

METHOD OF PRODUCING HIGH YIELD ATTENUATED SALMONELLA STRAINS**FIELD OF THE INVENTION**

[0001] This invention relates to a novel method for growing attenuated mutant *Salmonella typhi* strains lacking galactose epimerase activity and harboring a recombinant DNA molecule. The method comprises the step of culturing said *Salmonella typhi* strain without adding glucose to the medium during the fermentation with a starting glucose amount that is depleted before reaching the stationary phase. The invention further relates to attenuated mutant *Salmonella typhi* strains obtainable by said method and to an attenuated mutant *Salmonella typhi* strain harboring a recombinant DNA molecule encoding a VEGF receptor protein for use as a vaccine.

BACKGROUND OF THE INVENTION

[0002] Among the number of different approaches for vaccine development, live bacterial vaccines are one of the most promising, as they mimic the route of entry of many pathogens and are able to elicit effective humoral and cellular immune responses, at the level of both systemic and mucosal compartments. Live bacterial vaccines can be administered orally or nasally, which offers advantages of simplicity and safety compared to parental administration. Batch preparation costs are relatively low and formulations of live bacterial vaccines show high stability. Attenuation can be accomplished by deletion of various genes, including virulence, regulatory, and metabolic genes.

[0003] Attenuated bacterial vaccines can not only be used to induce immunity to their corresponding pathogenic strain, but they can also be modified to deliver one or more heterologous antigens.

[0004] Attenuated derivatives of *Salmonella enterica* are attractive as vehicles for the delivery of heterologous antigens to the mammalian immune system because *S. enterica* strains can potentially be delivered via mucosal routes of immunization and have the ability to invade host tissues and persist, while continuing to produce a heterologous antigen. Furthermore, *Salmonella* strains elicit strong humoral and cellular immune responses, at the level of both systemic and mucosal compartments.

[0005] Several *Salmonella typhimurium* strains attenuated by *aro* mutations have been shown to be safe and effective delivery vehicles for heterologous antigens in animal models.

[0006] Approaches of delivering DNA constructs encoding heterologous antigens, in particular VEGF receptor proteins, via live attenuated *Salmonella typhimurium* strains into mouse target cells are described in WO 03/073995. Niethammer et al., (Nature Medicine 2002, 8(12), 1369) demonstrated that the attenuated *S. typhimurium aroA* strain SL7207 harboring an expression vector encoding the murine vascular endothelial growth factor receptor 2 (VEGFR-2 or FLK-1), which is essential for tumor angiogenesis, is functional as a cancer vaccine.

[0007] There is however only one attenuated *Salmonella enterica* serovar strain, namely *Salmonella enterica* serovar typhi Ty21a (short: *S. typhi* Ty21a), which has been accepted for use in humans.

[0008] This well-tolerated, live oral vaccine against typhoid fever was derived by chemical mutagenesis of the wild-type virulent bacterial isolate *S. typhi* Ty2 and harbors a loss-of-function mutation in the *galE* gene, as well as other less defined mutations. It has been licensed as typhoid vaccine in many countries after it was shown to be efficacious and safe in field trials.

[0009] There is a strong demand for live attenuated bacterial vectors as delivery vehicles for heterologous antigens - especially cancer antigens - that are safe for use in humans. The provision of such an attenuated bacterial vector as DNA vaccine also calls for the efficient, high-yield cultivation of the attenuated bacterial strain transformed with said heterologous antigen DNA. Transformation of bacterial strains with recombinant DNA constructs often results in decreased cell growth. Thus, it is often necessary to improve the preferably large-scale cultivation process to obtain high yields of viable and functionally active cells.

OBJECTS OF THE INVENTION

[0010] It was an object of the present invention to improve the methods of the prior art for growing attenuated mutants strains of *Salmonella typhi*. In particular, it was an object of the present invention to develop an efficient cultivation method for obtaining high yields of viable bacterial cells harboring a recombinant DNA molecule encoding a heterologous antigen. Such a method and attenuated mutant strains of *Salmonella typhi* obtainable by such a method would serve to satisfy the great need for safe attenuated *Salmonella* strains as DNA vaccines for use in humans. Such a method would be particularly suitable for the commercial large-scale production of DNA vaccines based on attenuated *Salmonella* strains.

SUMMARY OF THE INVENTION

[0011] Surprisingly, it has been found that the cell yield of an attenuated *Salmonella typhi* strain lacking galactose epimerase activity can be remarkably increased, if the amount of glucose in the medium is reduced to zero before reaching the stationary phase. The inventors have shown that glucose addition to the medium during fermentation does not result in higher cell mass / higher OD values. On the contrary, omitting glucose addition during the cultivation of the attenuated *Salmonella* strain yields in optical densities of about 6 to about 8 at the onset of the stationary phase of cell growth, whereas identical culturing at a glucose level of about 1 to about 4 g / l, preferably of about 2 to about 3 g / l (e.g. achieved by sequentially pulsing the medium with glucose) results in optical densities of only about 3 to about 5.5.

[0012] The method for growing an attenuated mutant strain of *Salmonella typhi* according to the invention further yields cells of different morphology as compared to cells cultivated with glucose. *Salmonella typhi* cells grown without glucose are smaller and shorter than cells cultivated with glucose. However, these morphologically different cells, like the cells grown in a glucose containing medium, are fully biologically active and do not show any tendency for cell lysis.

[0013] The increase in cell yield obtained by omitting glucose-addition during cultivation according to the method of the present invention is observed regardless of whether the selected attenuated mutant strain of *Salmonella typhi* harbors a recombinant DNA molecule encoding a heterologous antigen (such as pcDNA3.1-FLK1, or a plasmid derived thereof, like pVAX10.VR2-1) or not (the empty attenuated strain).

[0014] Thus, in one aspect, the present invention relates to a method for growing an attenuated mutant strain of *Salmonella typhi* lacking galactose epimerase activity and comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette, comprising the step of culturing the strain in a buffered medium comprising peptone at approximately neutral starting pH value at fermentation scale, wherein the amount of glucose in the medium during the fermentation is adjusted such that the amount of glucose is reduced to zero before reaching the stationary phase,

wherein no glucose is added to the medium during the fermentation,

wherein the starting glucose concentration is from about 0 g/l to about 3 g/l,

and wherein the volume of the medium is at least about 10 l.

[0015] In a particular embodiment, no glucose is added to the medium during the fermentation and the starting amount of glucose is depleted before reaching the stationary phase.

[0016] In a particular embodiment, said attenuated mutant strain of *Salmonella typhi* is *Salmonella typhi* Ty21a.

[0017] In a particular embodiment, the expression cassette is a eukaryotic expression cassette. In a particular embodiment, the expression cassette encodes a VEGF receptor protein. In a particular embodiment, the VEGF receptor protein is selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith. In a preferred embodiment, human VEGFR-2 has the amino acid sequence as found in SEQ ID NO 1.

[0018] In a particular embodiment, the buffered medium comprises peptone of non-animal origin. In a preferred embodiment, said buffered medium is Tryptic Soy Broth (TSB) of non-animal origin.

[0019] In a particular embodiment, the volume of the medium is at least about 10 l, more particularly from about 10 l to about 10.000 l, more particularly from about 30 l to about 1.000 l, most particularly from about 100 l to about 500 l.

[0020] In a particular embodiment, the starting glucose concentration corresponds to that of bacterial minimal medium or less, particularly the starting glucose concentration is from about 0 g/l to about 4 g/l.

[0021] In a particular embodiment, the starting pH value is from about 5 to less than about 9, particularly from about 6 to about 8, more particularly from about 6.5 to about 7.5.

[0022] In a particular embodiment, the pH value is adjusted during said culturing to a pH value of about 6 to about 8, particularly to a pH value of about 6.5 to about 7.5.

[0023] In a particular embodiment, the progress of growth is determined by measuring the optical density (OD), particularly by in-situ monitoring of the optical density of the culture or by taking samples and measuring the optical density of the samples.

[0024] In another particular embodiment, the progress of growth is determined by measuring the cell density, particularly microscopically or by measuring the electrical resistance, or by flow cytometry.

[0025] In another particular embodiment, the progress of growth is determined by measuring the colony forming units (CFU) value by taking samples and plating on agar plates.

[0026] In a particular embodiment, the cells are harvested before reaching an optical density of about 6, particularly at an optical density of about 5 to about 6.

[0027] In a particular embodiment, the attenuated mutant strain of *Salmonella typhi* is *Salmonella typhi* Ty21a and the recombinant DNA molecule comprises the kanamycin resistance gene, the pMB1 ori, and a eukaryotic expression cassette encoding human VEGFR-2, under the control of the CMV promoter. In a particular embodiment, human VEGFR-2 has the nucleic acid sequence as found in SEQ ID NO 2.

[0028] In another aspect, the present invention relates to an attenuated mutant strain of *Salmonella typhi* lacking galactose epimerase activity and comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette,

obtainable by a method for growing the strain, comprising the step of culturing the strain in a buffered medium comprising peptone at approximately neutral starting pH at fermentation scale,

wherein the amount of glucose in the medium during the fermentation is adjusted such that the amount of glucose is reduced to zero before reaching the stationary phase,

wherein no glucose is added to the medium during the fermentation,

wherein the starting glucose concentration is from about 0 g/l to about 3 g/l,

wherein the volume of the medium is at least about 10 l.

[0029] In a particular embodiment, no glucose is added to the medium during the fermentation and the starting amount of glucose is depleted before reaching the stationary phase.

[0030] In a particular embodiment, the expression cassette is a eukaryotic expression cassette encoding a VEGF receptor protein. In a particular embodiment, the eukaryotic

expression cassette encodes a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least

about 80% homology therewith. In a particular embodiment, human VEGFR-2 has the amino acid sequence as found in SEQ ID NO 1.

[0031] In a particular embodiment, the attenuated mutant strain is *Salmonella typhi* Ty21a and the recombinant DNA molecule comprises the kanamycin resistance gene, the pMB1 ori, and a eukaryotic expression cassette encoding human VEGFR-2 under the control of the CMV promoter. In a particular embodiment, human VEGFR-2 has the nucleic acid sequence as found in SEQ ID NO 2.

[0032] In yet another aspect, the present invention relates to an attenuated mutant strain of *Salmonella typhi* Ty21a comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette encoding a VEGF receptor protein for use as a vaccine.

[0033] In certain embodiments, the VEGF receptor protein is selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith.

[0034] In a preferred embodiment, the human VEGFR-2 has the amino acid sequence as found in SEQ ID NO 1.

[0035] The fact that higher cell yields of attenuated mutant strains of *Salmonella typhi* can be obtained by the method according to the invention, is of importance with respect to the safe and economic manufacture of a commercial DNA vaccine based on an attenuated *Salmonella* strain, such as the approved *Salmonella typhi* Ty21a strain. *Salmonella typhi* Ty21a carrying a recombinant DNA molecule encoding a heterologous antigen, such as the expression plasmid pVAX10.VR2-1, can thus be cultured in higher yields, resulting in higher yields of the DNA vaccine for use in

humans. This is also in compliance with the strong safety rules which must be applied when manufacturing and cultivating *Salmonella*, even in an attenuated version.

DETAILED DESCRIPTION OF THE INVENTION

[0036] The present invention may be understood more readily by reference to the following detailed description of the invention and the examples included therein.

1) *Salmonella typhi* including wild-type *Salmonella typhi* Ty2 and attenuated *Salmonella typhi* Ty21a

[0037] Within the subject method any attenuated *Salmonella typhi* strain may be used. The attenuated *S. typhi* Ty21a strain is the active component of Typhoral L[®], also known as Vivotif[®] (manufactured by Berna Biotech Ltd., a Crucell Company, Switzerland). It is currently the only licensed live oral vaccine against typhoid fever. This vaccine is licensed in more than 40 countries. The Marketing Authorization number of Typhoral L[®] is PL 15747/0001 dated 16 December 1996. One dose of vaccine contains at least 2×10^9 viable *S. typhi* Ty21a colony forming units and at least 5×10^9 non-viable *S. typhi* Ty21a cells. The vaccine strain is grown in fermenters under controlled conditions in medium containing a digest of yeast extract, an acid digest of casein, glucose and galactose.

[0038] One of the biochemical properties of the *Salmonella typhi* Ty21a bacterial strain, as used according to this invention, is its inability to metabolize galactose. The recombinant attenuated bacterial strain is also not able to reduce sulfate to sulfide which differentiates it from the wild-type *Salmonella typhi* Ty2 strain. In regards to the serological characteristics of *Salmonella typhi* Ty21a strain, it contains the O9-

antigen which is a polysaccharide of the outer membrane of the bacteria and lacks the O5-antigen which is in turn a characteristic component of *Salmonella typhi* Ty2. Again, this serological characteristic supports the rationale for including the appropriate test in the panel of identity tests for batch release.

[0039] In a particular embodiment, the attenuated mutant strain of *Salmonella typhi* grown by the method according to the invention is *Salmonella typhi* Ty21a carrying at least one copy of a plasmid DNA, pVAX10.VR2-1, encoding a eukaryotic expression cassette of the human Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2). This attenuated mutant strain is designated VXM01 and can be used as an oral cancer vaccine.

[0040] According to the invention, the attenuated *Salmonella typhi* Ty21a strain functions as the bacterial carrier of the plasmid DNA encoding the heterologous antigen Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2), in the oral delivery of the DNA vaccine designated VXM01.

[0041] Delivery of vaccines based on plasmid DNA technology results in a broad spectrum of both mucosal and systemic immune responses. Live replicating vectors produce their own immunomodulatory factors such as lipopolysaccharides (LPS) *in situ* which may constitute an advantage over other forms of administration such as microencapsulation. Moreover, the use of the natural route of entry proves to be of benefit since many bacteria, like *Salmonella*, egress from the gut lumen via the M cells of Peyer's patches and migrate eventually into the lymph nodes and spleen, thus allowing targeting of vaccines to inductive sites of the immune system. The vaccine strain of *Salmonella typhi*, Ty21a, has been demonstrated to-date to have an excellent safety profile. Upon exit from the gut lumen via the M cells, the bacteria are taken up by phagocytic cells, such as macrophages and dendritic cells. These cells

are activated by the pathogen and start to differentiate, and probably migrate, into the lymph nodes and spleen. Due to their attenuating mutations, bacteria of the *S. typhi* Ty21 strain are not able to persist in these phagocytic cells but die at this time point. There is no data available to-date indicating that *S. typhi* Ty21a is able to enter the bloodstream systemically. The live attenuated *Salmonella typhi* Ty21a vaccine strain thus allows specific targeting of the immune system while exhibiting an excellent safety profile.

[0042] Live attenuated bacterial carriers that carry DNA encoding target antigens, can be used as vehicles for the oral delivery of these antigens. Live replicating vectors produce, *in situ*, their own immunomodulatory factors, such as lipopolysaccharides (LPS), which also constitutes an advantage over other forms of vaccine administration, like microencapsulation.

[0043] Genetic immunization might be advantageous over conventional vaccination. The target DNA can be detected for a considerable period of time thus acting as a depot of the antigen. Sequence motifs in some plasmids, like GpC islands, are immunostimulatory and can function as adjuvants furthered by the immunostimulation due to LPS and other bacterial components.

[0044] As indicated above, the recombinant *Salmonella* bacteria are taken up by phagocytic cells upon exit from the gut lumen via the M cells. These phagocytic cells are activated by the pathogen and start to differentiate, and probably migrate, into the lymph nodes and spleen. During this period, the bacteria die due to their attenuated mutation and release the plasmid-based eukaryotic expression vectors followed by a transfer of the plasmids into the cytosol, either via a specific transport system or by endosomal leakage. Finally, the vector enters the nucleus, where it is transcribed, leading to antigen expression in the cytosol of the host cell. Specific cytotoxic T cells

against the heterologous antigen, preferably human VEGFR-2, are induced by the activated antigen presenting cells (APCs).

[0045] In a particular embodiment, the recombinant DNA molecule carried by the *Salmonella typhi* Ty21a strain is a plasmid DNA, pVAX10.VR2-1 (7.58 kb), containing a eukaryotic Human Cytomegalovirus (CMV) immediate-early promoter, to ensure efficient transcription of the VEGFR-2 protein in the host cell, and a prokaryotic origin of replication (ori), to ensure multiplication within the bacterial host. The vector pcDNA3 is commercially available (Invitrogen) and was modified to comply with regulatory requirements whereby the sequences not necessary for replication in *E. coli* or for expression of the recombinant proteins in mammalian cells were removed to limit the DNA sequences with possible homology to the human genome and to minimize the possibility of chromosomal integration. Furthermore, the kanamycin resistance gene substituted the ampicillin resistant gene. For the attenuated mutant *Salmonella typhi* strain VXM01 produced according to the method of this invention, the high copy pUC origin of the pVAX1-Flk-1 plasmid was replaced by the low copy origin of replication of pBR322 in the pVAX10.VR2-1. The low copy modification was made in order to reduce the metabolic burden and to make the construct more stable. Details of the plasmid pVAX10.VR2-1 construct are depicted in Figure 2.

2) Vascular Endothelial Growth Factor Receptor:

[0046] Vascular Endothelial Growth Factor VEGF (Kd 75-760 pM) is a member of a family of six structurally related proteins (VEGF-A [also known as VEGF], -B, -C, -D, -E and PLGF [placental growth factor, also known as PGF or PIGF-2]) that regulates the growth and differentiation of multiple components of the vascular system, especially blood and lymph vessels. The role of VEGF in angiogenesis appears to be mediated through the interaction of this protein with VEGFR-2. VEGFR-2, also known

as kinase-insert-domain-containing receptor (KDR), is a 1356 amino acid long, 200-230 kDa molecular weight high-affinity receptor for VEGF, as well as for VEGF-C and VEGF-D. Identified in humans through the screening of endothelial cDNA for tyrosine kinase receptors, VEGFR-2 shares 85% sequence identity with the previously discovered murine VEGFR-2, also known as fetal liver kinase 1 (Flk-1). VEGFR-2 is normally expressed in endothelial and hematopoietic precursors, as well as in endothelial cells, nascent hematopoietic stem cells and the umbilical cord stroma. However, in quiescent adult vasculature, VEGFR-2 mRNA appears to be down regulated.

[0047] The extracellular domain of VEGFR-2 contains 18 potential N-linked glycosylation sites. VEGFR-2 is initially synthesized as a 150 kDa protein and rapidly glycosylated to a 200 kDa intermediate form, and then further glycosylated at a slower rate to a mature 230 kDa protein which is expressed on the cell surface.

[0048] The amino acid sequence of the human VEGFR-2 encoding cDNA sequence cloned into the pVAX10.VR2-1 plasmid is presented in Figure 1.

3) Manufacturing of empty and engineered *Salmonella typhi* Ty21a

[0049] The manufacturing process of the attenuated mutant strain of *Salmonella typhi* as carried out according to the invention comprises culturing the attenuated mutant strain of *Salmonella typhi* in medium which comprises peptone as a source for amino acids and peptides. Media suitable for the method of the present invention include, but are not limited to, standard TSB medium as well as TSB medium of non-animal origin. Both standard TSB as well as TSB of non-animal origin comprise 2.5 g/l glucose. Usually, the starting glucose amount in TSB, or TSB-like medium is more or less completely consumed after 3 – 5 h of cultivation of an attenuated *Salmonella*

typhi strain, and must be substituted by fresh glucose every 3 – 5 h in order to sustain a more or less constant glucose level in the culture medium. The observation that omitting glucose-addition during cultivation of attenuated mutant strains of *Salmonella typhi* optionally harboring a recombinant DNA molecule encoding a heterologous antigen leads to an increased cell growth compared to cultivation with glucose-addition is very surprising and suggests that specific metabolic pathways in the bacterial cells are triggered by the absence of glucose. The effect described can also be observed, if the TSB or TSB-like medium does not contain any glucose at the beginning of the cultivation process. Therefore, the method according to the invention has, apart from higher cell yields and thus higher yields of the final DNA vaccine, the further advantage of being cheaper and simpler by rendering the glucose feeding steps during fermentation unnecessary.

[0050] The manufacturing process of the attenuated mutant strain of *Salmonella typhi* Ty21a as carried out according to the invention is exemplarily described in the following Table 1:

Cells I (Salmonella Typhi Ty21a, WT) or Cells II (Salmonella Typhi Ty21a- pVAX10.VR2-1)	
↓ 2 x 500 ml TSB + kanamycin (500μL) (OD ₆₀₀ > 0.3) ↓ 10 x 1000 ml TSB + kanamycin (75mL) (OD ₆₀₀ > 0.5)	
100 L Fermentation volume TSB + 0.001 % galactose - 30°C - Airflow 100L/min (1 vvm) - pressure not controlled - pH 7.0 controlled with NaOH - foam controlled (Corning) - pO ₂ ≥ 40% regulated by stirrer - stirrer minimum 200 rpm - no glucose feeding - Final OD _{600nm} (end of exponential growth phase) - Cooling to at least 25°C before harvest	
Cross flow filtration - 10 fold concentration - 10 fold buffer exchange on diafiltration with 15% sucrose, 0.45% ascorbate solution pH 7.2, followed by further concentration to 1/20 vol. of original harvest Store at 2 – 8°C until filling about 24 hours	

[0051] In more detail: The cultures (TSB medium plus 25 μg/ml kanamycin) are inoculated each with different samples of the salmonella strains (empty Ty21a and recombinant Ty21a (pVAX10.VR2-1). In the production cell samples TSB medium is used containing 2.5 – 3.0 g/l glucose, preferably 2.5 g/l. In one control medium glucose is omitted. Furthermore, the medium contains kanamycin, preferably 25 μg/ml. The cultures are incubated at 30°C ± 2°C with shaking until an Optical Density OD_{600nm} >0.1 is reached. Further details are described in the Example section.

[0052] In-process controls for the first and second pre-culture steps, at the completion of the incubation times, includes analysis of bacterial growth by measuring OD_{600nm}.

pH and CFU/ml as well as, if applicable, determination of plasmid stability (PST) and bacterial examination. The latter analysis is based on a blood agar assay for determining hemolytic reactions of fastidious pathogenic microorganisms. The CFU value is assessed before and after the cross flow filtration (CFF). Upon formulation of the final bacterial concentrate, CFU and refractive index were measured on the formulation with the lowest bacterial concentration.

[0053] The method according to the invention, wherein glucose feeding is omitted, is less labor intensive and more efficient, resulting in higher cell yields.

4) Influence of glucose feeding during culturing cells

[0054] Growth of cells of empty and engineered *Salmonella typhi* Ty21a was tested in a TSB or TSB-like medium by culturing the cells between 0 and 30 hours at 25 – 35°C, preferably 30°C and a pH between 6.5 and 7.5, preferably 7.0. Growth was measured in OD or in CFU / ml (colony forming units).

[0055] The results of these experiments show, that glucose addition does not result in higher OD-values / cell mass yields of the wild type strain despite of glucose consumption. In contrast, flasks without glucose addition reached higher OD values (6 for preculture 1, 8 for preculture 2). Approx. 1 h after glucose addition OD remained static (or even slightly declined) compared to growth without glucose addition. On repeated pulses glucose consumption declined, no additional/additive effect on growth was observed. pH values were also monitored during fermentation, and in some experiments adjusted to the starting pH value, if shifts could be observed.

[0056] Without glucose addition a shift of pH to alkaline was observed after depletion of glucose, while with glucose pulse pH-value dropped (compare Figure 8 for preculture 1 and Figure 9 for preculture 2). Phenomena were observed in all precultures used, indicating no influence of generation number. Glucose pulsing was done 1 – 5 times (preferably 1 – 3 times) during an average culturing time of maximum 30 h. Usually, after approximately 5 - 15 hours cell growth entered the stationary phase after the exponential phase, depending on the starting conditions of the cell culture. If a preculture grown with glucose pulse was inoculated into fresh medium the same growth characteristics (higher OD values / pH shift to alkaline without glucose addition) were observed.

[0057] By omitting glucose feeding in the growth phase or even in the starting medium from the beginning, a shift of the pH values to alkaline (from ca. 7 to ca. 8) can be observed, although the medium system is buffered. It might be favorable to adjust the pH value during cell growth to the original starting pH of ca. 7.0.

[0058] Results indicated that glucose concentrations above a comparably low limit (approx. 2.5 g/l) trigger a reversible change in (glucose) metabolism. Without wishing to be bound by any theory, it is presumed that a substance, not yet identified, is then secreted into the medium which inhibits further growth, even if glucose declines again below trigger level. After inoculation in new medium this substance is diluted to a concentration beneath effectiveness. Very similar results can be obtained, if the starting medium does not contain any glucose.

[0059] Interestingly the same results can be obtained not only with the empty *Salmonella typhi* Ty21a but also with the engineered strain *Salmonella typhi* Ty21a - pVAX10.VR2-1 (VXM01), indicating that the surprising effect is not influenced by the artificially engineered bacterial construct. However, as it can be seen from Figure 12,

the cell growth of the engineered bacterium without glucose feeding, is -- as expected- slower than of the wild-type strain, but finally can gain the same high optical density values (OD 7 - 8) as compared with the wild-type strain, whereas the engineered Salmonella strain cultured in the presence of glucose by glucose pulsing does never gain these high optical density values and stops, as a rule at a cell density of OD 3 or less.

[0060] Both the empty as well as the engineered *S. typhi* Ty21a strain show comparable growth characteristics regarding growth with and without glucose addition.

[0061] To sum up, the results of the invention as described above and in the following show with both strains, that glucose addition does not result in higher OD-values / cell mass yields. In contrast, flasks without glucose addition reached higher OD values. Without glucose addition a shift of pH to alkaline is observed after depletion of glucose while with glucose pulse pH-value drops. One effect of glucose concentration dependent change in metabolism during cultivating of *Salmonella typhi* Ty21a consists presumably in excretion of an inhibitory substance into growth medium, which is not known so far.

[0062] In one aspect, the present invention relates to a method for growing an attenuated mutant strain of *Salmonella typhi* lacking galactose epimerase activity and comprising at least one copy of a recombinant DNA molecule comprising an expression cassette, comprising the step of culturing the strain in a buffered medium comprising peptone at approximately neutral starting pH value at fermentation scale, wherein the amount of glucose in the medium during the fermentations is adjusted such that the amount of glucose is reduced to zero before reaching the stationary phase.

[0063] In the context of the present invention, the term "comprises" or "comprising" means "including, but not limited to". The term is intended to be open-ended, to specify the presence of any stated features, elements, integers, steps or components, but not to preclude the presence or addition of one or more other features, elements, integers, steps, components or groups thereof. The term "comprising" thus includes the more restrictive terms "consisting of" and "essentially consisting of".

[0064] In the context of the present invention, the term "about" or "approximately" means within 20%, alternatively within 10%, including within 5% of a given value or range. Alternatively, especially in biological systems, the term "about" means within about a log (i.e. an order of magnitude), including within a factor of two of a given value.

[0065] In the context of the present invention, the terms "growing" and "culturing" are used synonymously and refer to propagation of microorganisms in media conducive to their growth.

[0066] In the context of the present invention, the term "fermentation" refers to large-scale culturing of microorganisms, typically performed in a fermenter, i.e. an apparatus that maintains optimal conditions for the growth of said microorganisms, for the high-yield production of a desired microbial product, including metabolites and the microorganisms themselves.

[0067] In the context of the present invention, the term "attenuated" refers to a bacterial strain of reduced virulence compared to the parental bacterial strain, not harboring the attenuating mutation. Attenuated bacterial strains have preferably lost

their virulence but retained their ability to induce protective immunity. Attenuated bacteria may be found naturally or they may be produced in the laboratory, for example by adaptation to a new medium or cell culture or they may be produced by recombinant DNA technology.

[0068] In the context of the present invention, the term "mutant strain" refers to a bacterial strain harboring a mutation in its genome. In this context, the term "mutation" refers to a change in a nucleic acid sequence, including point mutations, insertions, deletions, translocations and inversions.

[0069] In the context of the present invention, the term "recombinant DNA molecule" refers to an engineered DNA construct, preferably composed of DNA pieces of different origin. The recombinant DNA molecule can be a linear nucleic acid, or preferably, a circular recombinant DNA plasmid generated by introducing an open reading frame of interest into an expression vector plasmid. The open reading frame is preferably a heterologous antigen. The heterologous antigen is preferably a cancer antigen. The cancer antigen is preferably a VEGF receptor protein. In the context of the present invention, the term "heterologous antigen" refers to an antigen derived from a species other than *Salmonella typhi*.

[0070] In the context of the present invention, the term "expression cassette" refers to a nucleic acid unit comprising at least one gene under the control of regulatory sequences controlling its expression. Expression cassettes comprised in the attenuated mutant strain of *Salmonella typhi* can preferably mediate transcription of the included open reading frame in target cells. Expression cassettes typically comprise a promoter, at least one open reading frame and a transcription termination signal.

[0071] In the context of the present invention, the term "peptone" refers to a mixture of cleavage products comprising amino acids and peptides produced by hydrolysis of protein-containing materials, for example by partial acid or enzymatic hydrolysis of native protein mixtures.

[0072] In the context of the present invention, the term "approximately neutral pH value" refers to a pH value of from about 5 to about 9, preferably from about 6 to about 8, more preferably from about 6.5 to about 7.5, most preferably about 7.0.

[0073] Media suitable for the method of the present invention include, but are not limited to, standard TSB medium as well as TSB medium of non-animal origin. Standard TSB medium known in the art is comprised of pancreatic casein peptone, soybean meal peptone, di-potassium hydrogen phosphate (buffer), sodium chloride, and glucose (= dextrose) as energy source in a starting concentration of 2.5 g/l. An example of suitable TSB medium of non-animal origin is CASO Bouillon of non-animal origin comprised of non-animal derived peptone, di-potassium hydrogen phosphate (buffer), sodium chloride, and glucose in a starting concentration of 2.5 g/l.

[0074] In the context of the present invention, the term "stationary phase" refers to the stage of bacterial growth after the exponential or logarithmic phase, wherein the cell density in the growth medium remains approximately constant. The term "before reaching the stationary phase" thus refers to any time point before the stationary phase and includes the lag phase (i.e. the first phase of bacterial growth during which the bacteria adapt to the growth conditions) and the exponential phase. It was surprisingly found that cultivation of attenuated mutant strains of *Salmonella typhi* according to the method of the present invention prolongs the exponential growth phase. When growing the attenuated mutant strain *Salmonella typhi* Ty21a with a starting glucose amount that is depleted during the exponential growth phase without addition of glucose during the fermentation process, the stationary phase is reached

not earlier than after 9 hours of culturing, preferably after 9 to 20 hours of culturing, more preferably after 9 to 15 hours after culturing, most preferably after 9 to 12 hours after culturing.

[0075] In a particular embodiment, no glucose is added to the medium during the fermentation and the starting amount of glucose is depleted before reaching the stationary phase. It is however also within the subject method of growing an attenuated mutant strain of *Salmonella typhi*, to add glucose, as long as the amount of glucose is reduced to zero before reaching the stationary phase.

[0076] In the context of the present invention, the term "glucose is depleted" means that the starting amount of glucose in the medium is consumed (i.e. taken up and metabolized) by the bacteria.

[0077] In a particular embodiment, the attenuated mutant strain of *Salmonella typhi* is *Salmonella typhi* Ty21a.

[0078] In a particular embodiment, the expression cassette is a eukaryotic expression cassette. It has been shown that the amount of heterologous antigen required to induce an adequate immune response may be toxic for the bacterium and result in cell death, over-attenuation or loss of expression of the heterologous antigen. Using a eukaryotic expression cassette that is not expressed in the bacterial vector but only in the target cell overcomes this toxicity problem.

[0079] In the context of the present invention, the term "eukaryotic expression cassette" refers to an expression cassette which allows for expression of the open reading frame in a eukaryotic cell. A eukaryotic expression cassette comprises regulatory sequences that are able to control the expression of an open reading frame in a eukaryotic cell, preferably a promoter and a polyadenylation signal.

Promoters and polyadenylation signals included in the recombinant DNA molecules comprised by the *Salmonella typhi* strain for use as a vaccine of the present invention are preferably selected to be functional within the cells of the subject to be immunized. Examples of promoters useful in the attenuated mutant *Salmonella typhi* strain of the present invention, especially in the production of a DNA vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rouse Sarcoma Virus (RSV) as well as promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine, and human metallothionein. In a particular embodiment, the eukaryotic expression cassette contains the CMV promoter. In the context of the present invention, the term "CMV promoter" refers to the immediate-early cytomegalovirus promoter.

[0080] Examples of suitable polyadenylation signals, especially for the production of a DNA vaccine for humans, include but are not limited to the BGH polyadenylation site, SV40 polyadenylation signals and LTR polyadenylation signals. In a particular embodiment, the eukaryotic expression cassette included in the recombinant DNA molecule comprised by the attenuated mutant strain of *Salmonella typhi* of the present invention comprises the BGH polyadenylation site.

[0081] In addition to the regulatory elements required for expression of a heterologous gene, such as a heterologous antigen, like promoters and polyadenylation signals, other elements can also be included in the recombinant DNA molecule. Such additional elements include enhancers. The enhancer can be, for example, the enhancer of human actin, human myosin, human hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

[0082] Regulatory sequences and codons are generally species dependent, so in order to maximize protein production, the regulatory sequences and codons are preferably selected to be effective in the species to be immunized. The person skilled in the art can produce recombinant DNA molecules that are functional in a given subject species.

[0083] In a particular embodiment, the expression cassette encodes a VEGF receptor protein.

[0084] VEGF receptor proteins are endothelial cell-specific receptor-tyrosine kinases that can be bound by the ligand vascular endothelial growth factor (VEGF) which causes them to dimerize and become activated through transphosphorylation. There are three main subtypes of VEGFR, VEGFR-1 (or FLT1), VEGFR-2 (or KDR, FLK1) and VEGFR-3 (or FLT4). VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF. Membrane-bound VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain. VEGFR transcripts give also rise to alternative splice variants that encode soluble VEGF receptor proteins. The VEGF family of growth factors encompasses 6 family members, VEGF-A through E and PGF. In a preferred embodiment, the eukaryotic expression cassette encodes a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith.

[0085] In the context of the present invention, the term "homolog" of human VEGFR-2 refers to a VEGF receptor protein that differs in the amino acid sequence and/or the nucleic acid sequence encoding the amino acid sequence of human VEGFR-2. The

homolog of human VEGFR-2 may be of natural origin, e.g. a homolog of VEGFR-2 of a different species, or an engineered VEGFR-2 homolog. It is known that the usage of codons is different between species. Thus, when expressing a heterologous protein in a target cell, it may be necessary, or at least helpful, to adapt the nucleic acid sequence to the codon usage of the target cell. Methods for designing and constructing homologs of VEGF receptor proteins are well known to anyone of ordinary skill in the art.

[0086] A VEGFR-2 homolog may contain one or more mutations comprising an addition, a deletion and/or a substitution of one or more amino acids. According to the teaching of the present invention, said deleted, added and/or substituted amino acids may be consecutive amino acids or may be interspersed over the length of the amino acid sequence of the functional VEGFR-2 homolog. According to the teaching of the present invention, any number of amino acids may be added, deleted, and/or substitutes, as long as the homolog and human VEGFR-2 share at least about 80% homology. In particular embodiments, the homolog of human VEGFR-2 has a sequence homology of at least about 80%, at least about 85%, at least about 90%, or most particularly of at least about 95% and a sequence identity of at least about 60%, at least about 65%, at least about 70% and most particularly of at least about 75%. Methods and algorithms for determining sequence identity and/or homology, including the comparison of homologs having deletions, additions and/or substitutions relative to a parental sequence, are well known to the practitioner of ordinary skill in the art. On the DNA level, the nucleic acid sequences encoding the homolog of human VEGFR-2 may differ to a larger extent due to the degeneracy of the genetic code.

[0087] In yet another preferred embodiment, the eukaryotic expression cassette encodes human VEGFR-2 of the amino acid sequence as found in SEQ ID NO 1.

[0088] In certain embodiments, the buffered medium comprises peptone of non-animal origin. In a preferred embodiment, the buffered medium is Tryptic Soy Broth (TSB) of non-animal origin.

[0089] In the context of the present invention, "peptone of non-animal origin" refers to a mixture of cleavage products produced by partial acid or enzymatic hydrolysis of native protein which is not of animal origin. Preferably, the native protein is of plant origin. Peptone of non-animal origin solely comprises components that are not directly derived from eukaryotic animals.

[0090] In certain embodiments, the volume of the medium is at least about 10 l.

[0091] In certain embodiments, the volume of the medium is from at least about 10 l, or 30 l, or 50 l, or 100 l up to a maximum of about 10.000 l, or 1.000 l, or 800 l or 500 l.

[0092] In particular embodiments, the volume of the medium is from about 100 l to about 500 l, more particularly about 100 l, about 150 l, about 200 l, about 250 l, about 300 l, about 400 l or about 500 l.

[0093] In certain embodiments, the starting glucose concentration corresponds to that of bacterial minimal medium or less.

[0094] In certain embodiments, the starting glucose concentration is from about 0 g/l to about 4 g/l.

[0095] In particular embodiments, the starting glucose concentration is about 0 g/l, about 0.5 g/l, about 1 g/l, about 1.5 g/l, about 2 g/l, about 2.5 g/l, about 3 g/l, about 3.5 g/l or about 4 g/l.

[0096] In certain embodiments, the starting pH value is from at least about 5, about 6, or about 6.5 up to a maximum of less than about 9, about 8, or about 7.5.

[0097] In particular embodiments, the starting pH value is from about 6.5 to about 7.5, more particularly about 7.0.

[0098] In a particular embodiment, the pH value is adjusted during culturing to a pH value from about 6 to about 8, particularly to from about 6.5 to about 7.5.

[0099] In a particular embodiment, the pH value is adjusted to from at least about 6, or 6.5 up to a maximum of about 8, or about 7.5 during fermentation.

[00100] In a particular embodiment, the pH value is adjusted to from about 6.5 to about 7.5 during fermentation, more particularly to about 7.0.

[00101] In a particular embodiment, the progress of growth is determined by measuring the optical density (OD).

[00102] In the context of the present invention, the term "optical density" or "turbidity" of a material refers to the logarithmic ratio of the radiation falling upon said material to the radiation transmitted through said material for a given wavelength. The optical density is preferably measured using a spectrophotometer. Preferably, the optical density can be used as a measure of the concentration of cells, preferably bacteria, in a suspension. As visible light passes through a cell suspension, the light

is scattered. Greater scatter indicates higher cell numbers. Typically, the optical density at a wavelength of 600 nm (OD_{600}) is measured. The optical density at a particular wavelength of a bacterial culture plotted against the culturing time gives a growth curve which can be used to delineate the various phases of bacterial growth and to determine the doubling time of bacterial culture. The growth curve is characteristic for a given type of bacteria cultured under given conditions in a given culture medium. Measuring the optical density of a cell suspension can thus be used to monitor the stage of bacterial growth. Optical density measurement can be used to determine the end point of the culturing procedure, i.e. the stage of bacterial growth, in which the cells are to be harvested, typically the mid-log phase of growth.

[00103] In a preferred embodiment, the progress of growth is determined by in-situ monitoring of the optical density of the culture or by taking samples and measuring the optical density of the samples. In-situ monitoring of the optical density of a bacterial culture using on-line or in-situ devices allows for constant, continuous, non-invasive monitoring of cell growth, minimizes the risk of contamination and eliminates the need of cumbersome, labor intensive extraction of samples.

[00104] It was surprisingly found that growing attenuated mutant strains of *Salmonella typhi* according to the method of the present invention yields in optical density values of about 6.0 to about 8.0 at the onset of the stationary phase. In comparison, cultivation of the same strain in a similar fermentation process but wherein glucose is added during fermentation to sustain an approximately stable glucose level of about 1 g/l to about 4 g/l yields in optical density values of about 3 to about 5.5. Thus, it was surprisingly found that growing an attenuated mutant strain of *Salmonella typhi* without addition of glucose to the medium during the fermentation and with a starting glucose amount in the medium that is depleted before reaching

the stationary phase yields in an increased optical density at the onset of the stationary phase compared to a fermentation process with glucose addition.

[00105] In another embodiment, the progress of growth is determined by measuring the cell density.

[00106] In a preferred embodiment, cell density is determined microscopically or by measuring the electrical resistance or by flow cytometry. The cell density can be determined microscopically by manually counting the cells using a counting chamber or hemocytometer. Measuring cell density based on the electrical resistance is preferably performed using a Coulter counter. Measuring the cell density by flow cytometry is achieved by letting the cells pass a laser beam in a narrow stream, which hits them one by one. A light detector picks up the light that is reflected from the cells.

[00107] It was surprisingly found that growing attenuated mutant strains of *Salmonella typhi* according to the method of the present invention yields in cell density values of about 5×10^{14} to about 8×10^{14} at the onset of the stationary phase. In comparison, cultivation of the same strain in a similar fermentation process but wherein glucose is added during fermentation to sustain an approximately stable glucose level of about 1 g/l to about 4 g/l yields in cell density values of about 5×10^{13} to 1×10^{14} . Thus, it was surprisingly found that growing an attenuated mutant strain of *Salmonella typhi* without addition of glucose to the medium during the fermentation and with a starting glucose amount in the medium that is depleted before reaching the stationary phase yields in an increased cell density at the onset of the stationary phase compared to a fermentation process with glucose addition.

[00108] The optical density of a cell suspension below about 0.4 is directly proportional to its cell density. Thus, a calibration curve can be created by plotting the optical density against the cell density. Such a calibration curve can be used to estimate the cell density of a cell suspension by measuring its optical density.

[00109] In another embodiment, the progress of growth is determined by measuring the colony forming units (CFU) value by taking samples and plating on agar plate. By this method, only viable cells are counted as only viable cells are able to form colonies on an agar plate.

[00110] It was surprisingly found that growing attenuated mutant strains of *Salmonella typhi* according to the method of the present invention yields CFU values of about 6×10^9 to about 8×10^9 at the onset of the stationary phase. In comparison, cultivation of the same strain in a similar fermentation process but wherein glucose is added during fermentation to sustain an approximately stable glucose level of about 1 g/l to about 4 g/l yields in CFU values of about 2×10^9 to 5×10^9 . Thus, it was surprisingly found that growing an attenuated mutant strain of *Salmonella typhi* without addition of glucose to the medium during the fermentation and with a starting glucose amount in the medium that is depleted before reaching the stationary phase not only yields in an increased cell density at the onset of the stationary phase but also in an increased number of viable cells compared to a fermentation process with glucose addition.

[00111] In a particular embodiment, the cells are harvested before reaching an optical density of about 6.

[00112] In a preferred embodiment, the cells are harvested at an optical density from about 5 to about 6.

[00113] In a particular embodiment, the attenuated mutant strain of *Salmonella typhi* is *Salmonella typhi* Ty21a and the recombinant DNA molecule comprises the kanamycin resistance gene, the pMB1 ori, and a eukaryotic expression cassette encoding human VEGFR-2, under the control of the CMV promoter.

[00114] In a preferred embodiment, human VEGFR-2 has the nucleic acid sequence as found in SEQ ID NO 2.

[00115] In yet another aspect, the present invention relates to an attenuated mutant strain of *Salmonella typhi* lacking galactose epimerase activity and comprising at least one copy of a recombinant DNA molecule comprising an expression cassette, which is obtainable by a method for growing the strain, comprising the step of culturing the strain in a buffered medium comprising peptone at approximately neutral starting pH value at fermentation scale, wherein the amount of glucose in the medium during the fermentations is adjusted such that the amount of glucose is reduced to zero before reaching the stationary phase.

[00116] In a particular embodiment, no glucose is added to the medium during the fermentation and the starting amount of glucose is depleted before reaching the stationary phase.

[00117] In a particular embodiment, the expression cassette is a eukaryotic expression cassette.

[00118] In a particular embodiment, the expression cassette encodes a VEGF receptor protein.

[00119] In a preferred embodiment, the eukaryotic expression cassette encodes a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith.

[00120] In yet another preferred embodiment, the eukaryotic expression cassette encodes human VEGFR-2 of the amino acid sequence as found in SEQ ID NO 1.

[00121] In a particular embodiment, the attenuated mutant strain is *Salmonella typhi* Ty21a and the recombinant DNA molecule comprises the kanamycin resistance gene, the pMB1 ori, and a eukaryotic expression cassette encoding human VEGFR-2 under the control of the CMV promoter.

[00122] In a preferred embodiment, human VEGFR-2 has the nucleic acid sequence as found in SEQ ID NO 2.

[00123] In another aspect, the present invention relates to an attenuated mutant strain of *Salmonella typhi* Ty21a comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette encoding a VEGF receptor protein for use as a vaccine.

[00124] In the context of the present invention, the term "vaccine" refers to an agent which is able to induce an immune response in a subject upon administration. A vaccine can preferably prevent, ameliorate or treat a disease. Preferably, such a vaccine comprises an attenuated mutant strain of *Salmonella typhi*, preferably *S. typhi* Ty21a. Preferably, the vaccine further comprises at least one copy of a recombinant DNA molecule comprising an expression cassette, preferably encoding a heterologous antigen. Such a vaccine comprising a vector, for instance an

attenuated bacterial strain, as a delivery vehicle for a DNA encoding a heterologous antigen is termed DNA vaccine.

[00125] In a particular embodiment, the eukaryotic expression cassette encodes a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith.

[00126] In a preferred embodiment, the eukaryotic expression cassette encodes human VEGFR-2 of the amino acid sequence as found in SEQ ID NO 1.

[00127] In another aspect, the present invention relates to a method for increasing cell growth of an attenuated mutant vaccine strain of Salmonella, lacking galactose epimerase activity, by culturing the strain in a buffered medium essentially comprising enzymatic digests of casein and soybean meal and a starting glucose amount of not more than 2.5 - 3.0 g / L at a starting pH value of 6.5 – 7.5, wherein culturing is carried out by omitting any additional glucose feeding into the fermentation broth until stationary phase of cell growth, thus not sustaining the original glucose level in the broth, said increase of cell growth, which is solely achieved by fully depletion of glucose during fermentation, is measured by optical density (OD) and has a value of 7.5 – 8.0 in the finally obtainable stationary phase (preferably obtained after 18 – 20h), compared to OD 5.0 to 5.5 in a respective fermentation process under same conditions, wherein however glucose is fed during fermentation to sustain a permanent glucose level in the fermentation broth of 2.0 – 3.0 g / L.

[00128] In a particular embodiment, the maximum cell growth is obtained after 20 h of culturing.

[00129] In a particular embodiment, a cell density of 5×10^{14} – 8×10^{14} is achieved by omitting any glucose feeding during fermentation, compared to 5×10^{13} to 1×10^{14} when adding glucose during cultivation.

[00130] In a particular embodiment, a colony forming unit (CFU) value of 6×10^9 to 8×10^9 per ml is achieved after fermentation by omitting any glucose feeding during fermentation, compared to 2×10^9 to 5×10^9 after glucose feeding during fermentation.

[00131] In a particular embodiment, the pH value is adjusted during fermentation to 7.0. By omitting glucose feeding in the growth phase or even in the starting medium from the beginning, a shift of the pH values to alkaline (from ca. 7 to ca. 8) can be observed, although the medium system is buffered. It might be favorable to adjust the pH value during cell growth to the original starting pH of ca. 7.0.

[00132] In a particular embodiment of the invention, the medium is Tryptic Soy Broth (TSB) or a medium which provides the same or similar nutrients.

[00133] In a further preferred embodiment according to the invention the attenuated mutant vaccine strain of Salmonella is Salmonella Typhi Ty21a.

[00134] In another aspect, the invention relates to a method of producing of an attenuated mutant cancer vaccine strain of Salmonella typhi Ty21a, wherein the strain carries multiple copies of a plasmid DNA, encoding a eukaryotic expression cassette of human vascular endothelial growth factor receptor 2 (VEGFR-2 or FLK-1), the method comprising a method for increasing cell growth of an attenuated mutant vaccine strain of Salmonella of the present invention.

[00135] In a particular embodiment said plasmid DNA is a 7580 bp plasmid DNA that comprises the cDNA of VEGFR-2 that is under the control of the CMV promoter, the kanamycin resistance gene, and the pMB1 ori, and that is designated as pVAX10.VR2-1.

SHORT DESCRIPTION OF FIGURES AND TABLES

Figure 1: Amino acid sequence of VEGFR-2 encoded by cDNA cloned into plasmid pVAX10.VR2-1

Figure 2: Plasmid map of pVAX10.VR2-1

Figure 3: Description of manufacturing process of Salmonella typhi Ty2a (pVAX10.VR2-1)

Figure 4: Flow chart of isolation of S. typhi Ty21a

Figure 5: Growth of preculture 1 and 2 of Salmonella typhi Ty21a with/without kanamycin

Figure 6: Growth of cultures (preculture 1) of Salmonella typhi Ty21a (empty) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was determined by optical density measurement at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units and glucose (glc) concentration in g/l.

Figure 7: Growth of cultures (preculture 1) of Salmonella typhi Ty21a (empty) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was determined by optical density measurement at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units and pH value shift.

Figure 8: Growth of cultures (preculture 2) of *Salmonella typhi* Ty21a (empty) with glucose pulsing (adjusted to final glucose concentration of 2.5 g / L) and without pulsing of glucose. Cell growth was measured as optical density at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units and glucose (glc) concentration in g/l.

Figure 9: Growth of cultures (preculture 2) of *Salmonella typhi* Ty21a (empty) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was measured as optical density at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units and pH value shift.

Figure 10: Growth of cultures (preculture 3, glucose primed inoculums of preculture 1) of *Salmonella typhi* Ty21a (empty) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was determined by optical density measurement at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units and glucose (glc) concentration in g / L

Figure 11: Growth of cultures (preculture 3, glucose primed inoculums of preculture 1) of *Salmonella typhi* Ty21a (empty) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was determined by optical density measurement at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units and pH value shift.

Figure 12: Growth of wild-type strain (preculture 1) of *Salmonella typhi* Ty21a compared to engineered cancer vaccine production strain (*Salmonella typhi* Ty21a: pVAX10.VR2-1, VXM01) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was measured as optical density at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-

axis represents culturing time in hours (h); y-axis represents cell density measured in OD units.

Figure 13: Growth of wild-type strain (preculture 2) of *Salmonella typhi* Ty21a compared to engineered cancer vaccine production strain (*Salmonella typhi* Ty21a: pVAX10.VR2-1, VXM01) with glucose pulsing (adjusted to final glucose concentration of 2.5 g/l) and without pulsing of glucose. Cell growth was determined by optical density measurement at 600nm (OD600). The arrows indicate the addition of glucose (pulsing). X-axis represents culturing time in hours (h); y-axis represents cell density measured in OD units.

Table 1: Manufacturing process

Table 2: Manufacturing process

Table 3: Results of OD600 measurement during fermentation process

Table 4: Glucose concentration during fermentation process

Table 5: pH-value shift during fermentation process

EXAMPLES

Example 1: Isolation of *Salmonella typhi* Ty21a strain for the preparation of the Research Seed Lot (RSL)

[00136] The first step in the preparation of the RSL consisted of the isolation of the attenuated *Salmonella typhi* Ty21a strain from Typhoral L[®] capsules followed by the transformation of the attenuated bacteria with the plasmid DNA (pVAX10.VR2-1).

[00137] The commercially available Typhoral L[®] capsules, containing an attenuated *Salmonella enterica* serovar typhi Ty21a strain, were used to prepare the stock of *S. typhi* to be used in the recombinant studies indicated below. The process

consisted of inoculating a liquid culture medium with part of the content of the capsules and further plating the liquid culture onto an agar medium for the purpose of isolating single bacterial colonies. Single colonies were isolated and grown in liquid culture medium. Two cultures, namely VAX.Ty21-1 and VAX.Ty21-2, were then formulated with glycerol, aliquoted (1mL) and stored at $-75^{\circ}\text{C} \pm 5^{\circ}\text{C}$ pending use. Identity of each of the two cultures was further confirmed.

Example 2: Plasmid construction

[00138] The principle of plasmid synthesis is based on double strand *in vitro* gene synthesis with the following steps:

- The whole pVAX10-VR2.1 plasmid sequence of 7.58kB was subdivided (by software analysis) in 5 sections of ~1.5kB. Each section was subdivided into 40-50bp oligonucleotides each having overlapping regions between oligonucleotides of both strands
- The *in vitro* synthesized oligonucleotides were then phosphorylated by incubation with T4 polynucleotide kinase
- After the annealing process of overlapping oligonucleotides under appropriate conditions, the Taq DNA ligase enzyme connected the aligned oligonucleotides
- Upon completion of the ligation step, PCR was performed using primers annealed at outward positions, to increase the yield of the ligated plasmid fragments (~1.5kB)
- A preparative agarose gel electrophoresis was performed to isolate the PCR products
- The isolated PCR products were cloned into TOPO vectors (Invitrogen K#4575-40) and transformed into TOP10 E. coli cells for propagation
- After TOPO plasmid isolation, a restriction and sequence verification was

performed

- The isolated aligned fragments were assembled via overlap PCR. This process was followed by linearly assembling the pVAX10.VR2-1 plasmid
- After XhoI restriction digest (single restriction site is present in the pVAX10.VR2-1 plasmid, see Figure 2.1.S.1.2.2-1) and covalent binding via T4 ligase, *E. coli* was transformed with the circular plasmid for propagation
- After final plasmid sequence verification, the pVAX10.VR2-1 plasmid was transformed into the *S. typhi* Ty21a bacterial strain.

[00139] The plasmid pVAX10.VR2-1 was thus successfully synthesized (no deviation to reference sequence). This plasmid was further used to transform the *S. typhi* Ty21a bacterial strain isolates (Vax.Ty21-1 and Vax.Ty21-2).

Example 3: Manufacturing processes

[00140] The following Table 2 summarizes the processes of manufacture with /without glucose feeding during fermentation.

Production step	Process variant A: with glucose feeding	Process variant A: without glucose feeding
Preculture Clean room class D and A in D	CELLS (with/without plasmid pVAX10.VR2-1) ↓ 350 mL TSB + kanamycin (1mL) ↓ 5 x 550 mL TSB + kanamycin (50mL) (OD ₆₀₀ ≥ 0.5)	CELLS (with or without plasmid pVAX10.VR2-1) ↓ 2 x 500 ml TSB + kanamycin (500μL) (OD ₆₀₀ > 0.3) ↓ 10 x 1000 ml TSB + kanamycin (75mL) (OD ₆₀₀ ≥ 0.5)
Fermentation Clean room class D	30L Fermentation volume TSB + 0,001 % galactose 30°C - Airflow 2 l/min (0.07 vvm) - pressure 1 bar - pH 7.0 controlled with NaOH - pO ₂ ≥ 40% regulated by stirrer - glucose feeding Σ 5 – 8 g/l - Final OD _{600nm} ~ 2.7 (target 6 – 10) - Cooling to 15°C before harvest	100 L Fermentation volume TSB + 0,001 % galactose 30°C - Airflow 100L/min (1 vvm) - pressure not controlled - pH 7.0 controlled with NaOH - foam controlled (Corning) - pO ₂ ≥ 40% regulated by stirrer - stirrer minimum 200 rpm - no glucose feeding - Final OD _{600nm} (end of exponential growth phase) - Cooling to at least 25°C before harvest
Harvest / concentration / wash Clean room class D	Cross flow filtration - 10fold concentration - 10fold buffer exchange on diafiltration with 15 % sucrose solution, followed by further concentration to 1/15 vol. of original harvest - Formulation by adding ascorbate to final concentration of 0.45% Storage at 2–8°C until filling 24 hours	Cross flow filtration - 10fold concentration - 10fold buffer exchange on diafiltration with 15% sucrose, 0.45% ascorbate solution pH 7.2, followed by further concentration to 1/20 vol. of original harvest Storage* at 2 – 8°C until filling about 24 hours,
Dilution / filling of vials Clean room class A in B	Manual filling of 150 vials of 5 dilutions - Adjustment of CFU - 300mL suspension → filling of 150 vials - Dilution of 30mL suspension 270 mL formulation solution, filling of 150 2 R glass vials - Four dilutions and filling of 150 vials until 1:10.000 - Closing of vials with rubber stoppers and aluminium caps - Storage at -75°C ± 10°C	Manual filling of 150 vials of 5 dilutions - Adjustment of CFU - 300mL suspension → filling of 150 vials - Dilution of 30mL suspension 270 mL formulation solution, filling of 150 2 R glass vials - Four dilutions and filling of 150 vials until 1:10.000 - Closing of vials with rubber stoppers and aluminium caps - Storage at ≤ -70°C

[00141] The bacterial growth process was carried out in 2x350 ml TSB medium further containing 25 µg/ml kanamycin. Two 2-L-Erlenmeyer flasks with baffles were inoculated each with an aliquot (1ml) of *Salmonella typhi* Ty21a and Ty21a (pVAX10.VR2-1). The cultures were incubated at 30°C ± 2°C with shaking (140 rpm) until an Optical Density OD_{600nm} >0.1 was reached. The bacterial harvest (50 ml) of the first culture was used to inoculate a second culture (pre-culture 2), 6x550 ml TSB

medium with kanamycin (25 µg/ml) contained in 3-l-Fernbach flasks with baffles. The cultures were incubated at 30°C ± 2°C with shaking (180 rpm) until an OD at 600 nm of the bacterial culture reached a value of ≥0.3. Upon completion of the incubation time, the main culture (TSB medium with 0.001% galactose) prepared in a controlled stainless-steel fermenter with a working volume of 27 l, was inoculated with the pre-culture 2 (pooled 5 flasks of bacterial culture) and incubated at 30°C with the following settings: airflow at 2 l/min, pressure at 1 bar, pH 7.0, pO₂ ≥ 40%.

[00142] One half of the culture samples was fed with glucose in an amount that gave a final concentration of 2.0 g/l to 3.0 g/l in the starting culture medium. Glucose feeding was carried out after 3 – 5 h after onset of fermentation and was repeated several times each 3 – 5 h.

[00143] The other half of the culture samples was treated identically but without any feeding of glucose during cultivation of *Salmonella* cells. As a control, a culture medium was tested that did not contain any glucose from the very first beginning (TSB medium without glucose).

[00144] The bacterial culture was concentrated by cross-flow filtration (CFF) / diafiltration against a 15% sucrose solution. Five dilutions of the final bacterial concentrate were carried out. The final bacterial product was then aliquoted (1 ml) in 2R vials, the vials closed, labeled, capped and stored at -75°C ± 5°C.

[00145] In-process controls for the first and second pre-culture steps, at the completion of the incubation times, included analysis of bacterial growth by measuring OD_{600nm}, pH and CFU/mL as well as determination of plasmid stability (PST) and bacterial examination. The latter analysis was based on a blood agar assay for determining hemolytic reactions of fastidious pathogenic microorganisms.

The CFU value was assessed before and after the CFF. Upon formulation of the final bacterial concentrate, CFU and refractive index were measured on the formulation with the lowest bacterial concentration.

Example 4: Growth of Salmonella typhi TY21a (pVAX10.VR2-1) with and without kanamycin

[00146] The production of the live bacterial cancer vaccine is based on the fermentation of a Salmonella enterica serovar typhi strain Ty21a (comprising the plasmid pVAX10.VR2-1). After growth the cell suspension has to be concentrated by crossflow filtration and washed by diafiltration. Five different dilutions of washed cell suspension have to be filled in 2 R glass vials.

[00147] The aims of these flask growth tests were:

- verifying growth rate for preculture 1 and 2 for planning time table of production run
- investigating the influence of glucose feeding to achieve high cell concentration (OD600 values)

[00148] Experiments were performed starting from the master cell banks of Salmonella enterica typhi TY21a and Salmonella enterica typhi TY21a (pVAX10.VR2-1) (= strain VMX01). Inoculum for fermentation was prepared by a two-step preculture (VK): Preculture 1 was inoculated directly from MCB; preculture 2 was inoculated by a larger volume out of preculture 1. Growth tests were performed to evaluate time course for cultivating preculture in 2 l Erlenmeyer flasks (preculture 1) and 3 l Fernbach Corning flasks (preculture 2) growth tests. Preculture was done with

and without kanamycin to get information about growth / exponential growth phase at both conditions.

[00149] Growth of these five flasks is compared in Figure 5:

- VK1a: 500 ml TSB Medium in 3 l Corning flask + 0.5 ml MCB, 30 °C, 120 rpm
- VK1b: 500 ml TSB Medium in 3 l Corning flask + 0.5 ml MCB, 30 °C, 120 rpm
- VK2: 1000 ml TSB Medium in 3 l Corning flask + 75 ml VK1b (OD 1,6), 30 °C, 120 rpm
- VK1a k: 500 ml TSB Medium + 25 mg/l kanamycin sulfate in 2 l Corning flask + 0.5 ml MCB, 30 °C, 120 rpm
- VK1b k: 500 ml TSB Medium + 25 mg/l kanamycin sulfate in 3 l Corning flask + 0.5 ml MCB, 30 °C, 120 rpm
- VK2a k: 1000 ml TSB Medium + 25 mg/l kanamycin sulfate in 3 l Corning flask + 75 ml VK1a k (OD 1,8), 30 °C, 120 rpm
- VK2b k: 1000 ml TSB Medium + 25 mg/l kanamycin sulfate in 3 l Corning flask + 75 ml VK1a k (OD 1,8), 30 °C, 120 rpm

[00150] The results in Figure 5 show, that there are no significant differences between growth with and without kanamycin as well as in growth in 2 l or 3 l Corning flask respectively. Cultivation time for preculture 1 at 30 °C should be between about 15 to 23 hours ($OD_{600} \sim 1 - 4$) to inoculate preculture 2 with cells in exponential phase. Minimum OD_{600} for preculture 2 (> 0.5) was achieved after 2 – 3 hours; exponential growth phase is characterized by OD_{600} values between 0.5 and 3.0.

Example 5: Growth of Salmonella typhi TY21a (empty) with and without glucose feeding

[00151] Growth tests with three precultures (1, 2, 3) were performed in 2 l Erlenmeyer flasks and two step culture as done with the production strain.

[00152] Preculture 1 is inoculated directly from RCB; preculture 2 is inoculated by a larger volume out of preculture 1. Due to absence of plasmid encoded kanamycin resistance, selective antibiotic was omitted in culture media. To evaluate the influence of preculture step (generation number), repeated glucose additions were done in preculture 1 as well as in preculture 2, both in comparison to "unpulsed" cultures. In addition, glucose pulsed preculture 1 was used as inoculum for preculture 2 to follow up the reversibility of glucose concentration mediated metabolic impact. Preculture 3 represents preculture 1 grown with glucose feeding (pulsing) inoculated into fresh TSB medium (with or without glucose).

[00153] The sample designations as used in Figures 6 – 11 are as follows:

- VK1a: 500 ml TSB Medium in 2 l Corning flask + 0.5 ml RCB, 30 °C, 120 rpm
- VK1b: 500 ml TSB Medium in 2 l Corning flask + 0.5 ml RCB, 30 °C, 120 rpm + glucose addition
- VK2a: 500 ml TSB Medium in 2 l Corning flask + 38 ml VK1a (OD 5,4), 30 °C, 120 rpm
- VK2b : 500 ml TSB Medium in 2 l Corning flask + 38 ml VK1a (OD 5,4), 30 °C, 120 rpm + glucose addition
- VK3a: 500 ml TSB Medium in 2 L Corning flask + 38 ml VK1b (OD 5,1; 5 hours after first glucose addition) 30 °C, 120 rpm
- VK3b: 500 ml TSB Medium in 2 L Corning flask + 38 ml VK1b (OD 5,1; 5 hours after first glucose addition) 30 °C, 120 rpm + glucose addition.

[00154] The results of these experiments show that glucose addition does not result in higher OD-values / cell mass yields of the wild type strain despite of glucose consumption. In contrast, flasks without glucose addition reached higher OD values (6 for preculture 1, 8 for preculture 2). Approx. 1 h after glucose addition OD remained static (or even slightly declined) compared to growth without glucose addition. On repeated pulses glucose consumption declined, no additional/additive effect on growth was observed.

[00155] Without glucose addition a shift of pH to alkaline was observed after depletion of glucose while with glucose pulse pH-value dropped (compare Figure 8 for preculture 1 and Figure 9 for preculture 2). Phenomena were observed in both precultures (VK1, VK2), indicating no influence of generation number.

[00156] When a preculture grown with glucose pulse was inoculated into fresh medium (1:14 dilution; VK3; Figure 10, Figure 11), the same growth characteristics (higher OD values / pH shift to alkaline without glucose addition) were observed.

[00157] Results indicated that glucose concentrations above a comparably low limit (approx. 2.5 g/l) trigger a reversible change in (glucose) metabolism. Presumably, a substance is then secreted into medium which inhibits further growth, even if glucose declines again below trigger level. After inoculation in new medium (VK3) this substance is diluted to a concentration beneath effectiveness.

Example 6: Growth of engineered production cancer vaccine strain *Salmonella typhi* Ty21a (pVAX10.VR2-1) (=VXM01) with and without glucose feeding.

[00158] The same experimental approach as described in Example 5 was carried out with the cancer vaccine production strain VXM01. The only difference to

Example 5 is that the strain was transformed with plasmid pVAX10.VR2-1. These investigations were made to support the hypothesis, that the growth characteristics of the production strain *S. typhi* Ty21a:pVAX10-VR2.1 (p) regarding the glucose metabolism is not influenced by the plasmid but a characteristic of the empty strain.. Growth and glucose pulse of both strains are compared in Figure 11 and Figure 12.

[00159] The results show, that growth characteristics of both strains (*S. typhi* Ty21a empty and engineered production strain) are comparable. No indication for influence of the plasmid was seen. Moreover, although the cells grown without glucose show a different morphology compared to the cells grown in the presence of glucose, they show no increased cell lysis and no decreases plasmid stability (in case of the engineered cells) as compared to the cells grown with glucose.

Table 3: *Results of OD600 measurement during fermentation process*

Cultivation time [h]	OD ₆₀₀ -results						
	VK1 a	VK1b	VK2	VK1a k	VK1b k	VK2a k	VK2b k
0	0.001	0.003	0.1	0.0	0.0	0.2	0.2
1			0.3			0.3	0.3
2.0			0.5			0.7	0.6
3.0	0.003		1.0			1.2	1.2
4.0			1.6			1.7	1.6
5.0	0.005		2.4			2.5	2.3
6.0			3.4			3.1	3.1
7.0			3.8			3.7	3.7
7.5	0.010					3.7	4.5
8.0			4.5				
9.0	0.026						
10.0							
11.0							
12.0							
13.0							
14.0							
15.0		1.6		1.8	2.1		
16.0		2.2		2.6	2.8		
17.0		2.8		2.8	3		
18.0		3.2			3.1		
19.0		3.7			3.0		
20.0							
21.0		5.1		4.2	3		
22.0							
23.0		6.1		5.3	3.1		
24.0	5.9		7.3			3.8	8
25.0	6.3						
26.0	6.9						
27.0	7.3						
28.0	7.6						
30.0	7.5						
39.0		7.0		7.6	3.2		

Table 4: *Glucose concentration during fermentation process*

Cultivation time [h]	Glucose concentration [g/l]			
	VK1a k	VK1b k	VK2a k	VK2b k
0	2.4	2.4	2.5	2.6
1			2.5	
2.0			2.4	
3.0			2.3	
4.0			1.7	
5.0			4.8	1.0
6.0			3.9	0.1
7.0			3.1	
7.5				
8.0			2.2	
9.0				
10.0				
11.0				
12.0				
13.0				
14.0				
15.0	1.4	1.1		
16.0	0.4	0.1		
17.0		3.8		
18.0		3.2		
19.0		2.5		
20.0				
21.0		1.6		
22.0				
23.0		0.8		
24.0			0	
25.0				
26.0				
27.0				
28.0				
30.0				
39.0		0.0		

Table 5: pH-value shift during fermentation process

Incubation time [h]	pH-vales			
	VK1a k pH	VK1b k pH	VK2a k pH	VK2b k pH
0	6.8	6.8	6.5	6.5
1			6.4	6.4
2.0			6.4	6.4
3.0			6.5	6.5
4.0			6.0	6.0
5.0			6.2	6.2
6.0			5.8	5.91
7.0				
7.5				
8.0			5.4	5.9
9.0				
10.0				
11.0				
12.0				
13.0				
14.0				
15.0	5.7	5.6		
16.0	5.6	5.6		
17.0	5.7	5.4		
18.0	5.8	4.4		
19.0	5.7	5.3		
20.0				
21.0	6.1	5.2		
22.0				
23.0	6.6	5.1		
24.0			5.7	8.28
25.0				
26.0				
27.0				
28.0				
30.0				
39.0	8.0	5.1		

CLAIMS

1. A method for growing an attenuated mutant strain of *Salmonella typhi* lacking galactose epimerase activity and comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette, comprising the step of culturing the strain in a buffered medium comprising peptone at approximately neutral starting pH value at fermentation scale, wherein the amount of glucose in the medium during the fermentation is adjusted such that the amount of glucose is reduced to zero before reaching the stationary phase,
wherein no glucose is added to the medium during the fermentation,
wherein the starting glucose concentration is from about 0 g/l to about 3 g/l,
and wherein the volume of the medium is at least about 10 l.
2. The method of claim 1, wherein said attenuated mutant strain of *Salmonella typhi* is *Salmonella typhi* Ty21a.
3. The method of any one of claims 1 or 2, wherein the eukaryotic expression cassette encodes a VEGF receptor protein, preferably a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith, particularly wherein human VEGFR-2 has the amino acid sequence as found in SEQ ID NO 1.
4. The method of any one of claims 1 to 3, wherein the buffered medium comprises peptone of non-animal origin, preferably wherein said buffered medium is Tryptic Soy Broth (TSB) of non-animal origin.

5. The method of any one of claims 1 to 4, wherein the volume of the medium is from about 10 l to about 10.000 l, preferably from about 30 l to about 1.000 l, more preferably from about 100 l to about 500 l.
6. The method of any one of claims 1 to 5, wherein the starting glucose concentration corresponds to that of bacterial minimal medium or less.
7. The method of any one of claims 1 to 6, wherein the starting pH value is from about 5 to less than about 9, preferably from about 6 to about 8, more preferably from about 6.5 to about 7.5.
8. The method of any one of claims 1 to 7, wherein the pH value is adjusted during said culturing to a pH value of about 6 to about 8, preferably to a pH value of about 6.5 to about 7.5.
9. The method of any one of claims 1 to 8, wherein the progress of growth is determined by (i) measuring the optical density (OD), preferably by (ia) in-situ monitoring of the optical density of the culture or by (ib) taking samples and measuring the optical density of the samples, or by (ii) measuring the cell density, preferably (iia) microscopically or (iib) by measuring the electrical resistance, or (iic) by flow cytometry, or by (iii) measuring the colony forming units (CFU) value by taking samples and plating on agar plates.
10. The method of any one of claims 1 to 9, wherein the cells are harvested before reaching an optical density of about 6, preferably at an optical density of about 5 to about 6.

11. The method of any one of claims 1 to 10, wherein the attenuated mutant strain of *Salmonella typhi* is *Salmonella typhi* Ty21a and the recombinant DNA molecule comprises the kanamycin resistance gene, the pMB1 ori, and a eukaryotic expression cassette encoding human VEGFR-2, under the control of the CMV promoter, particularly wherein human VEGFR-2 has the nucleic acid sequence as found in SEQ ID NO 2.
12. An attenuated mutant strain of *Salmonella typhi* lacking galactose epimerase activity and comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette,
obtainable by a method for growing the strain, comprising the step of
culturing the strain in a buffered medium comprising peptone at approximately neutral starting pH at fermentation scale,
wherein the amount of glucose in the medium during the fermentation is adjusted such that the amount of glucose is reduced to zero before reaching the stationary phase,
wherein no glucose is added to the medium during the fermentation,
wherein the starting glucose concentration is from about 0 g/l to about 3 g/l,
wherein the volume of the medium is at least about 10 l.
13. The attenuated mutant strain of claim 12, wherein the eukaryotic expression cassette encodes a VEGF receptor protein, preferably a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith, particularly wherein human VEGFR-2 has the amino acid sequence as found in SEQ ID NO 1.
14. The attenuated mutant strain of claim 13, wherein the attenuated mutant strain is *Salmonella typhi* Ty21a and the recombinant DNA molecule comprises the kanamycin resistance gene, the pMB1 ori, and an eukaryotic expression cassette encoding human

VEGFR-2 under the control of the CMV promoter, particularly wherein human VEGFR-2 has the nucleic acid sequence as found in SEQ ID NO 2.

15. An attenuated mutant strain of *Salmonella typhi* Ty21a comprising at least one copy of a recombinant DNA molecule comprising a eukaryotic expression cassette encoding a VEGF receptor protein, preferably a VEGF receptor protein selected from the group consisting of human VEGFR-2 and a homolog thereof that shares at least about 80% homology therewith, particularly wherein human VEGFR-2 has the amino acid sequence as found in SEQ ID NO 1, for use as a vaccine.