to the schedule above. Two weeks after the third immunization, mice were challenged with PPSA 24 (see above) by two s.c. injection of $1x10^6$ cells into each flank of shaven abdominal skin. Mice were monitored over a period of 14 days for tumor appearance and tumor volume was assessed by measuring the largest (a) and smallest (b) tumor diameter. Tumor volume was calculated as rotation ellipsoid using the following formula:

$$V = \frac{\pi}{6} * a * b^2$$, a > b.

Results were analyzed for significance by one way ANOVA and Dunnett's multiple comparison post-test using the Graph Pad Prism software. The post test was only performed when ANOVA revealed significance.

Figure 13 displays the results as means +/- SD. At day 6, 9, 12 and 14 significant protective effects were observed. As expected, naked DNA vaccination included as a control completely protected mice from tumor growth. However, naked DNA vaccination shows at most moderate efficiency in humans. Regarding the bacterial constructs, the vaccine strain SL7207/pMKhly-CtxB-PSA (expressing and secreting the CtxB-PSA fusion protein) turned out to be most efficient. It significantly reduced tumor volume at day 9, 12 and 14 after tumor challenge. Of note, also the strain SL7207/pMKhly-CtxB reduced tumor growth with values comparable to SL7207/pMKhly-CtxB-PSA at day 14. Although not significant, this delayed effect is well compatible to the cellular response which was measured in the ELISPOT assay. Furthermore, also the SL7207/pMKhly-PSA strain achieved significant protection at day 14 which indicates, that also this strain induced a T-cell response which was below the detection threshold. In contrast, the SL7207/pMKhly-PSA/CxtB strain did not induce a relevant effect and remained in the same range as SL7207/pMKhly1 alone.

Example 3: Expression and secretion of an oncogen – toxin fusion protein in *S. typhi* Ty21a

Analogous to example 1, another tumor antigen, the kinase domain of the oncogen B-Raf V600E with a 10 aa deletion in the kinase domain was used (BRaf V600E kinase domain kinase dead (KD), or briefly BRaf* KD, more briefly BKD). This oncogen and protein toxin components, here CtxB, as adjuvant, a BKD–CtxB fusion protein was constructed as described herein. Expression and secretion was demonstrated in the human vaccine strain *S. typhi* Ty21a. The molecular cloning is based on the plasmid/expression vector pMOhly1, which has been previously described (Gentschev et al., 2005; Gentschev et al., 1996) and the cloning procedure is analogous to example 1 in this application. The resulting vector is named pMBKDC. The sequence of the fusion protein is given in **Figure 10.**

Figure 17 depicts the expression and secretion of the fusion protein using the vector pMBKDC electroporated into the strain *Salmonella typhi* Ty21a and other bacterial strains.

The Salmonella typhi Ty21a strain expressing and secreting BKD–CtxB fusion protein was deposited at German Collection of Microorganisms and Cell Cultures (DSMZ) under DSM 19245.

Example 4: Comparison of an OVA-CtxB fusion construct with different genetically encoded immunological adjuvants

To compare the immunological efficacy of toxin-antigen fusion proteins secreted by live vaccines, a fusion protein encompassing a widely used antigen for immunological studies, chicken ovalbumin (OVA) and CtxB was constructed.

Sense primer NsiI – OVA – forward 5´ - CAT GTA TGC ATT AGC CAT GGT ATA CCT GG – 3´ and anti-sense primer NsiI – OVA – reverse 5´ - TTT TTT ATG CAT AAG GG AAA CAC CAC ATC TGC C – 3´were used to amplify by PCR a 1033 bp DNA fragment representing the *ova* gene (NM205152). After purification with the QIAquick PCR Purification Kit (Qiagen, Germany) and digestion with the *NsiI* restriction enzyme, the DNA fragment, carrying the whole *ova* gene without the N-terminal signal sequence, was inserted into the single *NsiI* site of the export vector pMKhly1. The resulting plasmid pMKhly-Ova was isolated from *E. coli* DH5alpha (Life Technologies), analyzed and sequenced.

Sense primer 5'*ctxB* NsiI (5´-GCATATGCACATGCATCACCTCAAAATATTACTGAT-3´) and anti-sense primer 3' *ctxB* SrfI NsiI (5´-GGCTTTTTTATATCTTATGCAT**GCCC GGGC**ATTGCGGCAATCGC-3´) (*SrfI* site is bold) were used to amplify by PCR a ~300bp DNA fragment representing the *ctxB* gene from *V. cholerae* El tor. After purification with the QIAquick PCR Purification Kit (Qiagen, Germany) and digestion with the *NsiI* restriction enzyme, the DNA fragment, carrying the whole *ctxB* gene without the N-terminal signal sequence, was inserted into the single *NsiI* site of the export vector pMKhly1. The resulting plasmid pMKhly-CtxB was isolated from *E. coli* DH5alpha (Life Technologies), analyzed and sequenced. The ova gene was amplified from the pCI-OVA plasmid by PCR using 5-OVA-SfrI GCC ATC ATG TCA GCT CTA and 3-OVA-SfrI AGG GGA AAC ACA TCT GCC primers. The PCR was carried out in a Thermal Cycler 60 (Biometra, Göttingen, Germany) for 30 cycles of 1 min at 94°C, of 1 min at 49°C, and of 2 min. 30 sec at 72°C. The 1.1–kb DNA product was subsequently cloned into the *SrfI* site of pMKhly-CtxB. The resulting pMKhly-CtxB-OVA plasmid was examined by restriction analysis and sequencing.

The capacity of this fusion protein to elicit Th1 immune responses was compared with those of pMKhly vector encoded secreted OVA alone in combination with DNA delivery

plasmids (encoding for proteins under the control of a eukaryotic promotor) coding for interferons (IFN-gamma), interleukins (IL-12) and chemokines (IP-10).

Immunization procedures and ELISPOT analysis were performed as described above. In brief, C57BL/6 mice were immunized orally three times with the different strains. 7 days after final immunization, splenocytes were removed, restimulated for 5 days and analysed in an ELISPOT assay. For restimulation of splenocytes, cells pulsed with the SIINFEKL peptide (letters stand for amino acids), the H-2^b restricted MHC-I epitope of ovalbumin, were used.

Figure 14 shows the superior efficacy of the fusion protein comprising chicken ovalbumin (OVA) and CtxB in inducing OVA specific $CD8^+$ T-cell responses and potentially innate immune system) responses compared to constructs co-delivering IFN-gamma or IP-10. It exhibits a similar efficacy as the strain with co-delivered IL-12 construct.

Example 5: Expression and secretion of viral antigens

The suitability of the microorganisms of the invention for the expression and secretion of any (non-tumor) antigen was demonstrated through the expression of hemagglutinin H1 protein of the chicken influenza virus H5N1 fused to CtxB in a veterinary vaccine strain, Salmonella typhimurium VacT.

Sense primer 5`-HA-G: 5´- ATC TGT CAA ATG GAG AAA -3´ and anti-sense primer 3-HA12C: 5`-TAC TCC ACT TAT TTC CTC TCT-3` were used in PCR, amplifying a DNA fragment encoding the H5 without the C- terminal membrane domain (H12C). The PCR product was subsequently cloned into the *SrfI* site of pMKhly-CtxB. The resulting pMKhly-CtxB-H12C plasmid was examined by restriction analysis and sequencing. The *S. typhimurium* VacT/pMKhly-CtxB-H12C strain efficiently expressed and secreted the hybrid H5-protein, as shown by immunoblotting with polyclonal antibodies raised against CtxB and HlyA (Fig. 2). The amount of secreted H5 was 2-3 µg protein/ml supernatant under the experimental conditions.

Bacteria were grown in BHI medium to a density of $1x10^9$ cells per ml. 20 ml of the culture were centrifuged for 30' at 4000 rpm (3000 g) and 4°C in a Hereaus centrifuge. 18 ml of the supernatant were transferred into a fresh tube. Subsequently, 1.8 ml TCA (trichlor-acetic acid, Applichem, Germany) were added; the liquid was mixed and incubated on ice for at least 1 hour. After incubation, the suspension was centrifuged for 30' at 4000 rpm (3000 g) and 4°C in a Hereaus centrifuge. The supernatant was decanted and the pellet was washed with 1 ml Aceton p.a. (Applichem, Germany); the precipitate was centrifuged for 10' at 4000 rpm (3000 g) and 4°C in a Hereaus centrifuge. The pellet was air-dried and taken up in 150 µl 5x Laemmli-buffer. 20 µl of the solution were used for each lane in SDS PAGE. The separated proteins were electrophoretically transferred to Hybond ECL nitrocellulose membrane (Amersham-Pharmacia, Little Chalfont, U.K.) and blocked overnight with PBS containing 1% BSA. The membrane was washed in PBS-Tween 0.05%, incubated with polyclonal rabbit anti CtxB antibody (1:1000, Zytomed, Berlin, Germany) or HlyAs antibody (Gentschev et al., 1996) and subsequently incubated with HRP-coupled anti rabbit IgG (1/2,000; Dianova, Hamburg, Germany) for 1 h. The Western blot was carried out using the enhanced chemiluminescence kit (GE Healthcare Life Science, Germany).

Figure 15 depicts the efficient expression and functional secretion of HA12C-CtxB fusion protein in the veterinary vaccine strain, Salmonella typhimurium VacT.

Example 6: Expression and secretion of fusion proteins of toxin adjuvants and tumor antigens via type III secretion systems

Several Gram negative bacteria carry type III secretion systems which can be exploited for antigen secretion. Usually, these systems can be exploited to directly inject heterologous antigens into cells. In this example, a toxin component of Yersinia which serves as secretion signal (YopE) is genetically fused to the heat labile enterotoxin subunit B (LT-B) fragment serving as an adjuvant and a tumor antigen, PSA. This construct is to be expressed in suitable, preferably attenuated, Yersinia strains which leads to secretion of the fusion protein by the endogenous type III secretion machinery via the YopE signal sequence.

In this example, the genetic fusion YopE18-LT-B-PSA is cloned into the EcoRI site of the plasmid paCYC184, for use the construct with the same orientation as the cat gene is used yielding a product expressed by the plasmid encoded cat promoter (http://www.fermentas.com/techinfo/nucleicacids/mappacyc184.htm). For enhanced expression, any suitable promotor can be used.

In the following, a cloning procedure is exemplified. Chromosomal DNA from E. coli encompassing the heat labile enterotoxin subunit B or a vector encompassing this sequence (GenBank accession number AF242418) is amplified using the following primers in a PCR reaction:

Eco-yope18-f (encompassing EcoRI site + YopE18 signal sequence):

5'-CTGAATTCATGAAAATATCATCATTTATTTCTACATCACTGCCCCTGCCGGCA TCAGTGTCA*AATAAAGTAAAATGTTATGTTTTAT* 3'

(5' region black: EcoRI site+3 bases 5', red: sequence encoding for secretion signal for type III secretion of YopE protein (aa 1-18), GenBank Accession M92066; italic region: homology region of LT-B gene (GB accession AF242418), bases 4-28).

Phosphorylated reverse primer LT-B-r:

5' GTTTTCCATACTGATTGCCGCAATTGAATTGG-3'

(reverse strand 372-341 of GB AF242418).

In parallel, the PSA sequence as described in this application from the vector pMK CtxB-PSA is amplified using the following primers:

phosphorylated forward primer psa-f: 5'-GTGGGAGGCTGGGAGTGCGAG-3'

reverse primer psa-eco-r: 5' CCTGAATTCTTAGACGTGATACCTTGAAGCAC
(5' black: EcoRI site+3 bases 5', blue: stop codon, black: 3' region of PSA sequence, this application)

Subsequently, the two fragments are ligated with a DNA Ligase under the appropriate conditions. After ligation, the new fragment is amplified in a second PCR reaction using the Eco-yope18-f and PSA-Eco-R primers described above.

The resulting fragment is purified using a suitable PCR purification kit to remove buffer and oligonucleotides and subsequently digested with EcoRI enzyme in suitable reaction conditions. The resulting 1040 bp fragment is purified using agarose gel electrophoresis in ligated into the EcoRI digested vector paCYC184. After sequencing tetracyclin resistant, cm sensitive clones, a clone which has the correct sequence integrated with the same orientation than the cm gene is chosen and transformed into a suitable Yersinia strain using electroporation. This vaccine strain will secrete the LT-B-PSA fusion via its endogenous type III secretion system.

Example 7: Expression and secretion of Toxin-Antigen fusions in Gram positive bacteria

Gram positive and negative bacteria have different prerequisites for secretion. Gram negative bacteria possess two membranes and therefore a secretion machinery in addition to secretion signals is mandatory for full secretion. In the case of Gram positive bacteria, a secretion signal sequence is sufficient.

In this example a system for secretion of a Tetanus-toxoid PSA fusion in Listeria using the p60 secretion signal is described.

In a first step, the vector pUC18(PS)actAOVATinlA (Loeffler et al., 2006) is digested using NsiI and subsequently treated with 3'-5' Exonuclease in the appropriate conditions. Finally, the fragment is purified by agarose gel electrophoresis.

In parallel, the tetanus toxoid fragment C (GenBank accession no. M12739) is amplified from a suitable source, e.g. genomic DNA from a *Clostridium tetanii* strain.using the following phosphorylated primers:
tetc for: 5' – TAAAAATCTGGATTGTTGGGTTG – 3', whereas the underlined additional base is to ensure the correct frame of the fusion.
tetc rev: 5' – ATCATTTGTCCATCCTTCATCTG – 3'.
The BRAF KD fragment is amplified from the plasmid pMO BKDC described in this application using the following phosphorylated primers:
br for: 5' – GATTGGGAGATTCCTGATG – 3'
br rev: 5' – CCCGTGGACAGGAAACGCACC – 3'.
Both fragments are purified using a suitable PCR purification kit and subsequently ligated with DNA ligase under suitable conditions. After ligation, the fragment is purified again using a suitable PCR purification kit and amplified using the tetc for and the br rev primer. After amplification, the resulting 2280 bp fragment is purified by agarose gel electrophoreses and ligated into the purified NsiI – 3'-5' exonuclease fragment of pUC18(PS)actAOVATinlA. After ligation and transformation into *E.coli*, a clone is selected carrying the fragment in frame. Subsequently, the plasmid is cut using PstI and SacI and the 2837 bp fragment is purified using gel electrophoresis and ligated into the appropriately PstI and SacI digested and purified vector pSP118 (Loeffler et al., 2006). The resulting vector, pSP118 – act-TetC-BRaf*KD is transformed into a *Listeria* strain carrying a trpS deletion e.g. *Listeria monocytogenes* delta trps delta aroA/B (Loeffler et

al., 2006). In this setting, the plasmid is stabilized via the plasmid based trpS ("Balanced lethal system") and the plasmid encodes for a phage lysine which leads to intracellular lysis of the strain. The act-TetC-BRaf*KD cassette is expressed mainly in eukaryotic host cells under the control of the actA promoter, which has some extracellular leakiness (Loeffler et al., 2006). The actA signal sequence of the cassette leads to secretion of the TetC-BRaf*KD fusion protein.

Example 8: Eucaryotic cells secreting toxin-antigen fusions

In this example, a eukaryotic cell line, eg tumor cell line, is constructed which secretes a CtxB-PSA fusion protein. For this purpose, a universal secretion signal is employed (US 6,733,997), which, in principle, allows the secretion of the corresponding expression cassette in cells of different origin, including mammalian cells, yeast and procaryotic cells.

In a first step, the CtxB-BRaf* KD fusion is amplified from the plasmid pMO BKDC (this application) using the following primers:

CB-for: 5' – atcGGATCCTCAAAATATTACTGATTTGTGTGC – 3' (lower case: spacer, underlined: BamHI site, uppercase: CtxB 5')

CB-rev: 5' – tagGGATCC*TTA*GTGGACAGGAAACGCACCATATCC – 3' (lower case: spacer, underlined: BamHI site, italic: stop codon, uppercase: BRaf* KD 3').

Subsequently, the PCR product is purified using a suitable PCR purification kit and subsequently partially digested (fragment contains internal BamHI site) with BamHI. The 1231 bp fragment of the partial digest is isolated via agarose gel electrophoresis and subsequently ligated into the BamHI digested and gel purified plasmid pVtgEGFP (US Patent application 6733997). Subsequently, a clone carrying the in frame orientation with Vtgss is selected and named pVtgCtxBRAf. This plasmid can be transformed via electroporation into a eukaryotic cell line, which can be selected via the kan/neo selection cassette. The cell lines can be established cell lines like cancer cell lines for use as heterologuous cancer vaccine or patient derived tumor cells for use as autologuous cancer vaccines.

In any case, the secretion signal Vtgss encoded by the vector pVtgEGFP genetically fused to CtxB-BRaf* KD will result in the secretion of the fusion protein.

Using a similar approach, fusion proteins can also be expressed in yeast. As an example, a modified cloning strategy as depicted in fig. 11 of US 6,733,997 is demonstrated. In the first step, the cassette containing the Vtgss-CtxB-BRaf*KD fusion from plasmid pVtgCtxBRAf described above is excised via EagI and Eco47III and inserted into the vector pBSPGK (US 6,733,997, fig. 11). Subsequently, the resulting vector is digested with SacI and HindIII and the fragment encompassing the PGK element and the Vtgss-

CtxB-BRaf*KD fusion is integrated in the corresponding region of the vector pYEX-S1 analoguous to the description of US 6,733,997 (fig. 11). The resulting plasmid can be transformed by electroporation in yeast strains like Saccharomyces cerevisiae. The yeast will express and secrete the fusion protein and can be used for vaccination purposes.

Abbreviations

ABC	ATP binding cassette
AIDA	adhesin involved in diffuse adherence
APC	Antigen-presenting cell
aroA	aroA gene
AT	alphaToxin
ATP	adenosine triphosphate
B-Raf KD	B-Raf kinase domain
BSA	bovine serum albumin
Cag A	major disease-associated virulence protein of Helicobacter pylori
CPE	Clostridium perfringens enterotoxin
CpG	DNA sequences containing hypomethylated immunstimulatory DNA sequences encompassing CpG motives
CT/Ctx	Cholera Toxin
CTB/CtxB	Cholera Toxin subunit B
CTL	cytotoxic $CD8^+$ T cells (T killer cells)
CMV	Cytomegalievirus
DNA	desoxyribonucleic acid
ds	double-stranded
DT/Dtx	Dipheteria Toxin
E. coli	Escherichia coli
EBV	Ebstein Barr virus
GM-1 receptor	Monosialoganglioside receptor 1
HA	hemagglutinin
HCV	Hepatitis C virus
HIV	human immunodeficiency virus

Hly	hemolysin
HPV	Human Papilloma virus
HT	Hemorrhagic Toxin
i.d.	intradermally
i.m.	intramuscular
i.p.	intraperetoneally
IFN	interferon
IgA	Immunoglobulin isotype A
IgG	Immunoglobulin isotype G
IL	interleukin
KD	kinase dead
LPS	lipopolysaccharide
LT	E. coli heat labile enterotoxin
LT	Lethal toxin
LTB	E. coli heat labile enterotoxin subunit B
mAb	monoclonal antibody
MHC	major histocompatibility complex
NK cell	natural killer cell
NKT cell	natural killer T cell
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Pseudomonas Exotoxin A
PSA	prostate specific antigen
PT/Ptx	Pertussis Toxin
RNA	ribonucleic acid
s.c.	subcutaneous

SDS	sodium dodecylsulphate
Sec	general secretory (Sec) pathway
ss	single-stranded
ST	E. coli heat stabile enterotoxin
ST/Stx	Shiga Toxin
STB/StxB	Shiga Toxin subunit B
T3SS	type III secretion system
Tat	twin-arginine translocation
TCA	trichloroacetic acid
Th1 (cell)	inflammatory $CD4^+$ T cells
Th2 (cell)	helper $CD4^+$ T cells
TSST-1	Toxic shock syndrome toxin
TT	Tetanus Toxin
VT	Vero toxin

Literature

Agren, L. C., Ekman, L., Lowenadler, B., Nedrud, J. G., and Lycke, N. Y. (1999). Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. J Immunol 162, 2432-2440.

Anderson, R. J., Pasetti, M. F., Sztein, M. B., Levine, M. M., and Noriega, F. R. (2000). DeltaguaBA attenuated Shigella flexneri 2a strain CVD 1204 as a Shigella vaccine and as a live mucosal delivery system for fragment C of tetanus toxin. Vaccine 18, 2193-2202.

Austin, E. A., Graves, J. F., Hite, L. A., Parker, C. T., and Schnaitman, C. A. (1990). Genetic analysis of lipopolysaccharide core biosynthesis by Escherichia coli K-12: insertion mutagenesis of the rfa locus. J Bacteriol 172, 5312-5325.

Balsalobre, C., Silvan, J. M., Berglund, S., Mizunoe, Y., Uhlin, B. E., and Wai, S. N. (2006). Release of the type I secreted alpha-haemolysin via outer membrane vesicles from Escherichia coli. Mol Microbiol 59, 99-112.

Barry, E. M., Gomez-Duarte, O., Chatfield, S., Rappuoli, R., Pizza, M., Losonsky, G., Galen, J., and Levine, M. M. (1996). Expression and immunogenicity of pertussis toxin S1 subunit-tetanus toxin fragment C fusions in Salmonella typhi vaccine strain CVD 908. Infect Immun 64, 4172-4181.

Becerra, J. C., Arthur, J. F., Landucci, G. R., Forthal, D. N., and Theuer, C. P. (2003). CD8+ T-cell mediated tumor protection by Pseudomonas exotoxin fused to ovalbumin in C57BL/6 mice. Surgery 133, 404-410.

Boon, T., Coulie, P. G., Van den Eynde, B. J., and van der Bruggen, P. (2006). Human T cell responses against melanoma. Annu Rev Immunol 24, 175-208.

Boyd, A. P., Ross, P. J., Conroy, H., Mahon, N., Lavelle, E. C., and Mills, K. H. (2005). Bordetella pertussis adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. J Immunol 175, 730-738.

Brossier, F., Weber-Levy, M., Mock, M., and Sirard, J. C. (2000). Protective antigen-mediated antibody response against a heterologous protein produced in vivo by Bacillus anthracis. Infect Immun 68, 5731-5734.

Bumann, D. (2001). Regulated antigen expression in live recombinant Salmonella enterica serovar Typhimurium strongly affects colonization capabilities and specific CD4(+)-T-cell responses. Infect Immun 69, 7493-7500.

Busby, S., and Ebright, R. H. (1994). Promoter structure, promoter recognition, and transcription activation in prokaryotes. Cell 79, 743-746.

Butterton, J. R., Beattie, D. T., Gardel, C. L., Carroll, P. A., Hyman, T., Killeen, K. P., Mekalanos, J. J., and Calderwood, S. B. (1995). Heterologous antigen expression in Vibrio cholerae vector strains. Infect Immun 63, 2689-2696.

Byun, Y., Ohmura, M., Fujihashi, K., Yamamoto, S., McGhee, J. R., Udaka, S., Kiyono, H., Takeda, Y., Kohsaka, T., and Yuki, Y. (2001). Nasal immunization with E. coli verotoxin 1 (VT1)-B subunit and a nontoxic mutant of cholera toxin elicits serum neutralizing antibodies. Vaccine 19, 2061-2070.

Campos, J., Martinez, E., Marrero, K., Silva, Y., Rodriguez, B. L., Suzarte, E., Ledon, T., and Fando, R. (2003). Novel type of specialized transduction for CTX phi or its satellite phage RS1 mediated by filamentous phage VGJ phi in Vibrio cholerae. J Bacteriol 185, 7231-7240.

Cardenas, L., and Clements, J. D. (1992). Oral immunization using live attenuated Salmonella spp. as carriers of foreign antigens. Clin Microbiol Rev 5, 328-342.

Cardenas, L., and Clements, J. D. (1993). Development of mucosal protection against the heat-stable enterotoxin (ST) of Escherichia coli by oral immunization with a genetic fusion delivered by a bacterial vector. Infect Immun 61, 4629-4636.

Chabalgoity, J. A., Harrison, J. A., Esteves, A., Demarco de Hormaeche, R., Ehrlich, R., Khan, C. M., and Hormaeche, C. E. (1997). Expression and immunogenicity of an Echinococcus granulosus fatty acid-binding protein in live attenuated Salmonella vaccine strains. Infect Immun 65, 2402-2412.

Chabalgoity, J. A., Khan, C. M., Nash, A. A., and Hormaeche, C. E. (1996). A Salmonella typhimurium htrA live vaccine expressing multiple copies of a peptide comprising amino acids 8-23 of herpes simplex virus glycoprotein D as a genetic fusion to tetanus toxin fragment C protects mice from herpes simplex virus infection. Mol Microbiol 19, 791-801.

Chabalgoity, J. A., Moreno, M., Carol, H., Dougan, G., and Hormaeche, C. E. (2000). Salmonella typhimurium as a basis for a live oral Echinococcus granulosus vaccine. Vaccine 19, 460-469.

Chabalgoity, J. A., Villareal-Ramos, B., Khan, C. M., Chatfield, S. N., de Hormaeche, R. D., and Hormaeche, C. E. (1995). Influence of preimmunization with tetanus toxoid on immune responses to tetanus toxin fragment C-guest antigen fusions in a Salmonella vaccine carrier. Infect Immun 63, 2564-2569.

Chacon, M. R., Londono, P., Dougan, G., and Selkirk, M. E. (1996). Heterologous expression of the cuticular-glutathione peroxidase of lymphatic filariae in an attenuated vaccine strain of Salmonella typhimurium abrogates H-2 restriction of specific antibody responses. Parasite Immunol 18, 307-316.

Chen, I., Finn, T. M., Yanqing, L., Guoming, Q., Rappuoli, R., and Pizza, M. (1998). A recombinant live attenuated strain of Vibrio cholerae induces immunity against tetanus toxin and Bordetella pertussis tracheal colonization factor. Infect Immun 66, 1648-1653.

Cheng-hua, S., Cheng, C., Jing-sheng, Z., Jiezhi., L., and Qing-jun, M. (1995). Gene fusion of cholera toxin B subunit and HBV PreS2 epitope and the antigenicity of fusion protein. Vaccine 13, 933-937.

Clemens, J., Savarino, S., Abu-Elyazeed, R., Safwat, M., Rao, M., Wierzba, T., Svennerholm, A. M., Holmgren, J., Frenck, R., Park, E., and Naficy, A. (2004). Development of pathogenicity-driven definitions of outcomes for a field trial of a killed oral vaccine against enterotoxigenic Escherichia coli in Egypt: application of an evidence-based method. J Infect Dis 189, 2299-2307.

Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci USA 97, 6640-6645.

Dietrich, G., Bubert, A., Gentschev, I., Sokolovic, Z., Simm, A., Catic, A., Kaufmann, S. H., Hess, J., Szalay, A. A., and Goebel, W. (1998). Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide Listeria monocytogenes. Nat Biotechnol 16, 181-185.

Dietrich, G., Viret, JF, and Gentschev, I. (2003). Haemolysin A and listeriolysin - two vaccine delivery tools for the induction of cell-mediated immunity. Int. J. Parasitol 33, 495-505

Dunn, G. P., Old, L. J., and Schreiber, R. D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. Immunity 21, 137-148.

Dunstan, S. J., Simmons, C. P., and Strugnell, R. A. (2003). In vitro and in vivo stability of recombinant plasmids in a vaccine strain of Salmonella enterica var. Typhimurium. FEMS Immunol Med Microbiol 37, 111-119.

Eriksson, A. M., Schon, K. M., and Lycke, N. Y. (2004). The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues. J Immunol 173, 3310-3319.

Fath, M. J., Skvirsky, R. C., and Kolter, R. (1991). Functional complementation between bacterial MDR-like export systems: colicin V, alpha-hemolysin, and Erwinia protease. J Bacteriol 173, 7549-7556.

Fensterle, J., Grode, L., Hess, J., and Kaufmann, S. H. E. (1999). Effective DNA vaccination against listeriosis by prime/boost inoculation with the gene gun. J Immunol 163, 4510-4518.

Fensterle, J., Schwartz, V., Riedmiller, H., and Rapp, U. R. (2005). Animal models for DNA vaccination against prostate cancer using PSA encoding plasmids. Onkologie 28 (suppl 2), 52.

Freytag, L. C., and Clements, J. D. (1999). Bacterial toxins as mucosal adjuvants. Curr Top Microbiol Immunol 236, 215-236.

Freytag, L. C., and Clements, J. D. (2005). Mucosal adjuvants. Vaccine 23, 1804-1813.

Garmory, H. S., Brown, K. A., and Titball, R. W. (2002). Salmonella vaccines for use in humans: present and future perspectives. FEMS Microbiol Rev 26, 339-353.

Garmory, H. S., Titball, R. W., Griffin, K. F., Hahn, U., Bohm, R., and Beyer, W. (2003). Salmonella enterica serovar typhimurium expressing a chromosomally integrated copy of the Bacillus anthracis protective antigen gene protects mice against an anthrax spore challenge. Infect Immun 71, 3831-3836.

Gentschev, I., Dietrich, G., and Goebel, W. (2002a). The E. coli alpha-hemolysin secretion system and its use in vaccine development. Trends Microbiol 10, 39-45.

Gentschev, I., Dietrich, G., Spreng, S., Pilgrim, S., Stritzker, J., Kolb-Maurer, A., and Goebel, W. (2002b). Delivery of protein antigens and DNA by attenuated intracellular bacteria. Int J Med Microbiol 291, 577-582.

Gentschev, I., Fensterle, J., Schmidt, A., Potapenko, T., Troppmair, J., Goebel, W., and Rapp, U. R. (2005). Use of a recombinant Salmonella enterica serovar Typhimurium strain expressing C-Raf for protection against C-Raf induced lung adenoma in mice. BMC Cancer 5, 15.

Gentschev, I., Mollenkopf, H., Sokolovic, Z., Hess, J., Kaufmann, S. H., and Goebel, W. (1996). Development of antigen-delivery systems, based on the Escherichia coli hemolysin secretion pathway. Gene 179, 133-140.

Gentschev, I., Dietrich, G., Spreng, S., Neuhaus, B., Maier, E., Benz, R., Goebel, W., Fensterle, J., and Rapp, UR. (2004). Use of the alpha-hemolysin secretion system of Escherichia coli for antigen delivery in the Salmonella typhi Ty21a vaccine strain. Int J Med Microbiol 294, 363-371.

Gomez-Duarte, O. G., Galen, J., Chatfield, S. N., Rappuoli, R., Eidels, L., and Levine, M. M. (1995). Expression of fragment C of tetanus toxin fused to a carboxyl-terminal fragment of diphtheria toxin in Salmonella typhi CVD 908 vaccine strain. Vaccine 13, 1596-1602.

Hajishengallis, G., Harokopakis, E., Hollingshead, S. K., Russell, M. W., and Michalek, S. M. (1996). Construction and oral immunogenicity of a Salmonella typhimurium strain expressing a streptococcal adhesin linked to the A2/B subunits of cholera toxin. Vaccine 14, 1545-1548.

Hajishengallis, G., Nawar, H., Tapping, R. I., Russell, M. W., and Connell, T. D. (2004). The Type II heat-labile enterotoxins LT-IIa and LT-IIb and their respective B pentamers differentially induce and regulate cytokine production in human monocytic cells. Infect Immun 72, 6351-6358.

Harokopakis, E., Hajishengallis, G., Greenway, T. E., Russell, M. W., and Michalek, S. M. (1997). Mucosal immunogenicity of a recombinant Salmonella typhimurium-cloned heterologous antigen in the absence or presence of coexpressed cholera toxin A2 and B subunits. Infect Immun 65, 1445-1454.

Hess, J., Gentschev, I., Miko, D., Welzel, M., Ladel, C., Goebel, W., and Kaufmann, S. H. (1996). Superior efficacy of secreted over somatic antigen display in recombinant Salmonella vaccine induced protection against listeriosis. Proc Natl Acad Sci U S A 93, 1458-1463.

Holmgren, J., Adamsson, J., Anjuere, F., Clemens, J., Czerkinsky, C., Eriksson, K., Flach, C. F., George-Chandy, A., Harandi, A. M., Lebens, M., et al. (2005). Mucosal

adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. Immunol Lett 97, 181-188.

Holmgren, J., and Czerkinsky, C. (2005). Mucosal immunity and vaccines. Nat Med 11, S45-53.

Holmgren, J., Czerkinsky, C., Eriksson, K., and Mharandi, A. (2003). Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine 21 Suppl 2, S89-95.

Hormozi, K., Parton, R., and Coote, J. (1999). Adjuvant and protective properties of native and recombinant Bordetella pertussis adenylate cyclase toxin preparations in mice. FEMS Immunol Med Microbiol 23, 273-282.

Huang, Y., Hajishengallis, G., and Michalek, S. M. (2000). Construction and characterization of a Salmonella enterica serovar typhimurium clone expressing a salivary adhesin of Streptococcus mutans under control of the anaerobically inducible nirB promoter. Infect Immun 68, 1549-1556.

Hunt, P. D., and Hardy, S. J. (1991). Heat-labile enterotoxin can be released from Escherichia coli cells by host intestinal factors. Infect Immun 59, 168-171.

Jackson, R. J., Marinaro, M., VanCott, J. L., Yamamoto, M., Okahashi, N., Fujihashi, K., Kiyono, H., Chatfield, S. N., and McGhee, J. R. (1996). Mucosal immunity: regulation by helper T cells and a novel method for detection. J Biotechnol 44, 209-216.

Jagusztyn-Krynicka, E. K., Clark-Curtiss, J. E., and Curtiss, R., 3rd (1993). Escherichia coli heat-labile toxin subunit B fusions with Streptococcus sobrinus antigens expressed by Salmonella typhimurium oral vaccine strains: importance of the linker for antigenicity and biological activities of the hybrid proteins. Infect Immun 61, 1004-1015.

Khan, C. M., Villarreal-Ramos, B., Pierce, R. J., Demarco de Hormaeche, R., McNeill, H., Ali, T., Chatfield, S., Capron, A., Dougan, G., and Hormaeche, C. E. (1994a). Construction, expression, and immunogenicity of multiple tandem copies of the Schistosoma mansoni peptide 115-131 of the P28 glutathione S-transferase expressed as C-terminal fusions to tetanus toxin fragment C in a live aro-attenuated vaccine strain of Salmonella. J Immunol 153, 5634-5642.

Khan, C. M., Villarreal-Ramos, B., Pierce, R. J., Riveau, G., Demarco de Hormaeche, R., McNeill, H., Ali, T., Fairweather, N., Chatfield, S., Capron, A., and et al. (1994b). Construction, expression, and immunogenicity of the Schistosoma mansoni P28 glu-

tathione S-transferase as a genetic fusion to tetanus toxin fragment C in a live Aro attenuated vaccine strain of Salmonella. Proc Natl Acad Sci U S A 91, 11261-11265.

Konieczny, M. P., Suhr, M., Noll, A., Autenrieth, I. B., and Alexander Schmidt, M. (2000). Cell surface presentation of recombinant (poly-) peptides including functional T-cell epitopes by the AIDA autotransporter system. FEMS Immunol Med Microbiol 27, 321-332.

Koprowski, H., 2nd, Levine, M. M., Anderson, R. J., Losonsky, G., Pizza, M., and Barry, E. M. (2000). Attenuated Shigella flexneri 2a vaccine strain CVD 1204 expressing colonization factor antigen I and mutant heat-labile enterotoxin of enterotoxigenic Escherichia coli. Infect Immun 68, 4884-4892.

Kweon, M. N., Yamamoto, M., Watanabe, F., Tamura, S., Van Ginkel, F. W., Miyauchi, A., Takagi, H., Takeda, Y., Hamabata, T., Fujihashi, K., et al. (2002). A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. J Infect Dis 186, 1261-1269.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lahiri, S. S. (2000). Bacterial toxins--an overview. J Nat Toxins 9, 381-408.

Lavelle, E. C., McGuirk, P., and Mills, K. H. (2004). Molecules of infectious agents as immunomodulatory drugs. Curr Top Med Chem 4, 499-508.

Lee, J. J., Sinha, K. A., Harrison, J. A., de Hormaeche, R. D., Riveau, G., Pierce, R. J., Capron, A., Wilson, R. A., and Khan, C. M. (2000). Tetanus toxin fragment C expressed in live Salmonella vaccines enhances antibody responses to its fusion partner Schistosoma haematobium glutathione S-transferase. Infect Immun 68, 2503-2512.

Loeffler, D. I., Schoen, C. U., Goebel, W., and Pilgrim, S. (2006). Comparison of different live vaccine strategies in vivo for delivery of protein antigen or antigen-encoding DNA and mRNA by virulence-attenuated Listeria monocytogenes. Infect Immun 74, 3946-3957.

Loessner, M. J., Wendlinger, G., and Scherer, S. (1995). Heterogeneous endolysins in Listeria monocytogenes bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. Mol Microbiol 16, 1231-1241.

Lycke, N. (2005). From toxin to adjuvant: basic mechanisms for the control of mucosal IgA immunity and tolerance. Immunol Lett 97, 193-198.

Lycke, N., Tsuji, T., and Holmgren, J. (1992). The adjuvant effect of Vibrio cholerae and Escherichia coli heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol 22, 2277-2281.

McSorley, S. J., Rask, C., Pichot, R., Julia, V., Czerkinsky, C., and Glaichenhaus, N. (1998). Selective tolerization of Th1-like cells after nasal administration of a cholera toxoid-LACK conjugate. Eur J Immunol 28, 424-432.

Mendelson, I., Gat, O., Aloni-Grinstein, R., Altboum, Z., Inbar, I., Kronman, C., Bar-Haim, E., Cohen, S., Velan, B., and Shafferman, A. (2005). Efficacious, nontoxigenic Bacillus anthracis spore vaccines based on strains expressing mutant variants of lethal toxin components. Vaccine 23, 5688-5697.

Mesnage, S., Weber-Levy, M., Haustant, M., Mock, M., and Fouet, A. (1999). Cell surface-exposed tetanus toxin fragment C produced by recombinant Bacillus anthracis protects against tetanus toxin. Infect Immun 67, 4847-4850.

Michalkiewicz, J., Stachowski, J., Barth, C., Patzer, J., Dzierzanowska, D., and Madalinski, K. (1999). Effect of Pseudomonas aeruginosa exotoxin A on IFN-gamma synthesis: expression of costimulatory molecules on monocytes and activity of NK cells. Immunol Lett 69, 359-366.

Michl, P., and Gress, T. M. (2004). Bacteria and bacterial toxins as therapeutic agents for solid tumors. Curr Cancer Drug Targets 4, 689-702.

Morona, R., Manning, P. A., and Reeves, P. (1983). Identification and characterization of the TolC protein, an outer membrane protein from Escherichia coli. J Bacteriol 153, 693-699.

Muhlen, K. A., Schumann, J., Wittke, F., Stenger, S., Van Rooijen, N., Van Kaer, L., and Tiegs, G. (2004). NK cells, but not NKT cells, are involved in Pseudomonas aeruginosa exotoxin A-induced hepatotoxicity in mice. J Immunol 172, 3034-3041.

Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R. T., Linder, T., Spyr, C., and Steffen, R. (2004). Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 350, 896-903.

Orr, N., Galen, J. E., and Levine, M. M. (1999). Expression and immunogenicity of a mutant diphtheria toxin molecule, CRM(197), and its fragments in Salmonella typhi vaccine strain CVD 908-htrA. Infect Immun 67, 4290-4294.

Poggi, A., Spada, F., Costa, P., Tomasello, E., Revello, V., Pella, N., Zocchi, M. R., and Moretta, L. (1996). Dissection of lymphocyte function-associated antigen 1-dependent adhesion and signal transduction in human natural killer cells shown by the use of cholera or pertussis toxin. Eur J Immunol 26, 967-975.

Pogonka, T., Klotz, C., Kovacs, F., and Lucius, R. (2003). A single dose of recombinant Salmonella typhimurium induces specific humoral immune responses against heterologous Eimeria tenella antigens in chicken. Int J Parasitol 33, 81-88.

Ramirez, R., Carracedo, J., Zamzami, N., Castedo, M., and Kroemer, G. (1994). Pertussis toxin inhibits activation-induced cell death of human thymocytes, pre-B leukemia cells and monocytes. J Exp Med 180, 1147-1152.

Ranallo, R. T., Fonseka, C. P., Cassels, F., Srinivasan, J., and Venkatesan, M. M. (2005). Construction and characterization of bivalent Shigella flexneri 2a vaccine strains SC608(pCFAI) and SC608(pCFAI/LTB) that express antigens from enterotoxigenic Escherichia coli. Infect Immun 73, 258-267.

Reveneau, N., Geoffroy, M. C., Locht, C., Chagnaud, P., and Mercenier, A. (2002). Comparison of the immune responses induced by local immunizations with recombinant Lactobacillus plantarum producing tetanus toxin fragment C in different cellular locations. Vaccine 20, 1769-1777.

Roland, K. L., Tinge, S. A., Killeen, K. P., and Kochi, S. K. (2005). Recent advances in the development of live, attenuated bacterial vectors. Curr Opin Mol Ther 7, 62-72.

Rosenberg, S. A. (2001). Progress in human tumour immunology and immunotherapy. Nature 411, 380-384.

Ryan, E. T., Butterton, J. R., Smith, R. N., Carroll, P. A., Crean, T. I., and Calderwood, S. B. (1997a). Protective immunity against Clostridium difficile toxin A induced by oral immunization with a live, attenuated Vibrio cholerae vector strain. Infect Immun 65, 2941-2949.

Ryan, E. T., Butterton, J. R., Zhang, T., Baker, M. A., Stanley, S. L., Jr., and Calderwood, S. B. (1997b). Oral immunization with attenuated vaccine strains of Vibrio cholerae expressing a dodecapeptide repeat of the serine-rich Entamoeba histolytica pro-

tein fused to the cholera toxin B subunit induces systemic and mucosal antiamebic and anti-V. cholerae antibody responses in mice. Infect Immun 65, 3118-3125.

Sanchez, J., Wallerstrom, G., Fredriksson, M., Angstrom, J., and Holmgren, J. (2002). Detoxification of cholera toxin without removal of its immunoadjuvanticity by the addition of (STa-related) peptides to the catalytic subunit. A potential new strategy to generate immunostimulants for vaccination. J Biol Chem 277, 33369-33377.

Sanchez, AE., Aquino, G., Ostoa-Saloma, P., Laclette, JP., and Rocha-Zavaleta, L. (2004). Cholera toxin B-subunit gene enhances mucosal Immunoglobulin A, Th1-type and CD8+ cytotoxic responses when co-administered intradermally with a DNA vaccine. Clinic Diagnost Laborat Immun 11, 711-719.

Schmid-Grendelmeier, P., and Crameri, R. (2001). Recombinant allergens for skin testing. Int Arch Allergy Immunol 125, 96-111.

Schodel, F., Enders, G., Jung, M. C., and Will, H. (1990). Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of Escherichia coli heat labile enterotoxin in attenuated salmonellae. Vaccine 8, 569-572.

Shaw, C. A., and Starnbach, M. N. (2003). Using Modified Bacterial Toxins to Deliver Vaccine Antigens. ASM News 69, 384-389.

Silva, A. J., Mohan, A., and Benitez, J. A. (2003). Cholera vaccine candidate 638: intranasal immunogenicity and expression of a foreign antigen from the pulmonary pathogen Coccidioides immitis. Vaccine 21, 4715-4721.

Smerdou, C., Anton, I. M., Plana, J., Curtiss, R., 3rd, and Enjuanes, L. (1996). A continuous epitope from transmissible gastroenteritis virus S protein fused to E. coli heat-labile toxin B subunit expressed by attenuated Salmonella induces serum and secretory immunity. Virus Res 41, 1-9.

Somner, E. A., Ogun, S. A., Sinha, K. A., Spencer Valero, L. M., Lee, J. J., Harrison, J. A., Holder, A. A., Hormaeche, C. E., and Khan, C. M. (1999). Expression of disulphide-bridge-dependent conformational epitopes and immunogenicity of the carboxy-terminal 19 kDa domain of Plasmodium yoelii merozoite surface protein-1 in live attenuated Salmonella vaccine strains. Microbiology 145 (Pt 1), 221-229.

Sory, M. P., and Cornelis, G. R. (1990). Delivery of the cholera toxin B subunit by using a recombinant Yersinia enterocolitica strain as a live oral carrier. Res Microbiol 141, 921-929.

Spangrude, G. J., Sacchi, F., Hill, H. R., Van Epps, D. E., and Daynes, R. A. (1985). Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin. J Immunol 135, 4135-4143.

Su, G. F., Brahmbhatt, H. N., de Lorenzo, V., Wehland, J., and Timmis, K. N. (1992). Extracellular export of Shiga toxin B-subunit/haemolysin A (C-terminus) fusion protein expressed in Salmonella typhimurium aroA-mutant and stimulation of B-subunit specific antibody responses in mice. Microb Pathog 13, 465-476.

Thungapathra, M., Sharma, C., Gupta, N., Ghosh, R. K., Mukhopadhyay, A., Koley, H., Nair, G. B., and Ghosh, A. (1999). Construction of a recombinant live oral vaccine from a non-toxigenic strain of Vibrio cholerae O1 serotype inaba biotype E1 Tor and assessment of its reactogenicity and immunogenicity in the rabbit model. Immunol Lett 68, 219-227.

Todar, K. (2002). Mechanism of bacterial pathogenicity: protein toxins. Todar's Online Textbook of Bacteriology.: http://textbookofbacteriology.net/proteintoxins.html.

Tzschaschel, B. D., Guzman, C. A., Timmis, K. N., and de Lorenzo, V. (1996a). An Escherichia coli hemolysin transport system-based vector for the export of polypeptides: export of Shiga-like toxin IIeB subunit by Salmonella typhimurium aroA. Nat Biotechnol 14, 765-769.

Tzschaschel, B. D., Klee, S. R., de Lorenzo, V., Timmis, K. N., and Guzman, C. A. (1996b). Towards a vaccine candidate against Shigella dysenteriae 1: expression of the Shiga toxin B-subunit in an attenuated Shigella flexneri aroD carrier strain. Microb Pathog 21, 277-288.

van Ginkel, F. W., Jackson, R. J., Yuki, Y., and McGhee, J. R. (2000). Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol 165, 4778-4782.

Vertiev, Y. V., Zdanovsky, A. G., Shevelev, A. B., Borinskaya, S. A., Gening, E. L., Martin, T., Ivanov, P. A., and Yankovsky, N. K. (2001). Recombinant Listeria strains producing the nontoxic L-chain of botulinum neurotoxin A in a soluble form. Res Microbiol 152, 563-567.

Walker, M. J., Rohde, M., Timmis, K. N., and Guzman, C. A. (1992). Specific lung mucosal and systemic immune responses after oral immunization of mice with Salmonella typhimurium aroA, Salmonella typhi Ty21a, and invasive Escherichia coli expressing recombinant pertussis toxin S1 subunit. Infect Immun 60, 4260-4268.

Ward, S. J., Douce, G., Figueiredo, D., Dougan, G., and Wren, B. W. (1999). Immunogenicity of a Salmonella typhimurium aroA aroD vaccine expressing a nontoxic domain of Clostridium difficile toxin A. Infect Immun 67, 2145-2152.

Wiener, M. C. (2000). Bacterial export takes its Tol. Structure 8, R171-175.

Wu, S., Beier, M., Sztein, M. B., Galen, J., Pickett, T., Holder, A. A., Gomez-Duarte, O. G., and Levine, M. M. (2000). Construction and immunogenicity in mice of attenuated Salmonella typhi expressing Plasmodium falciparum merozoite surface protein 1 (MSP-1) fused to tetanus toxin fragment C. J Biotechnol 83, 125-135.

Zheng, J. P., Zhang, Z. S., Li, S. Q., Liu, X. X., Yuan, S. L., Wang, P., Zhan, D. W., Wang, L. C., and Huang, C. F. (2005). Construction of a novel Shigella live-vector strain co-expressing CS3 and LTB/STm of enterotoxigenic E.coli. World J Gastroenterol 11, 3411-3418.

Zhu, C., Ruiz-Perez, F., Yang, Z., Mao, Y., Hackethal, V. L., Greco, K. M., Choy, W., Davis, K., Butterton, J. R., and Boedeker, E. C. (2006). Delivery of heterologous protein antigens via hemolysin or autotransporter systems by an attenuated ler mutant of rabbit enteropathogenic Escherichia coli. Vaccine 24, 3821-3831.

Claims

1. A microorganism as a carrier of nucleotide sequences coding for antigens and protein toxins comprising the following components:

(I) at least one nucleotide sequence coding for at least one complete or partial antigen of at least one wild-type or mutated protein; and

(II) at least one nucleotide sequence coding for at least one protein toxin and/or at least one protein toxin subunit; and

(III) **a)** at least one nucleotide sequence coding for at least one transport system which enables the expression of the expression products of component (I) and component (II) on the outer surface of the microorganism and/or enables the secretion of the expression products of component (I) and component (II); and/or coding for at least one signal sequence which enables the secretion of the expression products of component (I) and component (II); and/or

b) optionally, at least one nucleotide sequence coding for at least one protein for lysing the microorganism in the cytosol of mammalian cells and for intracellularly releasing plasmids or expression vectors, which are contained in the lysed microorganism; and

(IV) at least one nucleotide sequence for at least one activation sequence for the expression of one or more of components (I) to (III), wherein said activation sequence can be activated in the microorganism and/or is tissue cell-specific, tumor cell-specific, macrophage-specific, dendrite-specific, lymphocyte-specific, function-specific or non-cell-specific";

wherein any of components (I) to (IV) can be present either once or several times and if a component of components (I) to (IV) is present several times it can independently from each other be either identical or different.

2. The microorganism according to claim 1, wherein component (I) and component (II) are <u>not</u> identical, that is component (I) does <u>not</u> code for at least one nucleotide sequence coding for at least one protein toxin and/or at least one protein toxin subunit.

3. The microorganism according to any of claims 1 to 2, wherein the microorganism is selected from the group consisting of “bacterium, gram-positive bacterium, gram-negative bacterium, eukaryotic cell” and preferably is selected from the group consisting of "Escherichia spp., Escherichia coli, Salmonella spp., Salmonella typhi, Salmonella typhimurium, Yersinia spp., Yersinia enterocolitica, Vibrio spp., Vibrio cholerae, Listeria spp., Listeria monocytogenes, Shigella spp., Shigella flexneri, Yersinia spp, Pseudomonas spp”, wherein preferably the virulence of the microorganism is attenuated.

4. The microorganism according to claim 3, wherein Vibrio cholerae is excluded as microorganism.

5. The microorganism according to any of claims 1 to 4, wherein the at least one complete or partial antigen of at least one wild-type or mutated protein according to component (I) is selected from the group consisting of the following wild-type proteins and their known mutants: “receptor; extracellular, transmembranic or intracellular part of a receptor; adhesion molecule; extracellular, transmembranic or intracellular part of an adhesion molecule; signal-transducing protein; cell-cycle protein; transcription factor; differentiation protein; embryonic protein; viral protein; allergen; protein of microbial pathogen; protein of eukaryotic pathogen; cancer testis antigen protein; tumor antigen protein; and/or tissue-cell specific protein”, wherein the tissue cell is selected from the group consisting of “glandula thyroidea, glandula mammaria, glandula salivaria, nodus lymphoideus, glandula mammaria, tunica mucosa gastris, kidney, ovarium, prostate, cervix, tunica serosa vesicae urinariae and nevus”.

6. The microorganism according to claim 5, wherein the at least one complete or partial antigen of at least one wild-type or mutated protein according to component (I) is selected from the group consisting of the following wild-type proteins and their known mutants: “Her-2/neu, androgen receptor, estrogen receptor, midkine receptor, EGF receptor, ERBB2, ERBB4, TRAIL receptor, FAS, TNFalpha receptor, TGF-beta receptor, lactoferrin receptor, basic myelin, alpha-lactalbumin, GFAP, fi-

brillary acid protein, tyrosinase, EGR-1, MUC1, c-Raf (Raf-1), A-Raf, B-Raf, B-Raf V599E, B-Raf V600E, B-Raf KD, B-Raf V600E kinase domain, B-Raf V600E KD, B-Raf V600E kinase domain KD, B-Raf kinase domain, B-Raf kinase domain KD, N-Ras, K-Ras, H-Ras, Bcl-2, Bcl-X, Bcl-W, Bfl-1, Brag-1, Mcl-1, A1, Bax, BAD, Bak, Bcl-Xs, Bid, Bik, Hrk, Bcr/abl, Myb, C-Met, IAP1, IAO2, XIAP, ML-IAP LIVIN, survivin, APAF-1, cyclin D(1-3), cyclin E, cyclin A, cyclin B, cyclin H, Cdk-1, Cdk-2, Cdk-4, Cdk-6, Cdk-7, Cdc25C, p16, p15, p21, p27, p18, pRb, p107, p130, E2F(1-5), GAAD45, MDM2, PCNA, ARF, PTEN, APC, BRCA, Akt, PI3K, mTOR, p53 and homologues, C-Myc, NFkB, c-Jun, ATF-2, Sp1, prostate specific antigen (PSA), carcinoembryonic antigen, alpha-fetoprotein, PAP; PSMA; STEAP; MAGE, MAGE-1, MAGE-3, NY-ESO-1, PSCA, MART, Gp100, tyrosinase, GRP, TCF-4, viral antigens of the viruses HIV, HPV, HCV, HPV, EBV, CMV, HSV, influenza virus, influenza virus type A, influenza virus type A (H5N1) and (H3N2), influenza virus type B, influenza virus type C; hemagglutinins, hemagglutinin H1, hemagglutinin H5, hemagglutinin H7, hemagglutinin HA1 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12C (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), neuramidase, p60, LLO, urease, CSP, calflagin and/or CPB" or wherein the at least one complete or partial antigen of at least one wild-type or mutated protein according to component (I) is selected from the group of kinases consisting of the following wild-type proteins and their known mutants (accession numbers in parantheses): AAK1 (NM 014911), AATK (NM 004920), ABL1 (NM 005157), ABL2 (NM 005158), ACK1 (NM 005781), ACVR1 (NM 001105), ACVR1B (NM 020328), ACVR2 (NM 001616), ACVR2B (NM 001106), ACVRL1 (NM 000020), ADCK1 (NM 020421), ADCK2 (NM 052853), ADCK4 (NM 024876), ADCK5 (NM 174922), ADRBK1 (NM 001619), ADRBK2 (NM 005160), AKT1 (NM 005163), AKT2 (NM 001626), AKT3 (NM 005465), ALK (NM 004304), ALK7 (NM 145259), ALS2CR2 (NM 018571), ALS2CR7 (NM 139158), AMHR2 (NM 020547), ANKK1 (NM 178510), ANKRD3 (NM 020639), APEG1 (NM 005876), ARAF (NM 001654), ARK5 (NM 014840), ATM (NM 000051), ATR (NM 001184), AURKA (NM 003600), AURKB (NM 004217), AURKC (NM 003160), AXL (NM 001699), BCKDK (NM 005881), BCR (NM 004327), BIKE (NM 017593), BLK (NM 001715), BMPR1A (NM 004329), BMPR1B (NM 001203), BMPR2 (NM 001204), BMX (NM 001721), BRAF (NM 004333), BRD2 (NM 005104), BRD3 (NM 007371), BRD4 (NM 014299), BRDT

(NM 001726), BRSK1 (NM 032430), BRSK2 (NM 003957), BTK (NM 000061), BUB1 (NM 004336), BUB1B (NM 001211), CABC1 (NM 020247), CAMK1 (NM 003656), CaMK1b (NM 198452), CAMK1D (NM 020397), CAMK1G (NM 020439), CAMK2A (NM 015981), CAMK2B (NM 001220), CAMK2D (NM 001221), CAMK2G (NM 001222), CAMK4 (NM 001744), CAMKK1 (NM 032294), CAMKK2 (NM 006549), CASK (NM 003688), CCRK (NM 012119), CDC2 (NM 001786), CDC2L1 (NM 001787), CDC2L5 (NM 003718), CDC42BPA (NM 014826), CDC42BPB (NM 006035), CDC7L1 (NM 003503), CDK10 (NM 003674), CDK11 (NM 015076), CDK2 (NM 001798), CDK3 (NM 001258), CDK4 (NM 000075), CDK5 (NM 004935), CDK6 (NM 001259), CDK7 (NM 001799), CDK8 (NM 001260), CDK9 (NM 001261), CDKL1 (NM 004196), CDKL2 (NM 003948), CDKL3 (NM 016508), CDKL4 (NM 001009565), CDKL5 (NM 003159), CHEK1 (NM 001274), CHUK (NM 001278), CIT (NM 007174), CLK1 (NM 004071), CLK2 (NM 003993), CLK3 (NM 003992), CLK4 (NM 020666), CRK7 (NM 016507), CSF1R (NM 005211), CSK (NM 004383), CSNK1A1 (NM 001892), CSNK1D (NM 001893), CSNK1E (NM 001894), CSNK1G1 (NM 022048), CSNK1G2 (NM 001319), CSNK1G3 (NM 004384), CSNK2A1 (NM 001895), CSNK2A2 (NM 001896), DAPK1 (NM 004938), DAPK2 (NM 014326), DAPK3 (NM 001348), DCAMKL1 (NM 004734), DCAMKL2 (NM 152619), DCAMKL3 (XM 047355), DDR1 (NM 013993), DDR2 (NM 006182), DMPK (NM 004409), DMPK2 (NM 017525.1), DYRK1A (NM 001396), DYRK1B (NM 006484), DYRK2 (NM 006482), DYRK3 (NM 003582), DYRK4 (NM 003845), EEF2K (NM 013302), EGFR (NM 005228), EIF2AK3 (NM 004836), EIF2AK4 (NM_001013703), EPHA1 (NM 005232), EPHA10 (NM 001004338), EPHA2 (NM 004431), EPHA3 (NM 005233), EPHA4 (NM 004438), EPHA5 (NM 004439), EPHA6 (XM 114973), EPHA7 (NM 004440), EPHA8 (NM 020526), EPHB1 (NM 004441), EPHB2 (NM 017449), EPHB3 (NM 004443), EPHB4 (NM 004444), EPHB6 (NM 004445), ERBB2 (NM 004448), ERBB3 (NM 001982), ERBB4 (NM 005235), ERK8 (NM 139021), ERN1 (NM 001433), ERN2 (NM 033266), FASTK (NM 025096), FER (NM 005246), FES (NM 002005), FGFR1 (NM 000604), FGFR2 (NM 022970), FGFR3 (NM 000142), FGFR4 (NM 022963), FGR (NM 005248), FLJ23074 (NM 025052), FLJ23119 (NM 024652), FLJ23356 (NM 032237), FLT1 (NM 002019), FLT3 (NM 004119), FLT4 (NM 002020), FRAP1 (NM 004958), FRK (NM 002031), FYN (NM 002037), GAK (NM 005255), GPRK5 (NM 005308), GPRK6 (NM 002082), GPRK7 (NM 139209), GRK4 (NM 005307), GSG2 (NM 031965), GSK3A (NM 019884), GSK3B (NM

002093), GUCY2C (NM 004963), GUCY2D (NM 000180), GUCY2F (NM 001522), H11 (NM 014365), HAK (NM 052947), HCK (NM 002110), HIPK1 (NM 152696), HIPK2 (NM 022740), HIPK3 (NM 005734), HIPK4 (NM 144685), HRI (NM 014413), HUNK (NM 014586), ICK (NM 016513), IGF1R (NM 000875), IKBKB (NM 001556), IKBKE (NM 014002), ILK (NM 004517), INSR (NM 000208), INSRR (NM 014215), IRAK1 (NM 001569), IRAK2 (NM 001570), IRAK3 (NM 007199), IRAK4 (NM 016123), ITK (NM 005546), JAK1 (NM 002227), JAK2 (NM 004972), JAK3 (NM 000215), KDR (NM 002253), KIS (NM 144624), KIT (NM 000222), KSR (XM 290793), KSR2 (NM 173598), LAK (NM 025144), LATS1 (NM 004690), LATS2 (NM 014572), LCK (NM 005356), LIMK1 (NM 016735), LIMK2 (NM 005569), LMR3 (XM 055866), LMTK2 (NM 014916), LOC149420 (NM 152835), LOC51086 (NM 015978), LRRK2 (XM 058513), LTK (NM 002344), LYN (NM 002350), MAK (NM 005906), MAP2K1 (NM 002755), MAP2K2 (NM 030662), MAP2K3 (NM 002756), MAP2K4 (NM 003010), MAP2K5 (NM 002757), MAP2K6 (NM 002758), MAP2K7 (NM 005043), MAP3K1 (XM 042066), MAP3K10 (NM 002446), MAP3K11 (NM 002419), MAP3K12 (NM 006301), MAP3K13 (NM 004721), MAP3K14 (NM 003954), MAP3K2 (NM 006609), MAP3K3 (NM 002401), MAP3K4 (NM 005922), MAP3K5 (NM 005923), MAP3K6 (NM 004672), MAP3K7 (NM 003188), MAP3K8 (NM 005204), MAP3K9 (NM 033141), MAP4K1 (NM 007181), MAP4K2 (NM 004579), MAP4K3 (NM 003618), MAP4K4 (NM 145686), MAP4K5 (NM 006575), MAPK1 (NM 002745), MAPK10 (NM 002753), MAPK11 (NM 002751), MAPK12 (NM 002969), MAPK13 (NM 002754), MAPK14 (NM 001315), MAPK3 (NM 002746), MAPK4 (NM 002747), MAPK6 (NM 002748), MAPK7 (NM 002749), MAPK8 (NM 002750), MAPK9 (NM 002752), MAPKAPK2 (NM 032960), MAPKAPK3 (NM 004635), MAPKAPK5 (NM 003668), MARK (NM 018650), MARK2 (NM 017490), MARK3 (NM 002376), MARK4 (NM 031417), MAST1 (NM 014975), MAST205 (NM 015112), MAST3 (XM 038150), MAST4 (XM 291141), MASTL (NM 032844), MATK (NM 139355), MELK (NM 014791), MERTK (NM 006343), MET (NM 000245), MGC33182 (NM 145203), MGC42105 (NM 153361), MGC43306 (C9orf96), MGC8407 (NM 024046), MIDORI (NM 020778), MINK (NM 015716), MKNK1 (NM 003684), MKNK2 (NM 017572), MLCK (NM 182493), MLK4 (NM 032435), MLKL (NM 152649), MOS (NM 005372), MST1R (NM 002447), MST4 (NM 016542), MUSK (NM 005592), MYLK (NM 053025), MYLK2 (NM 033118), MYO3A (NM 017433), MYO3B (NM 138995), NEK1 (NM 012224), NEK10 (NM 152534), NEK11 (NM 024800), NEK2 (NM 002497), NEK3

(NM 002498), NEK4 (NM 003157), NEK5 (MGC75495), NEK6 (NM 014397), NEK7 (NM 133494), NEK8 (NM 178170), NEK9 (NM 033116), NLK (NM 016231), NPR1 (NM 000906), NPR2 (NM 003995), NRBP (NM 013392), NRBP2 (NM 178564), NRK (NM 198465), NTRK1 (NM 002529), NTRK2 (NM 006180), NTRK3 (NM 002530), OBSCN (NM 052843), OSR1 (NM 005109), PACE-1 (NM 020423), PAK1 (NM 002576), PAK2 (NM 002577), PAK3 (NM 002578), PAK4 (NM 005884), PAK6 (NM 020168), PAK7 (NM 020341), PASK (NM 015148), PCTK1 (NM 006201), PCTK2 (NM 002595), PCTK3 (NM 212503), PDGFRA (NM 006206), PDGFRB (NM 002609), PDK1 (NM 002610), PDK2 (NM 002611), PDK3 (NM 005391), PDK4 (NM 002612), PDPK1 (NM 002613), PFTK1 (NM 012395), PHKG1 (NM 006213), PHKG2 (NM 000294), PIK3R4 (NM 014602), PIM1 (NM 002648), PIM2 (NM 006875), PIM3 (NM 001001852), PINK1 (NM 032409), PKE (NM 173575), PKMYT1 (NM 004203), pknbeta (NM 013355), PLK (NM 005030), PLK3 (NM 004073), PRKAA1 (NM 006251), PRKAA2 (NM 006252), PRKACA (NM 002730), PRKACB (NM 002731), PRKACG (NM 002732), PRKCA (NM 002737), PRKCB1 (NM 002738), PRKCD (NM 006254), PRKCE (NM 005400), PRKCG (NM 002739), PRKCH (NM 006255), PRKCI (NM 002740), PRKCL1 (NM 002741), PRKCL2 (NM 006256), PRKCM (NM 002742), PRKCN (NM 005813), PRKCQ (NM 006257), PRKCZ (NM 002744), PRKD2 (NM 016457), PRKDC (NM 006904), PRKG1 (NM 006258), PRKG2 (NM 006259), PRKR (NM 002759), PRKWNK1 (NM 018979), PRKWNK2 (NM 006648), PRKWNK3 (NM 020922), PRKWNK4 (NM 032387), PRKX (NM 005044), PRKY (NM 002760), PRPF4B (NM 003913), PSKH1 (NM 006742), PSKH2 (NM 033126), PTK2 (NM 005607), PTK2B (NM 004103), PTK6 (NM 005975), PTK7 (NM 002821), PTK9 (NM 002822), PTK9L (NM 007284), PXK (NM 017771), QSK (NM 025164), RAD53 (NM 007194), RAF1 (NM 002880), RAGE (NM 014226), RET (NM 020975), RHOK (NM 002929), RIOK1 (NM 031480), RIOK2 (NM 018343), RIPK1 (NM 003804), RIPK2 (NM 003821), RIPK3 (NM 006871), RIPK5 (NM 015375), RNASEL (NM 021133), ROCK1 (NM 005406), ROCK2 (NM 004850), ROR1 (NM 005012), ROR2 (NM 004560), ROS1 (NM 002944), RPS6KA1 (NM 002953), RPS6KA2 (NM 021135), RPS6KA3 (NM 004586), RPS6KA4 (NM 003942), RPS6KA5 (NM 004755), RPS6KA6 (NM 014496), RPS6KB1 (NM 003161), RPS6KB2 (NM 003952), RPS6KC1 (NM 012424), RPS6KL1 (NM 031464), RYK (NM 002958), SBK (XM 370948), SCYL1 (NM 020680), SCYL2 (NM 017988), SGK (NM 005627), SgK069 (SU SgK069), SgK085 (XM 373109), SgK110 (SU SgK110), SGK2 (NM 016276),

SgK223 (XM 291277), SgK269 (XM 370878), SgK424 (CGP SgK424), SgK493 (SU_SgK493), SgK494 (NM 144610), SgK495 (NM 032017), SGKL (NM 013257), SK681 (NM 001001671), SLK (NM 014720), SMG1 (NM 015092), SNARK (NM 030952), SNF1LK (NM 173354), SNF1LK2 (NM 015191), SNK (NM 006622), SNRK (NM 017719), SRC (NM 005417), SRMS (NM 080823), SRPK1 (NM 003137), SRPK2 (NM 003138), SSTK (NM 032037), STK10 (NM 005990), STK11 (NM 000455), STK16 (NM 003691), STK17A (NM 004760), STK17B (NM 004226), STK18 (NM 014264), STK19 (NM 032454), STK22B (NM 053006), STK22C (NM 052841), STK22D (NM 032028), STK23 (NM 014370), STK24 (NM 003576), STK25 (NM 006374), STK3 (NM 006281), STK31 (NM 031414), STK32B (NM 018401), STK33 (NM 030906), STK35 (NM 080836), STK36 (NM 015690), STK38 (NM 007271), STK38L (NM 015000), STK39 (NM 013233), STK4 (NM 006282), STLK5 (NM 001003787), STYK1 (NM 018423), SUDD (NM 003831), SYK (NM 003177), TAF1 (NM 138923), TAF1L (NM 153809), TAO1 (NM 004783), TAOK1 (NM 020791), TAOK3 (NM 016281), TBCK (NM 033115), TBK1 (NM 013254), TEC (NM 003215), TEK (NM 000459), TESK1 (NM 006285), TESK2 (NM 007170), TEX14 (NM 031272), TGFBR1 (NM 004612), TGFBR2 (NM 003242), TIE (NM 005424), TIF1 (NM 003852), TLK1 (NM 012290), TLK2 (NM 006852), TNIK (NM 015028), TNK1 (NM 003985), TOPK (NM 018492), TP53RK (NM 033550), TRAD (NM 007064), TRIB1 (NM 025195), TRIB2 (NM 021643), TRIB3 (NM 021158), TRIM28 (NM 005762), TRIM33 (NM 015906), TRIO (NM 007118), TRPM6 (NM 017662), TRPM7 (NM 017672), TRRAP (NM 003496), TSSK4 (NM 174944), TTBK1 (NM 032538), TTBK2 (NM 173500), TTK (NM 003318), TTN (NM 003319), TXK (NM 003328), TYK2 (NM 003331), TYRO3 (NM 006293), ULK1 (NM 003565), ULK2 (NM 014683), ULK3 (NM 015518), ULK4 (NM 017886), VRK1 (NM 003384), VRK2 (NM 006296), VRK3 (NM 016440), WEE1 (NM 003390), Wee1B (NM 173677), YANK1 (NM 145001), YES1 (NM 005433), ZAK (NM 016653), and/or ZAP70 (NM 001079)".

7. The microorganism according to any of claims 1 to 6, wherein component (II) is selected from the group consisting of "bacterial toxin, enterotoxin, exotoxin, type I toxin, type II toxin, type III toxin, type IV toxin, type V toxin, RTX toxin, AB toxin, A-B toxin, A/B toxin, A+B toxin, A-5B toxin and/or AB5 toxin".

8. The microorganism according to claim 7, wherein component (II) is selected from the group consisting of "Adenylate cyclase toxin, Anthrax toxin, Anthrax toxin (EF), Anthrax toxin (LF), Botulinum toxin, Cholera toxin (CT, Ctx), Cholera toxin subunit B (CTB, CtxB), Diphtheria toxin (DT, Dtx), E. coli LT toxin, E. coli heat labile enterotoxin (LT), E. coli heat labile enterotoxin subunit B (LTB), E. coli ST toxin, E. coli heat stabile enterotoxin (ST), Erythrogenic toxin, Exfoliatin toxin, Exotoxin A, Perfringens enterotoxin, Pertussis toxin (PT, Ptx), Shiga toxin (ST, Stx), Shiga toxin subunit B (STB, StxB), Shiga-like toxin, Staphylococcus enterotoxins, Tetanus toxin (TT), Toxic shock syndrome toxin (TSST-1), Vero toxin (VT), Toxin A (TA) and Toxin B (TB) of Clostridium difficile, Lethal Toxin (LT) and Hemorrhagic Toxin (HT) of Clostridium sordellii, alpha Toxin (AT) of Clostridium novyi".

9. The microorganism according to any of claims 1 to 8, wherein component (I) and component (II) are linked together to enable the expression and/or secretion of a fusion protein encoded by both components.

10. The microorganism according to claim 9, wherein the fusion protein is selected from the group consisting of "CtxB–PSA, CtxB–B-Raf V600E KD, CtxB–B-Raf V600E kinase domain, CtxB–B-Raf V600E kinase domain KD, CtxB-B-Raf, CtxB-B-Raf KD, CtxB B-Raf kinase domain KD, CtxB-HA1, CtxB-HA12C".

11. The microorganism according to any of claims 1 to 10, wherein component (III) a) is selected from the group consisting of "type I secretion system, type II secretion system, type III secretion system, type IV secretion system, type V secretion system, hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter), hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of a not hly-specific bacterial promoter), transport signal for the S-layer (Rsa A) protein of Caulobacter crescentus, transport signal for the TolC protein of Escherichia coli, secretion signal Vtgss and/or secretion signals derived from listeriolysin, p60 and/or ActA" and wherein component (III) b) is selected from the group consisting of "endolysins, lytic protein

of gram-positive bacteria, lytic protein of Listeria monocytogenes, PLY551 of Listeria monocytogenes and/or holin of Listeria monocytogenes".

12. The microorganism according to claim 11, wherein component (III) a) is at least one nucleotide sequence coding for only one transport system, which enables the concomitant expression of the expression products of component (I) and component (II) on the outer surface of the microorganism and/or enables the concomitant secretion of the expression products of component (I) and component (II), wherein such component (III) a) preferably is at least one nucleotide sequence coding for the hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter) or the hemolysin transport system (signal) of Escherichia coli (nucleotide sequences containing HlyA, HlyB and HlyD under the control of a not hly-specific bacterial promoter).

13. The microorganism according to any of claims 1 to 12, wherein according to component (III) a) the expression products of components (I) and component (II) are secreted.

14. The microorganism according to any of claims 1 to 13, wherein

component **(I)** is selected from the group consisting of B-Raf V600E, B-Raf V600E kinase domain, B-Raf V600E KD, B-Raf V600E kinase domain KD, B-Raf KD, B-Raf kinase domain, B-Raf kinase domain KD, prostate specific antigen (PSA), hemagglutinin HA1 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12 (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1), hemagglutinin HA12C (preferably from Influenza A virus (A/Thailand/1 (KAN-1)2004(H5N1)";

component **(II)** is selected from the group consisting of "Cholera toxin subunit B (CTB, CtxB), E. coli heat-labile enterotoxin subunit B (LTB), tetanus toxin (TT)";

component **(III) a)** is selected from the group consisting of "HlyA hemolysin transport signal of Escherichia coli together with components of the Hly secretion sys-

tem (nucleotide sequences containing HlyA, HlyB and HlyD under the control of the hly-specific promoter)";

component **(IV)** is selected from the group consisting of "endogenous promoter of the E. coli hly locus";

wherein component (I) and component (II) are linked together to enable the expression of a fusion protein encoded by both components and wherein the fusion protein is secreted.

15. Pharmaceutical composition comprising at least one microorganism, preferably at least one lyophilized microorganism, according to any of claims 1 to 14 and a pharmaceutically acceptable carrier, preferably capsules.

16. Medicament comprising at least one microorganism according to any of claims 1 to 14 or at least one pharmaceutical composition according to claim 15.

17. Use of a microorganism according to any of claims 1 to 14 for the production of a medicament for the treatment and/or prophylaxis of physiological and/or pathophysiological conditions selected from the group consisting of "uncontrolled cell division, malignant tumors, benign tumors, solid tumors, sarcomas, carcinomas, hyperproliferative disorders, carcinoids, Ewing sarcomas, Kaposi sarcomas, brain tumors, tumors originating from the brain and/or the nervous system and/or the meninges, gliomas, neuroblastomas, stomach cancer, kidney cancer, kidney cell carcinomas, prostate cancer, prostate carcinomas, connective tissue tumors, soft tissue sarcomas, pancreas tumors, liver tumors, head tumors, neck tumors, oesophageal cancer, thyroid cancer, osteosarcomas, retinoblastomas, thymoma, testicular cancer, lung cancer, bronchial carcinomas, breast cancer, mamma carcinomas, intestinal cancer, colorectal tumors, colon carcinomas, rectum carcinomas, gynecological tumors, ovary tumors/ovarian tumors, uterine cancer, cervical cancer, cervix carcinomas, cancer of body of uterus, corpus carcinomas, endometrial carcinomas, urinary bladder cancer, bladder cancer, skin cancer, basaliomas, spinaliomas, melanomas, intraocular melanomas, leukemia, chronic leukemia, acute leukemia, lymphomas, infection, viral or bacterial infection, influenza, chronic inflammation, organ rejection and/or autoimmune diseases".

18. Plasmid or expression vector comprising components (I) to (IV) according to any of claims 1 to 14.

19. Process for the production of a microorganism according to any of claims 1 to 14, wherein a plasmid or expression vector according to claim 18 is produced, and a microorganism is transformed with this plasmid or expression vector.

20. Pharmaceutical kit comprising at least one microorganism according to any of claims 1 to 14 or a pharmaceutical composition according to claim 15 or a medicament according to claim 16 and a pharmacologically acceptable buffer, preferably a carbonate buffer.