

Virulence attenuated bacteria for treatment of malignant solid tumors

The field of the invention

The present invention relates to recombinant virulence attenuated Gram-negative bacterial strains for use in a method of treating a malignant solid tumor in a subject.

Background of the invention

A major problem in treatment of malignant solid tumors is the delivery of therapeutic molecules to cancer cells in sufficient amounts, while reducing damage on non-related cells and tissue. The most common treatment approaches, surgery, chemo- and radiation-therapy, too often fail to cure patients, and side effects, like nausea and diarrhoea are massive. Thus, several other therapeutic strategies have been exploited, including angiogenesis inhibitors and immunotherapies.

To reduce damage to non-cancerogenic tissue, approaches allowing targeted drug delivery are of great interest. For example, antibodies recognizing surface structures of tumor cells and, in an optimal case, selectively bind to tumor cells are used. To improve the mechanism of such antibodies they can be conjugated to therapeutic agents or to lipid vesicles packed with drugs. One of the challenges with such vesicles is the proper release of the active reagent. Even more complex is the delivery of therapeutic proteins or peptides, especially when intracellular mechanisms are targeted. Many alternative ways have been tried to solve the problem of delivering therapeutic proteins into eukaryotic cells, among which are “cell penetrating peptides” (CPP) or similar technologies as well as various nanoparticle-based methodologies. All these technologies have the drawback of low efficacy and that the cargo taken up by the cell via endocytosis is likely to end up being degraded in lysosomes. Furthermore, the conflict between need for stability of cargo-carrier in the human body and the requirement for destabilization and liberation within the target cell constitutes an intrinsic problem of such technologies.

Various bacteria have been shown to replicate within malignant solid tumors when administered from a distal site, including *Escherichia coli*, *Vibrio cholerae*, *Salmonella enterica*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Bifidobacteria*. Currently, only bacillus Calmette-Guérin (BCG, derived from *Mycobacterium bovis*) is used in clinical practice. BCG is administrated to treat superficial bladder cancer, while the underlying molecular mechanism remains largely unknown.

An optimal bacterial strain for treatment of malignant solid tumors will specifically accumulate and proliferate at the site of the tumor, while being eradicated at unrelated sites. In this sense, an optimal bacterial vehicle for targeted cancer therapy should provide high accumulation at the desired site of action while being minimally present at non-related sites.

- 5 The development of such a bacterial strain for the treatment of malignant solid tumors which is capable e.g. to deliver cargo produced inside bacteria to its site of action inside cancer cells, i.e. outside of bacteria, remains a major challenge.

Summary of the invention

- 10 The present invention relates to recombinant virulence attenuated Gram-negative bacterial strains for use in a method of treating a malignant solid tumor in a subject. In some embodiments the present invention provides recombinant virulence attenuated Gram-negative bacterial strains and the use thereof for treating a malignant solid tumor in a subject wherein the recombinant virulence attenuated Gram-negative bacterial strains allow the translocation
15 of various type III effectors, but also of type IV effectors, of viral proteins and most importantly of functional eukaryotic proteins into cells of the malignant solid tumor.

In a first aspect the present invention relates to a recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:

- 20 a promoter;
a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;
a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence,
25 for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, the method comprising administering to the subject said recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is administered in an amount that is sufficient to treat the subject.
30 Likewise the present invention relates to a method of treating a malignant solid tumor in a subjects, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, comprising administering to the subject a

recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:

a promoter;

a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably

5 linked to said promoter;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence,

wherein the recombinant virulence attenuated Gram-negative bacterial strain is administered in an amount that is sufficient to treat the subject.

10 Likewise the present invention relates to the use of a recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:

a promoter;

a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;

15 a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence,

for the manufacture of a medicament for treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor.

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In a further aspect the present invention relates to a recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one bacterial

25 protein which is part of a secretion system machinery, for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, the method comprising administering to the subject said recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is
30 administered in an amount that is sufficient to treat the subject.

Likewise the present invention relates to a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, comprising administering to a subject a

recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one bacterial protein which is part of a secretion system machinery,

5 wherein the recombinant virulence attenuated Gram-negative bacterial strain is administered in an amount that is sufficient to treat the subject.

Likewise the present invention relates to the use of a recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is

10 virulent toward eukaryotic cells or is deficient in the production of at least one bacterial protein which is part of a secretion system machinery, for the manufacture of a medicament for treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor.

15 In a further aspect the present invention relates to a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:

a promoter;

a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably

20 linked to said promoter;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence, and a pharmaceutically acceptable carrier,

for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, 25 the method comprising administering to the subject said pharmaceutical composition, wherein the pharmaceutical composition is administered in an amount that is sufficient to treat the subject.

Likewise the present invention relates to a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain

30 accumulates in the malignant solid tumor, comprising administering to a subject a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:

a promoter;

a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence, and a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is administered in an amount that is sufficient to treat the subject.

Likewise the present invention relates to the use of a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:

a promoter;

a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence, and a pharmaceutically acceptable carrier,

for the manufacture of a medicament for treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor.

In a further aspect the present invention relates to a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain and a pharmaceutically acceptable carrier, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one bacterial protein which is part of a secretion system machinery, for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, the method comprising administering to the subject said pharmaceutical composition, wherein the pharmaceutical composition is administered in an amount that is sufficient to treat the subject.

Likewise the present invention relates to a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, comprising administering to a subject a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain which is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one

bacterial protein which is part of a secretion system machinery, and a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is administered in an amount that is sufficient to treat the subject.

Likewise the present invention relates to the use of a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain which is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one bacterial protein which is part of a secretion system machinery, and a pharmaceutically acceptable carrier, for the manufacture of a medicament for treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor.

Brief description of the figures

Fig. 1: Characterization of T3SS protein delivery. (A) Schematic representation of T3SS dependent protein secretion into the surrounding medium (in-vitro secretion)(left side) or into eukaryotic cells (right side). I: shows the type 3 secretion system. II indicates proteins secreted into the surrounding medium, III proteins translocated through the membrane into the cytosol of eukaryotic cells (VII). VI shows a stretch of the two bacterial membranes in which the T3SS is inserted and the bacterial cytosol underneath. IV is a fusion protein attached to the YopE₁₋₁₃₈ N-terminal fragment (V) (B) In-vitro secretion of I: *Y. enterocolitica* E40 wild type, II: *Y. enterocolitica* ΔHOPEMT *asd* or III: *Y. enterocolitica* ΔHOPEMT *asd* + pBadSi_2 as revealed by Western blotting on total bacterial lysates (IV) and precipitated culture supernatants (V) using an anti-YopE antibody.

Fig. 2: Characterization of T3SS protein delivery into epithelial cells. (A) Anti-Myc immunofluorescence staining on HeLa cells infected at an MOI of 100 for 1 h with I: *Y. enterocolitica* ΔHOPEMT *asd* or II: *Y. enterocolitica* ΔHOPEMT *asd* + pBad_Si2. (B) Quantification of anti-Myc immunofluorescence staining intensity from (A) within HeLa cells. Data were combined from n = 20 sites, error bars indicated are standard error of the mean. I: uninfected, II: *Y. enterocolitica* ΔHOPEMT *asd* or III: *Y. enterocolitica* ΔHOPEMT *asd* + pBad_Si2. Y-axis indicates anti-Myc staining intensity [arbitrary unit], x-axis indicates time of infection in minutes (C) Quantification of Anti-Myc immunofluorescence staining intensity within cells. HeLa cells were infected for 1h with *Y. enterocolitica* ΔHOPEMT *asd* + pBad_Si2 at an MOI indicated on the x-axis. Data were combined from n = 20 sites, error bars

indicated are standard error of the mean. Y-axis indicates anti-Myc staining intensity [a.u.].

Fig. 3: Modifications of the T3SS based protein delivery allow nuclear localization of a YopE₁₋₁₃₈ fusion protein (EGFP). EGFP signal in HeLa cells infected with I: *Y. enterocolitica* ΔHOPEMT *asd* or II: *Y. enterocolitica* ΔHOPEMT *asd* Δ*yopB* carrying the plasmids III: +YopE₁₋₁₃₈-EGFP or IV: +YopE₁₋₁₃₈-EGFP-NLS at an MOI of 100. EGFP signal is shown in “a”, for localization comparison nuclei were stained in “b”.

Fig. 4: Modifications of the T3SS based protein delivery allow removal of the YopE₁₋₁₃₈ appendage. HeLa cells are infected with two different *Y. enterocolitica* strains at the same time, which is reached by simple mixing of the two bacterial suspensions. One strain is

delivering the TEV protease fused to YopE₁₋₁₃₈, while the other strain delivers a protein of interest fused to YopE₁₋₁₃₈ with a linker containing a double TEV protease cleavage site. After protein delivery into the eukaryotic cell, the TEV protease will cleave the YopE₁₋₁₃₈ appendage from the protein of interest **(A)** Digitonin lysed HeLa cells uninfected (II) or after infection (MOI of 100) for 2h with I: *Y. enterocolitica* ΔHOPEMT *asd* and III: + pBadSi₂, IV: + YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C, V: + YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C and further overnight treatment with purified TEV protease and VI: + YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C and a second strain + YopE₁₋₁₃₈-TEV were analyzed by Western blotting anti-INK4C (shown in “a”) for the presence of YopE₁₋₁₃₈ – 2x TEV cleavage site –Flag-INK4C or its cleaved form Flag-INK4C. As a loading control western blotting anti-Actin was performed (shown in “b”). In one case (V) the lysed cells were incubated overnight with purified TEV protease. **(B)** Actin normalized quantification of anti-INK4C staining intensity (shown as [a.u.] on the y-axis) from (A) at the size of full length YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C, where sample IV is set to 100%. I: *Y. enterocolitica* ΔHOPEMT *asd* and IV: + YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C, V: + YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C and further overnight treatment with purified TEV protease and VI: + YopE₁₋₁₃₈-2x TEV cleavage site-Flag-INK4C and a second strain + YopE₁₋₁₃₈-TEV. Data were combined from n = 2 independent experiments, error bars indicated are standard error of the mean **(C)** Digitonin lysed HeLa cells uninfected (II) or after infection (MOI of 100) for 2h with I: *Y. enterocolitica* ΔHOPEMT *asd* and III: + pBadSi₂, IV: + YopE₁₋₁₃₈-2x TEV cleavage site-ET1-Myc, V: + YopE₁₋₁₃₈-2x TEV cleavage site-ET1-Myc and further overnight treatment with purified TEV protease and VI: + YopE₁₋₁₃₈-2x TEV cleavage site-ET1-Myc and a second strain + YopE₁₋₁₃₈-TEV were analyzed by Western blotting anti-Myc (shown in “a”) for the presence of YopE₁₋₁₃₈ – 2x TEV cleavage site - ET1-Myc or its cleaved form

ET1-Myc. As a loading control western blotting anti-Actin was performed (shown in “b”) In one case (V) the lysed cells were incubated overnight with purified TEV protease.

Fig. 5: Delivery of bacterial effector proteins into eukaryotic cells (A) HeLa cells were infected with I: *Y. enterocolitica* ΔHOPEMT *asd* carrying II: pBad_Si2 or III: YopE₁₋₁₃₈-SopE at an MOI of 100 for the time indicated above the images (2, 10 or 60 minutes). After fixation cells were stained for the actin cytoskeleton **(B)** HeLa cells were left uninfected (II) or infected with I: *Y. enterocolitica* ΔHOPEMT *asd* carrying III: YopE₁₋₁₃₈-SopE-Myc and in some cases coinfectd with IV: YopE₁₋₁₃₈-SptP at the MOI indicated below the strain (MOI 50; MOI50:MOI50 or MOI50:MOI100) for 1h. After fixation cells were stained for the actin cytoskeleton (shown in “a”) and the presence of the YopE₁₋₁₃₈-SopE-Myc fusion protein was followed via staining anti-Myc (shown in “b”).

Fig. 6: Delivery of bacterial effector proteins into eukaryotic cells (A) Phospho-p38 (“a”), total p38 (“b”) and actin (“c”) western blot analysis on HeLa cells left untreated (II) or infected for 75 min with I: *Y. enterocolitica* ΔHOPEMT *asd* carrying III: pBad_Si2 or IV: YopE₁₋₁₃₈-OspF at an MOI of 100. Cells were stimulated with TNFα for the last 30 min of the infection as indicated (+ stands for addition of TNFα, - represent no treatment with TNFα) **(B)** Phospho-Akt T308 (“a”) and S473 (“b”) and actin (“c”) western blot analysis on HeLa cells left untreated (II) or infected for 22.5 or 45 min (indicated below the blots) with I: *Y. enterocolitica* ΔHOPEMT *asd* carrying III: pBad_Si2, IV: YopE₁₋₁₃₈-SopE or V: YopE₁₋₁₃₈-SopB at an MOI of 100 **(C)** cAMP levels (in fmol/well shown on y-axis) in HeLa cells left untreated (I) or infected for 2.5h with V: *Y. enterocolitica* ΔHOPEMT *asd* + YopE₁₋₁₃₈-BepA, VI: *Y. enterocolitica* ΔHOPEMT *asd* + YopE₁₋₁₃₈-BepA_{E305-end}, VII: *Y. enterocolitica* ΔHOPEMT *asd* + YopE₁₋₁₃₈-BepG_{Bid} or VIII: *Y. enterocolitica* ΔHOPEMT *asd* + pBad_Si2 at an MOI of 100. Cholera toxin (CT) was added for 1h as positive control to samples II (1 μg/ml), III (25 μg/ml) or IV (50 μg/ml). Data were combined from n = 3 independent experiments, error bars indicated are standard error of the mean. Statistical analysis was performed using an unpaired two-tailed t-test (ns indicates a non significant change, ** indicates a p value < 0.01, *** indicates a p value < 0.001).

Fig. 7: Delivery of human tBid into eukaryotic cells induces massive apoptosis. (A) Cleaved Caspase 3 p17 (“a”) and actin (“b”) western blot analysis on HeLa cells left untreated (II) or infected for 60 min with I: *Y. enterocolitica* ΔHOPEMT *asd* carrying III: pBad_Si2, IV: YopE₁₋₁₃₈-Bid or V: YopE₁₋₁₃₈-t-Bid at an MOI of 100. In some cases, cells were treated

with VI: 0.5 μ M Staurosporine or VII: 1 μ M Staurosporine **(B)** Digitonin lysed HeLa cells left untreated (II) or after infection for 1h with I: *Y. enterocolitica* Δ HOPEMT *asd* carrying III: pBad_Si2, IV: YopE₁₋₁₃₈-Bid or V: YopE₁₋₁₃₈-t-Bid at an MOI of 100 were analyzed by Western blotting anti-Bid (“a”) allowing comparison of endogenous Bid levels (marked Z) to translocated YopE₁₋₁₃₈-Bid (marked X) or YopE₁₋₁₃₈-tBid (marked Y) levels. As a loading control western blotting anti-Actin was performed (shown in “b”). In some cases, cells were treated with VI: 0.5 μ M Staurosporine or VII: 1 μ M Staurosporine **(C)** HeLa cells were left untreated (I) or infected at an MOI of 100 for 1h with II: *Y. enterocolitica* Δ HOPEMT *asd* + pBad_Si2, III: *Y. enterocolitica* Δ HOPEMT *asd* + YopE₁₋₁₃₈-Bid, IV: *Y. enterocolitica* Δ HOPEMT *asd* + YopE₁₋₁₃₈-tBid. In some cases, cells were treated with V: 0.5 μ M Staurosporine or VI: 1 μ M Staurosporine. After fixation cells were stained for the actin cytoskeleton (gray).

Fig. 8: tBid dependent phosphoproteome: HeLa cells were infected for 30 min with *Y. enterocolitica* Δ HOPEMT *asd* + YopE₁₋₁₃₈-t-Bid at an MOI of 100 and as a control with *Y. enterocolitica* Δ HOPEMT *asd* + pBad_Si2. **(A)** Graphical representation of the tBid phosphoproteome. Proteins containing phosphopeptides that were significantly regulated in a tBid dependent manner (gray) (q-value <0.01) as well as known apoptosis related proteins (dark gray) are represented in a STRING network of known and predicted protein-protein interactions (high-confidence, score 0.7). Only proteins with at least one connection in STRING are represented. **(B)** Confocal images of HeLa cells infected with either *Y. enterocolitica* Δ HOPEMT *asd* + pBad_Si2 (I) or *Y. enterocolitica* Δ HOPEMT *asd* + YopE₁₋₁₃₈-t-Bid (II) reveal the induction of an apoptotic phenotype upon tBid delivery. Cells were stained for the nuclei with Hoechst (“a”), for F-actin with phalloidin (“b”), for tubulin with an anti-tubulin antibody (“c”) and for mitochondria with mitotracker (“d”). Scale bar represents 40 μ m.

Fig. 9: Description of the type III secretion-based delivery toolbox. **(A)** Vector maps of the cloning plasmids pBad_Si1 and pBad_Si2 used to generate fusion constructs with YopE₁₋₁₃₈. The chaperone SycE and the YopE₁₋₁₃₈-fusion are under the native *Y. enterocolitica* promoter. The two plasmids only differ in presence of an arabinose inducible EGFP present on pBad_Si1 **(B)** Multiple cloning site directly following the yopE₁₋₁₃₈ fragment on pBad_Si1 and pBad_Si2 plasmids.

Fig. 10: Characterization of T3SS protein delivery into various cell lines. Anti-Myc immunofluorescence staining on Swiss 3T3 fibroblasts (“a”), Jurkat cells (“b”) and HUVEC

cells (“c”) left untreated (II) or infected with *Y. enterocolitica* Δ HOPEMT *asd* + pBad_Si2 (I) at the MOI indicated above the images (MOI 25, 50, 100, 200 and 400 for HUVECs) for 1 h.

Fig. 11: T3SS dependency of delivery of bacterial effector proteins into eukaryotic cell.

Digitonin lysed HeLa cells after infection at an MOI of 100 for time indicated above the blots (0, 5, 15, 10, 60 and 120 minutes) with *Y. enterocolitica* Δ HOPEMT *asd* Δ yopB + YopE₁₋₁₃₈-SopE-Myc (I) or *Y. enterocolitica* Δ HOPEMT *asd* + YopE₁₋₁₃₈-SopE-Myc (II) were analyzed by Western blotting anti-Myc. The size corresponding to YopE₁₋₁₃₈-SopE-Myc is marked with “a”, while the size of the endogenous c-Myc protein is marked with “b”.

Fig. 12 and 13: T3SS dependent secretion of various other proteins into the culture supernatant.

In-vitro secretion experiment of I: *Y. enterocolitica* Δ HOPEMT *asd* + YopE₁₋₁₃₈ fused to the protein as indicated. Protein content of total bacterial lysates (“A”) and precipitated culture supernatants (“B”) was analyzed by Western blotting using an anti-YopE antibody. Numbers written indicate molecular weight in kDa at the corresponding height.

Figs. 14A to N: *Y. enterocolitica* and *S. enterica* strains used in this study.

List of *Y. enterocolitica* and *S. enterica* strains used in this study providing information on background strains, plasmids and proteins for T3SS dependent delivery encoded on corresponding plasmids. Further, information on oligonucleotides used for construction of the corresponding plasmid, the backbone plasmid and antibiotic resistances is provided.

Fig. 15: Delivery of murine tBid, murine Bid BH3 and murine Bax BH3 into B16F10

cells induces massive apoptosis. B16F10 cells uninfected (I) or after infection (MOI of 50) for 2.5h with *Y. enterocolitica* Δ HOPEMT *asd* and II: + pBadSi_2, III: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine tBid, IV: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bid BH3 or V: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bax BH3. After fixation cells were stained for the actin cytoskeleton and nuclei (both in gray).

Fig. 16: Delivery of murine tBid, murine Bid BH3 and murine Bax BH3 into D2A1 cells

induces massive apoptosis. D2A1 cells uninfected (I) or after infection (MOI of 50) for 2.5h with *Y. enterocolitica* Δ HOPEMT *asd* and II: + pBadSi_2, III: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine tBid, IV: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bid BH3 or V: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bax BH3. After fixation cells were stained for the actin cytoskeleton and nuclei (both in gray).

Fig. 17: Delivery of murine tBid, murine Bid BH3 and murine Bax BH3 into HeLa cells

induces massive apoptosis. HeLa cells uninfected (I) or after infection (MOI of 50) for 2.5h with *Y. enterocolitica* Δ HOPEMT *asd* and II: + pBadSi_2, III: + YopE₁₋₁₃₈-*Y. enterocolitica*

codon optimized murine tBid, IV: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bid BH3 or V: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bax BH3. After fixation cells were stained for the actin cytoskeleton and nuclei (both in gray).

Fig. 18: Delivery of murine tBid, murine Bid BH3 and murine Bax BH3 into 4T1 cells

induces massive apoptosis. 4T1 cells uninfected (I) or after infection (MOI of 50) for 2.5h with *Y. enterocolitica* ΔHOPEMT *asd* and II: + pBadSi_2, III: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine tBid, IV: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bid BH3 or V: + YopE₁₋₁₃₈-*Y. enterocolitica* codon optimized murine Bax BH3. After fixation cells were stained for the actin cytoskeleton and nuclei (both in gray).

Fig. 19: Delivery of murine tBid by *S. enterica* grown under SPI-1 T3SS inducing conditions into eukaryotic cells induces apoptosis.

Cleaved Caspase 3 p17 western blot analysis on HeLa cells left untreated (I) or infected for 4h with III: *S. enterica* *aroA* carrying IV: SteA₁₋₂₀-t-Bid, V: SteA_{FL}-Bid, VI: SopE₁₋₈₁-t-Bid or VII: SopE₁₋₁₀₅-t-Bid at an MOI of 100. For this experiment, all *S. enterica* *aroA* strains were grown under SPI-1 T3SS inducing conditions. In some cases, cells were treated with II: 1 μM Staurosporine. Numbers written indicate molecular weight in kDa at the corresponding height.

Fig. 20: Delivery of murine tBid by *S. enterica* grown under SPI-2 T3SS inducing conditions into eukaryotic cells induces apoptosis.

Cleaved Caspase 3 p17 western blot analysis on HeLa cells left untreated (I) or infected for 4h with III: *S. enterica* *aroA* carrying IV: SteA₁₋₂₀-t-Bid, V: SteA_{FL}-Bid, VI: SopE₁₋₈₁-t-Bid or VII: SopE₁₋₁₀₅-t-Bid at an MOI of 100. For this experiment, all *S. enterica* *aroA* strains were grown under SPI-2 T3SS inducing conditions. In some cases, cells were treated with II: 1 μM Staurosporine. Numbers written indicate molecular weight in kDa at the corresponding height.

Fig. 21: *S. enterica* T3SS dependent secretion of various cell cycle proteins into the culture supernatant.

In-vitro secretion experiment of *S. enterica* *aroA* + either SteA_{FL} (I, III, V, VII) or SopE₁₋₁₀₅ (II, IV, VI, VIII) fused to proteins as listed following. I and II: Ink4a-MycHis; III and IV: Ink4c-MycHis; V and VI: Mad2-MycHis; VII and VIII: Cdk1-MycHis. Protein content of precipitated culture supernatants (“A”) and total bacterial lysates (“B”) was analyzed by Western blotting using an anti-myc antibody. Numbers written indicate molecular weight in kDa at the corresponding height.

Fig. 22: T3SS dependent secretion of various known cell cycle interfering peptides into the culture supernatant.

In-vitro secretion experiment of I: *Y. enterocolitica* ΔHOPEMT *asd* + pBad_Si2. II-VII: *Y. enterocolitica* ΔHOPEMT *asd* + YopE₁₋₁₃₈ fused to peptides as listed

following: II: Ink4A₈₄₋₁₀₃; III: p107/RBL1₆₅₇₋₆₆₂; IV: p21₁₄₁₋₁₆₀D149A; V: p21₁₄₅₋₁₆₀D149A; VI: p21₁₇₋₃₃; VII: cyclin D2₁₃₉₋₁₄₇. Protein content of precipitated culture supernatants (“A”) and total bacterial lysates (“B”) was analyzed by Western blotting using an anti-YopE antibody. Numbers written indicate molecular weight in kDa at the corresponding height.

5 **Fig. 23: Fusion of the T3SS delivered protein to Ubiquitin allows removal of the YopE₁₋₁₃₈ appendage.** HeLa cells are infected with a strain delivering a protein of interest fused to YopE₁₋₁₃₈ with a directly fused Ubiquitin (YopE₁₋₁₃₈-Ubi). After protein delivery into the eukaryotic cell, endogenous Ubiquitin specific proteases will cleave the YopE₁₋₁₃₈-Ubi appendage from the protein of interest. Digitonin lysed HeLa cells uninfected (I) or after
10 infection (MOI of 100) for 1h with II: *Y. enterocolitica* ΔHOPEMT *asd* + YopE₁₋₁₃₈-Flag-INK4C-MycHis or III: + YopE₁₋₁₃₈-Flag-Ubiquitin-INK4C-MycHis were analyzed by Western blotting anti-INK4C for the presence of IV: YopE₁₋₁₃₈-Flag-Ubiquitin-INK4C-MycHis or V: YopE₁₋₁₃₈-Flag-INK4C-MycHis, the cleaved form VI: INK4C-MycHis and VII: the endogenous INK4C.

15 **Fig. 24: The *Yersinia enterocolitica* W227 virulence plasmid, pYV.** The 69’673 bp plasmid of *Yersinia* virulence (pYV) of strain W227 drawn to scale. T3SS effector proteins, origin of replication and the arsenic resistance (encoded by genes *arsC*, *B*, *R* and *H*) are indicated: I: origin of replication, II: *yopO*, III: *yopP*, IV: *yopQ*, V: *yopT*, VI: *sycT*, VII: *yopM*, VIII: *yopD*, IX: *yopB*, X: *sycD*, XII: *yopH*, XIII: *sycH*, XIV: *sycE*,
20 XV: *yopE*, XVI: *yadA*, XVII-XVXX: *arsC*, *B*, *R* and *H*.

Fig. 25: Validation of *yop* deletion by in vitro secretion analysis.

In vitro secretion analysis of I: *Y. enterocolitica* Δ*yopH,O,P,E,M,T asd*, II: *Y. enterocolitica* MRS40 wt, III: *Y. enterocolitica* Δ*yopH,O,P,E,M,T asd* + p-*yopE*. Marks at the right side indicate height of specific Yop proteins: IV: YopO, V: YopH, VI: YopM, VII: YopE.

25 **Fig. 26: Dose-escalation study in immuno-competent mice: weight of mice.** Weight of mice was assessed daily following i.v. infection with bacteria. I: Days, II: weight in gram, III: *Y. enterocolitica* Δ*yopH,O,P,E,M,T asd*, IV: *S. enterica* Δ*aroA* administered i.v. in the amount indicated (10⁵, 10⁶, 10⁷, 10⁸ cfu per animal).

Fig. 27: Dose-escalation study in immuno-competent mice: counts in blood. Counts in the
30 blood at the time indicated were assessed by serial dilution and counting of resulting colony forming units (CFU). I: Days, II: CFU per ml, III: *Y. enterocolitica* Δ*yopH,O,P,E,M,T asd*, IV: *S. enterica* Δ*aroA* administered i.v. in the amount indicated (10⁵, 10⁶, 10⁷, 10⁸ cfu per animal).

Fig. 28: Dose-escalation study in immuno-competent mice: counts in liver. Counts in the liver at the time indicated were assessed by organ homogenization, serial dilution and counting of resulting colony forming units (CFU). I: Days, II: CFU per g III: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ *asd*, IV: *S. enterica* $\Delta aroA$ administered i.v. in the amount indicated (10^5 , 10^6 , 10^7 , 10^8 cfu per animal).

Fig. 29: Dose-escalation study in immuno-competent mice: counts in lung. Counts in the lung at the time indicated were assessed by organ homogenization, serial dilution and counting of resulting colony forming units (CFU). I: Days, II: CFU per g III: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ *asd*, IV: *S. enterica* $\Delta aroA$ administered i.v. in the amount indicated (10^5 , 10^6 , 10^7 , 10^8 cfu per animal).

Fig. 30: Dose-escalation study in immuno-competent mice: counts in spleen. Counts in the spleen at the time indicated were assessed by organ homogenization, serial dilution and counting of resulting colony forming units (CFU). I: Days, II: CFU per g III: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ *asd*, IV: *S. enterica* $\Delta aroA$ administered i.v. in the amount indicated (10^5 , 10^6 , 10^7 , 10^8 cfu per animal).

Fig. 31: Biodistribution of *Y. enterocolitica* subsp. *paleartica* in the B16F10 melanoma mouse allograft model: scoring for physical appearance. I: Days, II: score, III: *Y. enterocolitica* MRS40 wt, IV: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$. The arrow indicates the day of i.v. infection with 2×10^5 bacteria.

Fig. 32: Biodistribution of *Y. enterocolitica* subsp. *paleartica* in the B16F10 melanoma mouse allograft model: scoring for behavior. I: Days, II: score, III: *Y. enterocolitica* MRS40 wt, IV: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$. The arrow indicates the day of i.v. infection with 2×10^5 bacteria.

Fig. 33: Biodistribution of *Y. enterocolitica* subsp. *paleartica* in the B16F10 melanoma mouse allograft model: weights of mice. Weight of mice was assessed daily following i.v. infection with bacteria. I: Days, II: body weight in gram, III: *Y. enterocolitica* MRS40 wt, IV: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$. The arrow indicates the day of i.v. infection with 2×10^5 bacteria.

Fig. 34: Biodistribution of *Y. enterocolitica* subsp. *paleartica* in the B16F10 melanoma mouse allograft model: biodistribution of *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$. Counts in the organs at the time indicated were assessed by organ homogenization, serial dilution and counting of resulting colony forming units (CFU). I: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$, II:

CFU per gram tissue, III: day 1, IV: day 4, V: blood, VI: spleen, VII: liver, VIII: lung, IX: tumor. * indicates a mouse with no visible tumor.

Fig. 35: Biodistribution of *Y. enterocolitica* subsp. *palearctica* in the B16F10 melanoma mouse allograft model: biodistribution of *Y. enterocolitica* MRS40 wt. Counts in the organs at the time indicated were assessed by organ homogenization, serial dilution and counting of resulting colony forming units (CFU). I: *Y. enterocolitica* MRS40 wt, II: CFU per gram tissue, III: day 1, IV: day 4, V: blood, VI: spleen, VII: liver, VIII: lung, IX: tumor.

Fig. 36: Biodistribution of *Y. enterocolitica* subsp. *palearctica* in the 4T1 breast cancer mouse allograft model: biodistribution of *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$. Counts in the organs at day 8 post infection were assessed by organ homogenization, serial dilution and counting of resulting colony forming units (CFU). I: *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$, II: CFU per gram tissue, III: blood, IV: spleen, V: liver, VI: lung, VII: tumor.

Fig. 37: Delivery of synthetic increased pro-apoptotic proteins. Delivery of single synthetic proteins consisting of single or tandem repeats of BH3 domains originating from pro-apoptotic proteins t-BID or BAX leads to enhanced apoptosis induction in 4T1 and B16F10 cancerous cells. 4T1 (I) or B16F10 (II) cells were infected with *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ encoding on pBad-MycHisA IV: YopE₁₋₁₃₈-tBID BH3 extended domain, V: YopE₁₋₁₃₈-linker-tBID BH3, VI: YopE₁₋₁₃₈-tBID BH3, VII: YopE₁₋₁₃₈-(tBID BH3)₂, VIII: YopE₁₋₁₃₈-tBID BH3 - BAX BH3 or IX: YopE₁₋₁₃₈-BAX BH3 - tBID BH3. A titration of the bacteria added to the cells (MOI) was performed for each strain, cell counts determined and IC50 calculated using non-linear regression. IC50 MOI is indicated (III).

Fig. 38: Induction of apoptosis by pYV-encoded synthetic pro-apoptotic proteins. Delivery of a single or a tandem repeat of BID BH3 domain encoded on the pYV leads to apoptosis induction in 4T1 and B16F10 cancerous cells. 4T1 (I) or B16F10 (II) cells were infected with *Y. enterocolitica* $\Delta HOPEMT$ + IV: pYV-YopE₁₋₁₃₈-BH3-Bid, or V: + pYV-YopE₁₋₁₃₈-(BH3-Bid)₂ or VI: with *Y. enterocolitica* $\Delta HOPEMT$ pBad-MycHisA-YopE₁₋₁₃₈-(BH3-Bid)₂ for 3 hours. A titration of the bacteria added to the cells (MOI) was performed for each strain, cell counts determined and IC50 (III) calculated using non-linear regression.

Fig. 39: Tumor colonization of i.v. injected *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ in the 4T1 breast cancer allograft model. Bacterial counts in tumors are indicated as colony forming units (CFU) per gram of tissue (III). Counts were assessed in tumors at day 8 (I) and 14 (II) post infection. Each dot represents an individual mouse. The horizontal dashed line indicates the detection limit.

Fig. 40: Biodistribution of i.v. injected *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ in the 4T1 breast cancer allograft model. Bacterial counts in blood (I), spleen (II), liver (III), lung (IV) and tumor (V) are indicated as colony forming units (CFU) per gram of tissue or per ml of blood (VI). Counts were assessed at day 14 post infection. Each dot represents an individual mouse. The horizontal dashed line indicates the detection limit. * indicates a mouse with large metastases found on lung.

Fig. 41: Delay of tumor progression in wildtype Balb/C mice allografted s.c. with 4T1 breast cancer cells. Wildtype Balb/C mice allografted s.c. with 4T1 breast cancer cells were i.v. injected with I: PBS or II: 1×10^7 *Y. enterocolitica* dHOPEMT + pYV-YopE₁₋₁₃₈(BH3-Bid)₂, once the tumor had reached a size of 150-250 mm³. The day of the i.v. injection of bacteria was defined as day 0. Tumor volume was measured over the following days (III; day 0 to day 9 post i.v. injection of bacteria) with calipers. The relative tumor volume, normalized to the tumor volume at day 0, is indicated (IV) as mm³. The mean is indicated with symbols, error bars depicted show the standard error of the mean. Statistical significance is measured with a 2way ANOVA, * indicates p value <0.05, ** a p value < 0.005.

Fig. 42: Tumor progression in wildtype Balb/C mice allografted s.c. with 4T1 breast cancer cells. Wildtype Balb/C mice allografted s.c. with 4T1 breast cancer cells were i.v. injected with I: PBS or II: 1×10^7 *Y. enterocolitica* dHOPEMT, once the tumor had reached a size of 150-250 mm³. The day of the i.v. injection of bacteria was defined as day 0. Tumor volume was measured over the following days (III; day 0 to day 9 post i.v. injection of bacteria) with calipers. The relative tumor volume, normalized to the tumor volume at day 0, is indicated (IV) as mm³. The mean is indicated with symbols, error bars depicted show the standard error of the mean.

Detailed description of the invention

The present invention relates to recombinant virulence attenuated Gram-negative bacterial strains for use in a method of treating a malignant solid tumor in a subject.

For the purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

The term "Gram-negative bacterial strain" as used herein includes the following bacteria:

Aeromonas salmonicida, *Aeromonas hydrophila*, *Aeromonas veronii*, *Anaeromyxobacter dehalogenans*, *Bordetella bronchiseptica*, *Bordetella parapertussis*, *Bordetella pertussis*, *Bradyrhizobium japonicum*, *Burkholderia cenocepacia*, *Burkholderia cepacia*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Chlamydia muridarum*, *Chlamydia trachomatis*, *Chlamydophila abortus*, *Chlamydophila pneumoniae*, *Chromobacterium violaceum*, *Citrobacter rodentium*, *Desulfovibrio vulgaris*, *Edwardsiella tarda*, *Endozoicomonas elysicola*, *Erwinia amylovora*, *Escherichia albertii*, *Escherichia coli*, *Lawsonia intracellularis*, *Mesorhizobium loti*, *Myxococcus xanthus*, *Pantoea agglomerans*, *Photobacterium damsela*, *Photobacterium luminescens*, *Photobacterium temperate*, *Pseudoalteromonas spongiae*, *Pseudomonas aeruginosa*, *Pseudomonas plecoglossicida*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Rhizobium* sp, *Salmonella enterica* and other *Salmonella* sp, *Shigella flexneri* and other *Shigella* sp, *Sodalis glossinidius*, *Vibrio alginolyticus*, *Vibrio azureus*, *Vibrio campellii*, *Vibrio caribbenthicus*, *Vibrio harvey*, *Vibrio parahaemolyticus*, *Vibrio tasmaniensis*, *Vibrio tubiashii*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Xanthomonas oryzae*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*. Preferred Gram-negative bacterial strains of the invention are Gram-negative bacterial strains comprised by the family of *Enterobacteriaceae* and *Pseudomonadaceae*. The Gram-negative bacterial strain of the present invention is normally used for delivery of heterologous proteins by the bacterial T3SS into eukaryotic cells in vitro and/or in vivo, preferably in vivo.

The term "recombinant virulence attenuated Gram-negative bacterial strain" as used herein refers to a recombinant virulence attenuated Gram-negative bacterial strain genetically transformed with a vector. A useful vector of the present invention is e.g an expression vector, a vector for chromosomal or virulence plasmid insertion or a DNA or RNA fragment for chromosomal or virulence plasmid insertion or modification. Virulence of such a recombinant Gram-negative bacterial strain is usually attenuated by deletion of bacterial effector proteins having virulence activity which are transported by one or more bacterial proteins, which are part of a secretion system machinery. Such effector proteins are delivered by a secretion system machinery into a host cells where they exert their virulence activity toward various host proteins and cellular machineries. Many different effector proteins are known, transported by various secretion system types and displaying a large repertoire of biochemical

activities that modulate the functions of host regulatory molecules. Virulence of the recombinant Gram-negative bacterial strain used herein can be attenuated additionally by lack of a siderophore normally or occasionally produced by the Gram-negative bacterial strain so that the strain does not produce the siderophore e.g. is deficient in the production of the siderophore. Thus in a preferred embodiment a recombinant virulence attenuated Gram-negative bacterial strain is used in the methods of the invention which lacks of a siderophore normally or occasionally produced by the Gram-negative bacterial strain so that the strain does not produce the siderophore e.g. is deficient in the production of a siderophore, more preferably a *Yersinia* strain, in particular *Y. enterocolitica* MRS40 $\Delta yopH,O,P,E,M,T$, is used in the methods of the invention which lacks of a siderophore normally or occasionally produced by the Gram-negative bacterial strain so that the strain does not produce the siderophore e.g. is deficient in the production of a siderophore. The recombinant virulence attenuated Gram-negative bacterial strain used in the methods of the invention preferably does not produce at least one, preferably at least two siderophores e.g. is deficient in the production of at least one, preferably at least two siderophores, more preferably the recombinant virulence attenuated Gram-negative bacterial strain used in the methods of the invention does not produce any siderophore.

The term “siderophore”, “iron siderophore” or “iron chelator” which are used interchangeably herein refer to compounds with high affinity for iron e.g. small compounds with high affinity for iron.

Siderophores of Gram-negative bacteria are e.g. Enterobactin and dihydroxybenzoylserine synthesized by *Salmonella*, *Escherichia*, *Klebsiella*, *Shigella*, *Serratia* (but used by all enterobacteria), Pyoverdins synthesized by *Pseudomonas*, Vibriobactin synthesized by *Vibrio*, Acinetobactin and Acinetoferrin by *Acinetobacter*, Yersiniabactin and Aerobactin synthesized by *Yersinia*, Ornibactin synthesized by *Burkholderia*, Salmochelin synthesized by *Salmonella*, Aerobactin synthesized by *Escherichia*, *Shigella*, *Salmonella*, and *Yersinia*, Alcaligin synthesized by *Bordetella*, Bisucaberin synthesized by *Vibrio*.

Siderophores include hydroxamate, catecholate and mixed ligand siderophores. Several siderophores have to date been approved for use in humans, mainly with the aim of treating iron overload. Preferred siderophores are Deferoxamine (also known as desferrioxamine B, desferoxamine B, DFO-B, DFOA, DFB or desferal), Desferrioxamine E, Deferasirox (Exjade, Desirox, Defrijet, Desifer) and Deferiprone (Ferriprox).

The terms “Gram-negative bacterial strain deficient to produce an amino acid essential for growth” and “auxotroph mutant” are used herein interchangeably and refer to Gram-negative bacterial strains which can not grow in the absence of at least one exogenously provided essential amino acid or a precursor thereof. The amino acid the strain is deficient to produce is e.g. aspartate, *meso*-2,6-diaminopimelic acid, aromatic amino acids or leucine-arginine [1]. Such a strain can be generated by e.g. deletion of the aspartate-beta-semialdehyde dehydrogenase gene (Δ asd). Such an auxotroph mutant cannot grow in absence of exogenous *meso*-2,6-diaminopimelic acid [2]. The mutation, e.g. deletion of the aspartate-beta-semialdehyde dehydrogenase gene is preferred herein for a Gram-negative bacterial strain deficient to produce an amino acid essential for growth of the present invention.

The term “Gram-negative bacterial strain deficient to produce adhesion proteins binding to the eukaryotic cell surface or extracellular matrix” refers to mutant Gram-negative bacterial strains which do not express at least one adhesion protein compared to the adhesion proteins expressed by the corresponding wild type strain. Adhesion proteins may include e.g. extended polymeric adhesion molecules like pili/fimbriae or non-fimbrial adhesins. Fimbrial adhesins include type-1 pili (such as *E. coli* Fim-pili with the FimH adhesin), P-pili (such as Pap-pili with the PapG adhesin from *E. coli*), type 4 pili (as pilin protein from e.g. *P. aeruginosa*) or curli (Csg proteins with the CsgA adhesin from *S. enterica*). Non-fimbrial adhesions include trimeric autotransporter adhesins such as YadA from *Y. enterocolitica*, BpaA (*B. pseudomallei*), Hia (*H. influenzae*), BadA (*B. henselae*), NadA (*N. meningitidis*) or UspA1 (*M. catarrhalis*) as well as other autotransporter adhesins such as AIDA-1 (*E. coli*) as well as other adhesins/invasins such as InvA from *Y. enterocolitica* or Intimin (*E. coli*) or members of the Dr-family or Afa-family (*E. coli*). The terms YadA and InvA as used herein refer to proteins from *Y. enterocolitica*. The autotransporter YadA [3] binds to different forms of collagen as well as fibronectin, while the invasin InvA [4] binds to β -integrins in the eukaryotic cell membrane. If the Gram-negative bacterial strain is a *Y. enterocolitica* strain the strain is preferably deficient in InvA and/or YadA.

As used herein, the term “family of *Enterobacteriaceae*” comprises a family of gram-negative, rod-shaped, facultatively anaerobic bacteria found in soil, water, plants, and animals, which frequently occur as pathogens in vertebrates. The bacteria of this family share a similar physiology and demonstrate a conservation within functional elements and genes of

the respective genomes. As well as being oxidase negative, all members of this family are glucose fermenters and most are nitrate reducers.

Enterobacteriaceae bacteria of the invention may be any bacteria from that family, and specifically includes, but is not limited to, bacteria of the following genera: *Escherichia*,

- 5 *Shigella*, *Edwardsiella*, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Erwinia*, *Morganella*, *Providencia*, or *Yersinia*. In more specific embodiments, the bacterium is of the *Escherichia coli*, *Escherichia blattae*, *Escherichia fergusonii*, *Escherichia hermannii*, *Escherichia vulneris*, *Salmonella enterica*, *Salmonella bongori*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, *Enterobacter aerogenes*, *Enterobacter gergoviae*,
10 *Enterobacter sakazakii*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Yersinia pseudotuberculosis*, *Yersinia pestis*, *Yersinia enterocolitica*, *Erwinia amylovora*, *Proteus mirabilis*, *Proteus vulgaris*, *Proteus penneri*, *Proteus hauseri*, *Providencia alcalifaciens*, or *Morganella morganii* species. Preferably the Gram-negative bacterial strain is selected from the group consisting of the
15 genera *Yersinia*, *Escherichia*, *Salmonella*, *Shigella*, *Pseudomonas*, *Chlamydia*, *Erwinia*, *Pantoea*, *Vibrio*, *Burkholderia*, *Ralstonia*, *Xanthomonas*, *Chromobacterium*, *Sodalis*, *Citrobacter*, *Edwardsiella*, *Rhizobiae*, *Aeromonas*, *Photobacterium*, *Bordetella* and *Desulfovibrio*, more preferably from the group consisting of the genera *Yersinia*, *Escherichia*, *Salmonella*, and *Pseudomonas*, most preferably from the group consisting of the genera
20 *Yersinia* and *Salmonella*.

The term "*Yersinia*" as used herein includes all species of *Yersinia*, including *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*. Preferred is *Yersinia enterocolitica*.

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The term "*Salmonella*" as used herein includes all species of *Salmonella*, including *Salmonella enterica* and *S. bongori*. Preferred is *Salmonella enterica*.

- 30 "Promoter" as used herein refers to a nucleic acid sequence that regulates expression of a transcriptional unit. A "promoter region" is a regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. Within the promoter region will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains

(consensus sequences) responsible for the binding of RNA polymerase such as the putative -35 region and the Pribnow box. The term "operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other and they are located on the same nucleic acid fragment. A promoter is operably linked to a structural gene if it controls the transcription of the gene and it is located on the same nucleic acid fragment as the gene. Usually the promoter is functional in said Gram-negative bacterial strain, i.e. the promoter is capable of expressing the fusion protein of the present invention, i.e. the promoter is capable of expressing the fusion protein of the present invention without further genetic engineering or expression of further proteins. Furthermore, a functional promoter must not be naturally counter-regulated to the bacterial T3SS.

The term "delivery" used herein refers to the transportation of a protein from a recombinant virulence attenuated Gram-negative bacterial strain to a eukaryotic cell, including the steps of expressing the heterologous protein in the recombinant virulence attenuated Gram-negative bacterial strain, secreting the expressed protein(s) from such recombinant virulence attenuated Gram-negative bacterial strain and translocating the secreted protein(s) by such recombinant virulence attenuated Gram-negative bacterial strain into the cytosol of the eukaryotic cell. Accordingly, the terms "delivery signal" or "secretion signal" which are used interchangeably herein refer to a polypeptide sequence which can be recognized by the secretion and translocation system of the Gram-negative bacterial strain and directs the delivery of a protein from the Gram-negative bacterial strain to eukaryotic cells.

The term "delivery signal from a bacterial effector protein" used herein refers to a delivery signal from a bacterial effector protein functional in the recombinant Gram-negative bacterial strain, i.e. which allows an expressed heterologous protein in the recombinant Gram-negative bacterial strain to be secreted from such recombinant Gram-negative bacterial strain by a secretion system such as the type III secretion system or to be translocated by such recombinant Gram-negative bacterial strain into the cytosol of a eukaryotic cell by a secretion system such as the type III secretion system. The term "delivery signal from a bacterial effector protein" used herein also comprises a fragment of a delivery signal from a bacterial effector protein i.e. shorter versions of a delivery signal e.g. a delivery signal comprising up to 10, preferably up to 20, more preferably up to 50, even more preferably up to 100, in particular up to 140 amino acids of a delivery signal e.g. of a naturally occurring delivery

signal. Thus a nucleotide sequence such as e.g. a DNA sequence encoding a delivery signal from a bacterial effector protein may encode a full length delivery signal or a fragment thereof wherein the fragment usually comprises usually up to 30, preferably up to 60, more preferably up to 150, even more preferably up to 300, in particular up to 420 nucleic acids.

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As used herein, the "secretion" of a protein refers to the transportation of a heterologous protein outward across the cell membrane of a recombinant virulence attenuated Gram-negative bacterial strain. The "translocation" of a protein refers to the transportation of a heterologous protein from a recombinant virulence attenuated Gram-negative bacterial strain across the plasma membrane of a eukaryotic cell into the cytosol of such eukaryotic cell.

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The term "bacterial protein, which is part of a secretion system machinery" as used herein refers to bacterial proteins constituting essential components of the bacterial type 3 secretion system (T3SS), type 4 secretion system (T4SS) and type 6 secretion system (T6SS), preferably T3SS. Without such proteins, the respective secretion system is non-functional in translocating proteins to host cells, even if all other components of the secretion system and the bacterial effector protein to be translocated are still encoded and produced.

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The term "bacterial effector protein" as used herein refers to bacterial proteins transported by secretion systems e.g. by bacterial proteins, which are part of a secretion system machinery into host cells. Such effector proteins are delivered by a secretion system into a host cell where they exert e.g. virulence activity toward various host proteins and cellular machineries. Many different effector proteins are known, transported by various secretion system types and displaying a large repertoire of biochemical activities that modulate the functions of host regulatory molecules. Secretion systems include type 3 secretion system (T3SS), type 4 secretion system (T4SS) and type 6 secretion system (T6SS). Some effector proteins (as *Shigella flexneri* IpaC) as well belong to the class of bacterial protein, which are part of a secretion system machinery and allow protein translocation. The recombinant virulence attenuated Gram-negative bacterial strain used herein usually comprises bacterial proteins constituting essential components of the bacterial type 3 secretion system (T3SS), type 4 secretion system (T4SS) and/or the type 6 secretion system (T6SS), preferably of the type 3 secretion system (T3SS).

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The term "T6SS effector protein" or "bacterial T6SS effector protein" as used herein refers to proteins which are naturally injected by T6S systems into the cytosol of eukaryotic cells or bacteria and to proteins which are naturally secreted by T6S systems that might e.g form translocation pores into the eukaryotic membrane. The term "T4SS effector protein" or

"bacterial T4SS effector protein" as used herein refers to proteins which are naturally injected by T4S systems into the cytosol of eukaryotic cells and to proteins which are naturally secreted by T4S systems that might e.g form the translocation pore into the eukaryotic membrane.

The term "T3SS effector protein" or "bacterial T3SS effector protein" as used herein refers to proteins which are naturally injected by T3S systems into the cytosol of eukaryotic cells and to proteins which are naturally secreted by T3S systems that might e.g form the translocation pore into the eukaryotic membrane (including pore-forming translocators (as *Yersinia* YopB and YopD) and tip-proteins like *Yersinia* LcrV). Preferably proteins which are naturally injected by T3S systems into the cytosol of eukaryotic cells are used. These virulence factors

will paralyze or reprogram the eukaryotic cell to the benefit of the pathogen. T3S effectors display a large repertoire of biochemical activities and modulate the function of crucial host regulatory molecules [5,6] and include AvrA, AvrB, AvrBs2, AvrBS3, AvrBsT, AvrD, AvrD1, AvrPphB, AvrPphC, AvrPphEPto, AvrPpiBPto, AvrPto, AvrPtoB, AvrRpm1, AvrRpt2, AvrXv3, CigR, EspF, EspG, EspH, EspZ, ExoS, ExoT, GogB, GtgA, GtgE, GALA family of proteins, HopAB2, HopAO1, HopI1, HopM1, HopN1, HopPtoD2, HopPtoE, HopPtoF, HopPtoN, HopU1, HsvB, IcsB, IpaA, IpaB, IpaC, IpaH, IpaH7.8, IpaH9.8, IpgB1, IpgB2, IpgD, LcrV, Map, OspC1, OspE2, OspF, OspG, OspI, PipB, PipB2, PopB, PopP2, PthXo1, PthXo6, PthXo7, SifA, SifB, SipA/SspA, SipB, SipC/SspC, SipD/SspD, SlrP, SopA, SopB/SigD, SopD, SopE, SopE2, SpiC/SsaB, SptP, SpvB, SpvC, SrfH, SrfJ, Sse, SseB, SseC, SseD, SseF, SseG, SseI/SrfH, SseJ, SseK1, SseK2, SseK3, SseL, SspH1, SspH2, SteA, SteB, SteC, SteD, SteE, TccP2, Tir, VirA, VirPphA, VopF, XopD, YopB, YopD YopE, YopH, YopJ, YopM, YopO, YopP, YopT, YpkA.

The term "recombinant virulence attenuated Gram-negative bacterial strain accumulating in a malignant solid tumor " or "the recombinant virulence attenuated Gram-negative bacterial strain accumulates in a malignant solid tumor" as used herein refers to a recombinant virulence attenuated Gram-negative bacterial strain which replicates within a malignant solid tumor thereby increasing the bacterial count of this recombinant virulence attenuated Gram-

negative bacterial strain inside the malignant solid tumor. Surprisingly it has been found that the recombinant virulence attenuated Gram-negative bacterial strain after administration to the subject accumulates specifically in the malignant solid tumor i.e. accumulates specifically in the organ where the malignant tumor is present, wherein the bacterial counts of the recombinant virulence attenuated Gram-negative bacterial strain in organs where no malignant solid tumor is present is low or not detectable.

In case of extracellular residing bacteria as *Yersinia*, the bacteria mostly accumulate within the intercellular space formed between tumor cells. Intracellular growing bacteria as *Salmonella* will mostly invade tumor cells and reside inside such cells, while extracellular accumulations might still occur. Bacterial counts of the recombinant virulence attenuated Gram-negative bacterial strain accumulated inside the malignant solid tumor can be e.g. in the range of 10^4 to 10^9 bacteria per gram of tumor tissue.

The term "malignant solid tumor" or "malignant solid tumor indication" used herein refers to an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors may be benign (not cancer), or malignant (cancer). Malignant solid tumors are treated with the methods of the present invention. Different types of malignant solid tumors are named for the type of cells that form them. Examples of malignant solid tumors are sarcomas, carcinomas, and lymphomas. Leukemias (cancers of the blood) generally do not form malignant solid tumors (definition according to the national cancer institute of the NIH). Malignant solid tumors include, but are not limited to, abnormal mass of cells which may stem from different tissue types such as liver, colon, colorectum, skin, breast, pancreas, cervix uteri, corpus uteri, bladder, gallbladder, kidney, larynx, lip, oral cavity, oesophagus, ovary, prostate, stomach, testis, thyroid gland or lung and thus include malignant solid liver, colon, colorectum, skin, breast, pancreas, cervix uteri, corpus uteri, bladder, gallbladder, kidney, larynx, lip, oral cavity, oesophagus, ovary, prostate, stomach, testis, thyroid gland or lung tumors. Preferred malignant solid tumors which can be treated with the methods of the present invention are malignant solid tumors which stem from skin, breast, liver, pancreas, bladder, prostate and colon and thus include malignant solid skin, breast, liver, pancreas, bladder, prostate and colon tumors. Equally preferred malignant solid tumors which can be treated with the methods of the present invention are malignant solid tumors associated with liver cancer, such as hepatocellular carcinoma.

The term "bacterial effector protein which is virulent toward eukaryotic cells" as used herein refers to bacterial effector proteins, which are transported by secretion systems into host cells where they exert their virulence activity toward various host proteins and cellular machineries. Many different effector proteins are known, transported by various secretion system types and displaying a large repertoire of biochemical activities that modulate the functions of host regulatory molecules. Secretion systems include type 3 secretion system (T3SS), type 4 secretion system (T4SS) and type 6 secretion system (T6SS). Importantly, some effector proteins which are virulent toward eukaryotic cells (as *Shigella flexneri* IpaC) as well belong to the class of bacterial proteins, which are part of a secretion system

machinery. In case the bacterial effector protein which is virulent toward eukaryotic cells is as well essential for the function of the secretion machinery, such a protein is excluded from this definition. T3SS effector proteins which are virulent towards eukaryotic cells refers to proteins as *Y. enterocolitica* YopE, YopH, YopJ, YopM, YopO, YopP, YopT or *Shigella flexneri* OspF, IpgD, IpgB1 or *Salmonella enterica* SopE, SopB, SptP or *P. aeruginosa* ExoS, ExoT, ExoU, ExoY or *E. coli* Tir, Map, EspF, EspG, EspH, EspZ. T4SS effector proteins which are virulent towards eukaryotic cells refers to proteins as *Legionella pneumophila* LidA, SidC, SidG, SidH, SdhA, SidJ, SdjA, SdeA, SdeA, SdeC, LepA, LepB, WipA, WipB, YlfA, YlfB, VipA, VipF, VipD, VpdA, VpdB, DrrA, LegL3, LegL5, LegL7, LegLC4, LegLC8, LegC5, LegG2, Ceg10, Ceg23, Ceg29 or *Bartonella henselae* BepA, BepB, BepC, BepD, BepE, BepF BepG or *Agrobacterium tumefaciens* VirD2, VirE2, VirE3, VirF or *H. pylori* CagA or *Bordetella pertussis* pertussis toxin. T6SS effector proteins which are virulent towards eukaryotic cells refers to proteins as *Vibrio cholerae* VgrG proteins (as VgrG1).

The term "T3SS effector protein which is virulent toward eukaryotic cells" or "bacterial T3SS effector protein which is virulent toward eukaryotic cells" as used herein refers to proteins which are naturally injected by T3S systems into the cytosol of eukaryotic cells and to proteins which are naturally secreted by T3S systems that might e.g form the translocation pore into the eukaryotic membrane, which are virulence factors toward eukaryotic cells i.e. to proteins which paralyze or reprogram the eukaryotic cell to the benefit of the pathogen.

Effectors display a large repertoire of biochemical activities and modulate the function of crucial host regulatory mechanisms such as e.g. phagocytosis and the actin cytoskeleton, inflammatory signaling, apoptosis, endocytosis or secretory pathways[5,6] and include AvrA, AvrB, AvrBs2, AvrBS3, AvrBsT, AvrD, AvrD1, AvrPphB, AvrPphC, AvrPphEPto,

AvrPpiB_{Pto}, AvrP_{to}, AvrP_{to}B, AvrRpm1, AvrRpt2, AvrXv3, CigR, EspF, EspG, EspH, EspZ, ExoS, ExoT, GogB, GtgA, GtgE, GALA family of proteins, HopAB2, HopAO1, HopI1, HopM1, HopN1, HopP_{to}D2, HopP_{to}E, HopP_{to}F, HopP_{to}N, HopU1, HsvB, IcsB, IpaA, IpaH, IpaH7.8, IpaH9.8, IpgB1, IpgB2, IpgD, LcrV, Map, OspC1, OspE2, OspF, OspG, 5 OspI, PipB, PipB2, PopB, PopP2, PthXo1, PthXo6, PthXo7, SifA, SifB, SipA/SspA., SlrP, SopA, SopB/SigD, SopD, SopE, SopE2, SpiC/SsaB, SptP, SpvB, SpvC, SrfH, SrfJ, Sse, SseB, SseC, SseD, SseF, SseG, SseI/SrfH, SseJ, SseK1, SseK2, SseK3, SseL, SspH1, SspH2, SteA, SteB, SteC, SteD, SteE, TccP2, Tir, VirA, VirPphA, VopF, XopD, YopE, YopH, YopJ, YopM, YopO, YopP, YopT, YpkA.

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T3SS effector genes of *Yersinia* which are virulent to a eukaryotic cell and can be deleted/mutated from e.g. *Y. enterocolitica* are YopE, YopH, YopM, YopO, YopP (also named YopJ), and YopT [7]. The respective effector genes which are virulent to a eukaryotic cell can be deleted/mutated from *Shigella flexneri* (e.g. OspF, IpgD, IpgB1), *Salmonella enterica* (e.g. SopE, SopB, SptP), *P. aeruginosa* (e.g. ExoS, ExoT, ExoU, ExoY) or *E. coli* (e.g. Tir, Map, EspF, EspG, EspH, EspZ). The nucleic acid sequences of these genes are available to those skilled in the art, e.g., in the Genebank Database (yopH, yopO, yopE, yopP, yopM, yopT from NC_002120 GI:10955536; *S. flexneri* effector proteins from AF386526.1 GI:18462515; *S. enterica* effectors from NC_016810.1 GI:378697983 or FQ312003.1 15 GI:301156631; *P. aeruginosa* effectors from AE004091.2 GI:110227054 or CP000438.1 GI:115583796 and *E. coli* effector proteins from NC_011601.1 GI:215485161).

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For the purpose of the present invention, genes are denoted by letters of lower case and italicised to be distinguished from proteins. In case the genes (denoted by letters of lower case and italicised) are following a bacterial species name (like *E. coli*), they refer to a mutation of 25 the corresponding gene in the corresponding bacterial species. For example, YopE refers to the effector protein encoded by the *yopE* gene. *Y. enterocolitica yopE* represents a *Y. enterocolitica* having a mutation in the *yopE* gene.

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As used herein, the terms “polypeptide”, “peptide”, “protein”, “polypeptidic” and “peptidic” 30 are used interchangeably to designate a series of amino acid residues connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. Preferred are proteins which have an amino acid sequence comprising at least 10 amino acids, more

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preferably at least 20 amino acids.

According to the present invention, "a heterologous protein" includes naturally occurring proteins or parts thereof and also includes artificially engineered proteins or parts thereof. As used herein, the term "heterologous protein" refers to a protein or a part thereof other than the T3SS effector protein or N-terminal fragment thereof to which it can be fused. In particular the heterologous protein as used herein refers to a protein or a part thereof, which do not belong to the proteome, i.e. the entire natural protein complement of the specific recombinant virulence attenuated Gram-negative bacterial strain provided and used by the invention, e.g. which do not belong to the proteome, i.e. the entire natural protein complement of a specific bacterial strain of the genera *Yersinia*, *Escherichia*, *Salmonella* or *Pseudomonas*. Usually the heterologous protein is of animal origin including human origin. Preferably the heterologous protein is a human protein. More preferably the heterologous protein is selected from the group consisting of proteins involved in apoptosis or apoptosis regulation, cell cycle regulators, ankyrin repeat proteins, cell signaling proteins, reporter proteins, transcription factors, proteases, small GTPases, GPCR related proteins, nanobody fusion constructs and nanobodies, bacterial T3SS effectors, bacterial T4SS effectors and viral proteins. Particular preferably the heterologous protein is selected from the group consisting of proteins involved in apoptosis or apoptosis regulation, cell cycle regulators, ankyrin repeat proteins, reporter proteins, small GTPases, GPCR related proteins, nanobody fusion constructs, bacterial T3SS effectors, bacterial T4SS effectors and viral proteins. Even more particular preferred are heterologous proteins selected from the group consisting of proteins involved in apoptosis or apoptosis regulation, cell cycle regulators, and ankyrin repeat proteins. Most preferred are proteins involved in apoptosis or apoptosis regulation, like animal, preferably human heterologous proteins involved in apoptosis or apoptosis regulation

In some embodiments the vector of the Gram-negative bacterial strain of the present invention comprises two second DNA sequences encoding the identical or two different heterologous proteins fused independently from each other in frame to the 3' end of said first DNA sequence.

In some embodiments the vector of the Gram-negative bacterial strain of the present invention comprises three second DNA sequences encoding the identical or three different heterologous proteins fused independently from each other in frame to the 3' end of said first DNA sequence.

The heterologous protein expressed by the recombinant virulence attenuated Gram-negative bacterial strain has usually a molecular weight of between 1 and 150kD, preferably between 1 and 120kD, more preferably between 1 and 100kDa, most preferably between 15 and 100kDa.

- 5 In some embodiments the vector of the Gram-negative bacterial strain of the present invention comprises repeated domains of a heterologous protein or two or more domains of different heterologous proteins fused in frame to the 3' end of said first DNA sequence.

10 The term "heterologous proteins which belong to the same functional class of proteins" as used herein refers to heterologous proteins which have the same function e.g. heterologous proteins having enzymatic activity, heterologous proteins which act in the same pathway such as e.g. cell cycle regulation, or share a common specific feature as e.g. belonging to the same class of bacterial effector proteins. Functional classes of proteins are e.g. proteins involved in apoptosis or apoptosis regulation, proteins which act as cell cycle regulators, ankyrin repeat
15 proteins, cell signaling proteins, reporter proteins, transcription factors, proteases, small GTPases, GPCR related proteins, nanobody fusion constructs and nanobodies, bacterial T3SS effectors, bacterial T4SS effectors or viral proteins which act jointly in the biological process of establishing virulence to eukaryotic cells.

- 20 According to the present invention, "a domain of a heterologous protein" includes domains of naturally occurring proteins and also includes domains of artificially engineered proteins. As used herein, the term "domain of a heterologous protein" refers to a domain of a heterologous protein other than a domain of a T3SS effector protein or a domain other than a domain comprising the N-terminal fragment thereof to which it can be fused to achieve a fusion
25 protein. In particular the domain of a heterologous protein as used herein refers to a domain of a heterologous protein, which do not belong to the proteome, i.e. the entire natural protein complement of the specific recombinant Gram-negative bacterial strain provided and used by the invention, e.g. which do not belong to the proteome, i.e. the entire natural protein complement of a specific bacterial strain of the genera *Yersinia*, *Escherichia*, *Salmonella* or
30 *Pseudomonas*. Usually the domain of the heterologous protein is of animal origin including human origin. Preferably the domain of the heterologous protein is a domain of a human protein. More preferably the domain of the heterologous protein is a domain of a protein selected from the group consisting of proteins involved in apoptosis or apoptosis regulation,

- cell cycle regulators, ankyrin repeat proteins, cell signaling proteins, reporter proteins, transcription factors, proteases, small GTPases, GPCR related proteins, nanobody fusion constructs and nanobodies, bacterial T3SS effectors, bacterial T4SS effectors and viral proteins. Particular preferably the domain of the heterologous protein is a domain of a protein selected from the group consisting of proteins involved in apoptosis or apoptosis regulation, cell cycle regulators, ankyrin repeat proteins, reporter proteins, small GTPases, GPCR related proteins, nanobody fusion constructs, bacterial T3SS effectors, bacterial T4SS effectors and viral proteins. Even more particular preferred are domains of heterologous proteins selected from the group consisting of proteins involved in apoptosis or apoptosis regulation, cell cycle regulators, and ankyrin repeat proteins. Most preferred are domains of proteins involved in apoptosis or apoptosis regulation, like animal proteins involved in apoptosis or apoptosis regulation, preferably domains of human heterologous proteins involved in apoptosis or apoptosis regulation.
- 15 The term "repeated domains of a heterologous protein" as used herein refers to a fusion protein consisting of several repetitions of a domain of a heterologous protein, where these domains might either be directly fused to each other or where a variable linker e.g. a linker between 1 and 30, preferably between 2 and 15, more preferably between 3 and 10 amino acids might be introduced in between the domains. Preferably repeated identical domains or repeated domains which have an amino acid sequence identity of more than 80 %, usually more than 85 %, preferably more than 90 %, even more preferably more than 95 %, in particular more than 96%, more particular more than 97%, even more particular more than 98 %, most particular more than 99% are used. Also preferred are identical domains which have an amino acid identity of 100 %. Preferably two repeated domains, more preferably two repeated identical domains or two repeated domains having an amino acid sequence identity of more than 90 %, preferably more than 95%, most preferably 100 % are comprised by the fusion protein as referred herein. More than two, e.g. three, four, five or six repeated domains are also contemplated by the present invention.
- 30 The term "two or more domains of different heterologous proteins" as used herein refers to a fusion protein consisting of one or several repetitions of at least two domains of different heterologous proteins e.g. at least two domains of heterologous proteins having an amino acid sequence identity of 80% or less, preferably 60% or less, more preferably 40% or less, where

these different domains might either be directly fused to each other or where a variable linker e.g. a linker between 1 and 30, preferably between 2 and 15, more preferably between 3 and 10 amino acids might be introduced in between the domains. Preferably two domains of different heterologous proteins are comprised by the fusion protein as referred herein. More than two, e.g. three, four, five or six domains of different heterologous proteins are also contemplated by the present invention.

The domain of a heterologous protein expressed by the recombinant Gram-negative bacterial strain has usually a molecular weight of between 1-50 kDa, preferably between 1-30 kDa, more preferably between 1-20 kDa, most preferably between 1-10 kDa.

According to the present invention " proteins involved in apoptosis or apoptosis regulation" include, but are not limited to, Bad, Bcl2, Bak, Bmt, Bax, Puma, Noxa, Bim, Bcl-xL, Apaf1, Caspase 9, Caspase 3, Caspase 6, Caspase 7, Caspase 10, DFFA, DFFB, ROCK1, APP, CAD, ICAD, CAD, EndoG, AIF, HtrA2, Smac/Diablo, Arts, ATM, ATR, Bok/Mtd, Bmf, Mcl-1(S), IAP family, LC8, PP2B, 14-3-3 proteins, PKA, PKC, PI3K, Erk1/2, p90RSK, TRAF2, TRADD, FADD, Daxx, Caspase8, Caspase2, RIP, RAIDD, MKK7, JNK, FLIPs, FKHR, GSK3, CDKs and their inhibitors like the INK4-family (p16(Ink4a), p15(Ink4b), p18(Ink4c), p19(Ink4d)), and the Cip1/Waf1/Kip1-2-family (p21(Cip1/Waf1), p27(Kip1), p57(Kip2)). Preferably Bad, Bmt, Bcl2, Bak, Bax, Puma, Noxa, Bim, Bcl-xL, Caspase9, Caspase3, Caspase6, Caspase7, Smac/Diablo, Bok/Mtd, Bmf, Mcl-1(S), LC8, PP2B, TRADD, Daxx, Caspase8, Caspase2, RIP, RAIDD, FKHR, CDKs and their inhibitors like the INK4-family (p16(Ink4a), p15(Ink4b), p18(Ink4c), p19(Ink4d)), most preferably BIM, Bid, truncated Bid, FADD, Caspase 3 (and subunits thereof), Bax, Bad, Akt, CDKs and their inhibitors like the INK4-family (p16(Ink4a), p15(Ink4b), p18(Ink4c), p19(Ink4d)) are used [8-10]. Additionally proteins involved in apoptosis or apoptosis regulation include DIVA, Bcl-Xs, Nbk/Bik, Hrk/Dp5, Bid and tBid, Egl-1, Bcl-Gs, Cytochrome C, Beclin, CED-13, BNIP1, BNIP3, Bcl-B, Bcl-W, Ced-9, A1, NR13, Bfl-1, Caspase 1, Caspase 2, Caspase 4, Caspase 5, Caspase 8. Proteins involved in apoptosis or apoptosis regulation are selected from the group consisting of pro-apoptotic proteins, anti-apoptotic proteins, inhibitors of apoptosis-prevention pathways and inhibitors of pro-survival signalling or pathways. Pro-apoptotic proteins comprise proteins selected from the group consisting of Bax, Bak, Diva, Bcl-Xs, Nbk/Bik, Hrk/Dp5, Bmf, Noxa, Puma, Bim, Bad, Bid and tBid, Bok, Apaf1, Smac/Diablo, BNIP1, BNIP3, Bcl-

Gs, Beclin 1, Egl-1 and CED-13, Cytochrome C, FADD, the Caspase family, and CDKs and their inhibitors like the INK4-family (p16(Ink4a), p15(Ink4b), p18(Ink4c), p19(Ink4d)) or selected from the group consisting of Bax, Bak, Diva, Bcl-Xs, Nbk/Bik, Hrk/Dp5, Bmf, Noxa, Puma, Bim, Bad, Bid and tBid, Bok, Egl-1, Apaf1, Smac/Diablo, BNIP1, BNIP3, Bcl-Gs, Beclin 1, Egl-1 and CED-13, Cytochrome C, FADD, and the Caspase family. Preferred are Bax, Bak, Diva, Bcl-Xs, Nbk/Bik, Hrk/Dp5, Bmf, Noxa, Puma, Bim, Bad, Bid and tBid, Bok, Egl-1, Apaf1, BNIP1, BNIP3, Bcl-Gs, Beclin 1, Egl-1 and CED-13, Smac/Diablo, FADD, the Caspase family, CDKs and their inhibitors like the INK4-family (p16(Ink4a), p15(Ink4b), p18(Ink4c), p19(Ink4d)). Equally preferred are Bax, Bak, Diva, Bcl-Xs, Nbk/Bik, Hrk/Dp5, Bmf, Noxa, Puma, Bim, Bad, Bid and tBid, Bok, Apaf1, BNIP1, BNIP3, Bcl-Gs, Beclin 1, Egl-1 and CED-13, Smac/Diablo, FADD, the Caspase family.

Anti-apoptotic proteins comprise proteins selected from the group consisting of Bcl-2, Bcl-Xl, Bcl-B, Bcl-W, Mcl-1, Ced-9, A1, NR13, IAP family and Bfl-1. Preferred are Bcl-2, Bcl-Xl, Bcl-B, Bcl-W, Mcl-1, Ced-9, A1, NR13 and Bfl-1.

Inhibitors of apoptosis-prevention pathways comprise proteins selected from the group consisting of Bad, Noxa and Cdc25A. Preferred are Bad and Noxa.

Inhibitors of pro-survival signalling or pathways comprise proteins selected from the group consisting of PTEN, ROCK, PP2A, PHLPP, JNK, p38. Preferred are PTEN, ROCK, PP2A and PHLPP.

In some embodiments the heterologous proteins involved in apoptosis or apoptosis regulation are selected from the group consisting of BH3-only proteins, caspases and intracellular signalling proteins of death receptor control of apoptosis. BH3-only proteins are preferred. BH3-only proteins comprise proteins selected from the group consisting of Bad, BIM, Bid and tBid, Puma, Bik/Nbk, Bod, Hrk/Dp5, BNIP1, BNIP3, Bmf, Noxa, Mcl-1, Bcl-Gs, Beclin 1, Egl-1 and CED-13. Preferred are Bad, BIM, Bid and tBid, in particular tBid.

Caspases comprise proteins selected from the group consisting of Caspase 1, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Caspase 10. Preferred are Caspase 3, Caspase 8 and Caspase 9.

Intracellular signalling proteins of death receptor control of apoptosis comprise proteins selected from the group consisting of FADD, TRADD, ASC, BAP31, GULP1/CED-6, CIDEA, MFG-E8, CIDEA, RIPK1/RIP1, CRADD, RIPK3/RIP3, Crk, SHB, CrkL, DAXX, the 14-3-3 family, FLIP, DFF40 and 45, PEA-15, SODD. Preferred are FADD and TRADD.

In some embodiments two heterologous proteins involved in apoptosis or apoptosis regulation are comprised by the Gram-negative bacterial strain and/or the vector of the present invention, wherein one protein is a pro-apoptotic protein and the other protein is an inhibitor of

apoptosis-prevention pathways or wherein one protein is a pro-apoptotic protein and the other protein is an inhibitor of pro-survival signalling or pathways.

Pro-apoptotic proteins encompassed by the present invention have usually an alpha helical structure, preferably a hydrophobic helix surrounded by amphipathic helices and usually comprise at least one of BH1, BH2, BH3 or BH4 domains, preferably comprise at least one BH3 domain. Usually pro-apoptotic proteins encompassed by the present invention have no enzymatic activity.

Anti-apoptotic proteins encompassed by the present invention have usually an alpha helical structure, preferably a hydrophobic helix surrounded by amphipathic helices and comprises a combination of different BH1, BH2, BH3 and BH4 domains, preferably a combination of different BH1, BH2, BH3 and BH4 domains wherein a BH1 and a BH2 domain is present, more preferably BH4-BH3-BH1-BH2, BH1-BH2, BH4-BH1-BH2 or BH3-BH1-BH2 (from N- to the C-terminus). Additionally, proteins containing at least one BIR domain are also encompassed.

Inhibitors of apoptosis-prevention pathways encompassed by the present invention have usually an alpha helical structure, preferably a hydrophobic helix surrounded by amphipathic helices and usually comprise one BH3 domain.

BH1, BH2, BH3 or BH4 domains are each usually between about 5 to about 50 amino acids in length. Thus in some embodiments the heterologous proteins involved in apoptosis or apoptosis regulation is selected from the group consisting of heterologous proteins involved in apoptosis or apoptosis regulation which are about 5 to about 200, preferably about 5 to about 150, more preferably about 5 to about 100, most preferably about 5 to about 50, in particular about 5 to about 25 amino acids in length.

In some embodiments the Gram-negative bacterial strain of the present invention is transformed with two domains of a heterologous proteins involved in apoptosis or apoptosis regulation, preferably two repeated, more preferably two identical repeated domains of a protein involved in apoptosis or apoptosis regulation or two domains of different proteins involved in apoptosis or apoptosis regulation, most preferably two identical repeated domains

of a protein involved in apoptosis or apoptosis regulation. In some embodiments the Gram-negative bacterial strain of the present invention is transformed with two domains of a heterologous proteins involved in apoptosis or apoptosis regulation, wherein one is a domain of a pro-apoptotic protein and the other is a domain of a protein which is an inhibitor of apoptosis-prevention pathways or wherein one is a domain of a pro-apoptotic protein and the other domain is a domain of a protein which is an inhibitor of pro-survival signalling or pathways.

A particular preferred domain is the BH3 domain of apoptosis inducer tBID, more particular the BH3 domain comprising a sequence selected from the group consisting of SEQ ID NOs: 217, 218, 219 and 220, preferably SEQ ID NO: 219 or SEQ ID NO: 220.

Equally preferred is the BH3 domain of apoptosis regulator BAX, more particular the BAX domain comprising a sequence selected from the group consisting of SEQ ID NOs: 221, 222, 223 and 224, preferably SEQ ID NO: 223 or SEQ ID NO: 224. The human and murine sequences are given in SEQ ID NOs 217-224, but tBID and BAX BH3 domains of all other species are equally included.

In some embodiments the repeated domains of the heterologous proteins are the BH3 domain, preferably repeated BH3 domains of apoptosis inducer tBID, more preferably repeated BH3 domains of the apoptosis inducer tBID comprised by SEQ ID NO: 219 or SEQ ID NO: 220 or SEQ ID NO: 202, even more preferably two repeated BH3 domains of apoptosis inducer tBID, most preferably two repeated BH3 domains of the apoptosis inducer tBID comprised by SEQ ID NO: 219 or SEQ ID NO: 220 or SEQ ID NO: 202, in particular two repeated BH3 domains of apoptosis inducer tBID comprised by the sequence of SEQ ID NO: 202. Thus in a preferred embodiment the Gram-negative bacterial strain and/or the vector of the present invention comprises a second DNA sequence encoding two repeated domains of a BH3 domain, more preferably two repeated BH3 domains of apoptosis inducer tBID. The two repeated domains may be connected by a linker of 1-30 amino acid length, preferably 2-15 amino acids, more preferred 3-10 amino acids long.

In some embodiments the two or more domains of different heterologous proteins are domains of heterologous proteins which belong to the same functional class of proteins, preferably the different heterologous proteins of the two or more domains are different heterologous proteins from the class of proteins involved in apoptosis or apoptosis regulation. In a preferred embodiment the two or more domains of different heterologous proteins are the

BH3 domain of apoptosis inducer tBID and the BH3 domain of apoptosis regulator BAX, in particular the fused BH3 domains comprised by the sequence of SEQ ID NO: 203 and 211. The two domains of different heterologous proteins may be connected by a linker of 1-30 amino acid length, preferably 2-15 amino acids, more preferred 3-10 amino acids long.

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In some embodiments the heterologous proteins is a pro-drug converting enzyme. In these embodiments the recombinant virulence attenuated Gram-negative bacterial strain expresses, preferably expresses and secretes a pro-drug converting enzyme. A prodrug converting enzyme as referred herein comprises enzymes converting non-toxic prodrugs into a toxic

10 drug, preferably enzymes selected from the group consisting of cytosine deaminase, purine nucleoside phosphorylase, thymidine kinase, beta-galactosidase, carboxylesterases, nitroreductase, carboxypeptidases and beta-glucuronidases., more preferably enzymes selected from the group consisting of cytosine deaminase, purine nucleoside phosphorylase, thymidine kinase, and beta-galactosidase.

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The term "protease cleavage site" as used herein refers to a specific amino acid motif within an amino acid sequence e.g. within an amino acid sequence of a protein or a fusion protein, which is cleaved by a specific protease, which recognizes the amino acid motif. For review see [11]. Examples of protease cleavage sites are amino acid motifs, which are cleaved by a

20 protease selected from the group consisting of enterokinase (light chain), enteropeptidase, prescission protease, human rhinovirus protease (HRV 3C), TEV protease, TVMV protease, FactorXa protease and thrombin.

The following amino acid motif is recognized by the respective protease:

- Asp-Asp-Asp-Asp-Lys: Enterokinase (light chain) / Enteropeptidase
- 25 - Leu-Glu-Val-Leu-Phe-Gln/Gly-Pro: PreScission Protease/human Rhinovirus protease (HRV 3C)
- Glu-Asn-Leu-Tyr-Phe-Gln-Ser and modified motifs based on the Glu-X-X-Tyr-X-Gln-Gly/Ser (where X is any amino acid) recognized by TEV protease (tobacco etch virus)
- Glu-Thr-Val-Arg-Phe-Gln-Ser: TVMV protease
- 30 - Ile-(Glu or Asp)-Gly-Arg: FactorXa protease
- Leu-Val-Pro-Arg/Gly-Ser: Thrombin.

Encompassed by the protease cleavage sites as used herein is ubiquitin. Thus in some preferred embodiments ubiquitin is used as protease cleavage site, i.e. the third DNA

sequence encodes ubiquitin as protease cleavage site, which can be cleaved by a specific ubiquitin processing proteases at the N-terminal site, e.g. which can be cleaved by a specific ubiquitin processing proteases called Deubiquitinating enzymes at the N-terminal site endogeneously in the cell where the fusion protein has been delivered to. Ubiquitin is processed at its C-terminus by a group of endogenous Ubiquitin-specific C-terminal proteases (Deubiquitinating enzymes, DUBs). The cleavage of Ubiquitin by DUBs is supposed to happen at the very C-terminus of Ubiquitin (after G76).

An "individual," "subject" or "patient" is a vertebrate. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, primates (including human and non-human primates) and rodents (e.g., mice and rats). In preferred embodiments, a subject is a human.

The term "mutation" is used herein as a general term and includes changes of both single base pair and multiple base pairs. Such mutations may include substitutions, frame-shift mutations, deletions, insertions and truncations.

The term "nuclear localization signal" as used herein refers to an amino acid sequence that marks a protein for import into the nucleus of a eukaryotic cell and includes preferably a viral nuclear localization signal such as the SV40 large T-antigen derived NLS (PPKKKRKV).

The term "multiple cloning site" as used herein refers to a short DNA sequence containing several restriction sites for cleavage by restriction endonucleases such as AclI, HindIII, SspI, MluCI, Tsp509I, PciI, AgeI, BspMI, BfuAI, SexAI, MluI, BceAI, HpyCH4IV, HpyCH4III, BaeI, BsaXI, AflIII, SpeI, BsrI, BmrI, BglII, AfeI, AluI, StuI, ScaI, ClaI, BspDI, PI-SceI, NsiI, AseI, SwaI, CspCI, MfeI, BssSI, BmgBI, PmlI, DraIII, AleI, EcoP15I, PvuII, AlwNI, BtsIMutI, TspRI, NdeI, NlaIII, CviAII, FatI, MslI, FspEI, XcmI, BstXI, PflMI, BccI, NcoI, BseYI, FauI, SmaI, XmaI, TspMI, Nt.CviPII, LpnPI, AciI, SacII, BsrBI, MspI, HpaII, ScrFI, BssKI, StyD4I, BsaJI, BslI, BtgI, NciI, AvrII, MnlI, BbvCI, Nb.BbvCI, Nt.BbvCI, SbfI, Bpu10I, Bsu36I, EcoNI, HpyAV, BstNI, PspGI, StyI, BcgI, PvuI, BstUI, EagI, RsrII, BsiEI, BsiWI, BsmBI, Hpy99I, MspAII, MspJI, SgrAI, BfaI, BspCNI, XhoI, EarI, AcuI, PstI, BpmlI, DdeI, SfcI, AflII, BpuEI, SmlI, AvaI, BsoBI, MboII, BbsI, XmnI, BsmI, Nb.BsmI, EcoRI, HgaI, AatII, ZraI, Tth111I, PflFI, PshAI, AhdI, DrdI, Eco53kI, SacI, BseRI, PleI, Nt.BstNBI,

MlyI, HinfI, EcoRV, MboI, Sau3AI, DpnII BfuCI, DpnI, BsaBI, TfiI, BsrDI, Nb.BsrDI, BbvI, BtsI, Nb.BtsI, BstAPI, SfaNI, SphI, NmeAIII, NaeI, NgoMIV, BglI, AsiSI, BtgZI, HinPII, HhaI, BssHII, NotI, Fnu4HI, Cac8I, MwoI, NheI, BmtI, SapI, BspQI, Nt.BspQI, BlpI, TseI, ApeKI, Bsp1286I, AlwI, Nt.AlwI, BamHI, FokI, BtsCI, HaeIII, PhoI, FseI, SfiI, NarI, KasI, SfoI, PluTI, AscI, EciI, BsmFI, ApaI, PspOMI, Sau96I, NlaIV, KpnI, Acc65I, BsaI, HphI, BstEII, AvaII, BanI, BaeGI, BsaHI, BanII, RsaI, CviQI, BstZ17I, BciVI, SalI, Nt.BsmAI, BsmAI, BcoDI, ApaLI, BsgI, AccI, Hpy166II, Tsp45I, HpaI, PmeI, HincII, BsiHKAII, ApoI, NspI, BsrFI, BstYI, HaeII, CviKI-1, EcoO109I, PpuMI, I-CeuI, SnaBI, I-SceI, BspHI, BspEI, MmeI, TaqαI, NruI, Hpy188I, Hpy188III, XbaI, BclI, HpyCH4V, FspI, PI-PspI, MscI, BsrGI, MseI, PacI, PsiI, BstBI, DraI, PspXI, BsaWI, BsaAI, EaeI, preferably XhoI, XbaI, HindIII, NcoI, NotI, EcoRI, EcoRV, BamHI, NheI, SacI, SalI, BstBI. The term “multiple cloning site” as used herein further refers to a short DNA sequence used for recombination events as e.g in Gateway cloning strategy or for methods such as Gibson assembly or topo cloning.

The term “wild type strain” or “wild type of the Gram-negative bacterial strain” as used herein refers to a naturally occurring variant or a naturally occurring variant containing genetic modifications allowing the use of vectors, such as deletion mutations in restriction endonucleases or antibiotic resistance genes. These strains contain chromosomal DNA as well as in some cases (e.g. *Y. enterocolitica*, *S. flexneri*) an unmodified virulence plasmid.

The term “*Yersinia* wild type strain” as used herein refers to a naturally occurring variant (as *Y. enterocolitica* E40) or a naturally occurring variant containing genetic modifications allowing the use of vectors, such as deletion mutations in restriction endonucleases or antibiotic resistance genes (as *Y. enterocolitica* MRS40, the Ampicillin sensitive derivate of *Y. enterocolitica* E40) These strains contain chromosomal DNA as well as an unmodified virulence plasmid (called pYV).

Y. enterocolitica subspecies *paleartica* refers to the low-pathogenic *Y. enterocolitica* strains, which are in contrast to the higher virulent strains of subspecies *enterocolitica* [12,13]. *Y. enterocolitica* subsp. *paleartica* lack, in comparison to *Y. enterocolitica* subsp. *enterocolitica*, a high-pathogenicity island (HPI). This HPI encodes the iron siderophore called yersiniabactin [14]. The lack of yersiniabactin in *Y. enterocolitica* subsp. *paleartica* renders this subspecies less pathogenic and dependent on induced systemic accessible iron for persistent infection in e.g. liver or spleen [14]. Iron can be made accessible for the bacteria in

an individual e.g by pretreatment with deferoxamine, an iron chelator used to treat iron overload in patients [15].

The term “comprise” is generally used in the sense of include, that is to say permitting the presence of one or more features or components.

The term "about" refers to a range of values $\pm 10\%$ of a specified value. For example, the phrase "about 200" includes $\pm 10\%$ of 200, or from 180 to 220.

- 10 In one aspect the present invention provides a recombinant virulence attenuated Gram-negative bacterial strain transformed with a vector which comprises in the 5' to 3' direction:
a promoter;
a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;
15 a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence,
for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, the method comprising administering to the subject said recombinant virulence attenuated
20 Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is administered in an amount that is sufficient to treat the subject.

- In a further aspect, the present invention provides a recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative
25 bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one bacterial protein which is part of a secretion system machinery, for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, the method comprising
30 administering to the subject said recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is administered in an amount that is sufficient to treat the subject. Preferred is a recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence

attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells, for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor, the method comprising administering to the subject said recombinant virulence attenuated Gram-negative bacterial strain, wherein the recombinant virulence attenuated Gram-negative bacterial strain is administered in an amount that is sufficient to treat the subject.

In one embodiment of the present invention the recombinant virulence attenuated Gram-negative bacterial strain which is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or which is deficient in the production of at least one bacterial protein which is part of a secretion system machinery is transformed with a vector which comprises in the 5' to 3' direction:

a promoter;

a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence.

In one embodiment of the present invention the recombinant virulence attenuated Gram-negative bacterial strain or the recombinant virulence attenuated Gram-negative bacterial strain which is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or which is deficient in the production of at least one bacterial protein which is part of a secretion system machinery, is transformed with a vector which comprises in the 5' to 3' direction:

a first DNA sequence encoding a delivery signal or a fragment thereof from a bacterial effector protein;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence. Preferably the DNA sequence encoding a heterologous protein is flanked on its 3' end by a DNA sequence homologous to the DNA sequence of the chromosome or of the endogenous virulence plasmid at the 3' end of the delivery signal from a bacterial effector protein or to a fragment thereof. More preferably, this DNA sequence flanking the homologous protein on its 3' end is homologous to the DNA sequence and is lying within 10kbp on the

chromosome or on an endogenous virulence plasmid at the 3' end of the delivery signal from a bacterial effector protein or to a fragment thereof. In particular, this nucleotide sequence flanking the homologous protein on its 3' end is homologous to the DNA sequence and is within the same operon on the chromosome or on an endogenous virulence plasmid as the delivery signal from a bacterial effector protein or a fragment thereof. In this embodiment, transformation is usually performed so that the fused first and the second DNA sequence are inserted by homologous recombination on an endogenous virulence plasmid or a chromosome, preferably on an endogenous virulence plasmid, of the recombinant virulence attenuated Gram-negative bacterial strain, and the fused first and the second DNA sequence is operably linked to a promoter of an endogenous virulence plasmid or of a chromosome e.g. of a chromosomal pathogenicity island. Preferably the fused first and the second DNA sequence is operably linked to a promoter of an endogenous virulence plasmid. In this embodiment the first DNA sequence comprises a delivery signal or fragment thereof from a bacterial effector protein, preferably a fragment thereof, which provides for homologous recombination at the homologous site at the chromosome or at an endogenous virulence plasmid to result in the second DNA sequence be placed in frame to the 3' end of the chromosomal or endogenous virulence plasmid delivery signal which is operatively linked to the endogenous promoter.

20 In a further embodiment of the present invention the recombinant virulence attenuated Gram-negative bacterial strain or the recombinant virulence attenuated Gram-negative bacterial strain which is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or which is deficient in the production of at least one bacterial protein which is part of a secretion system machinery, is transformed with a nucleotide molecule, preferably a DNA nucleotide molecule, comprising a nucleotide sequence encoding a heterologous protein and a nucleotide sequence which is homologous or identical to a nucleotide sequence encoding a delivery signal from a bacterial effector protein or which is homologous or identical to a nucleotide sequence encoding a fragment of a delivery signal from a bacterial effector protein, wherein the delivery signal from a bacterial effector protein is encoded on the chromosome or on an endogenous virulence plasmid of the recombinant virulence attenuated Gram-negative bacterial strain. Preferably the nucleotide sequence which is homologous or identical to a nucleotide sequence of a delivery signal from a bacterial effector protein or to a fragment thereof is located on the 5' end of the nucleotide sequence

encoding a heterologous protein. More preferably the nucleotide sequence encoding a heterologous protein is flanked on its 3' end by a nucleotide sequence homologous to the DNA sequence of the chromosome or of the endogenous virulence plasmid at the 3' end of the delivery signal from a bacterial effector protein or to a fragment thereof. Even more preferably, this nucleotide sequence flanking the homologous protein on its 3' end is homologous to the DNA sequence lying within 10kbp on the chromosome or on an endogenous virulence plasmid at the 3' end of the delivery signal from a bacterial effector protein or to a fragment thereof. In particular, this nucleotide sequence flanking the homologous protein on its 3' end is homologous to the DNA sequence is within the same operon on the chromosome or on an endogenous virulence plasmid as the delivery signal from a bacterial effector protein or a fragment thereof. In this embodiment, transformation is usually performed so that the nucleotide sequence encoding a heterologous protein is inserted on an endogenous virulence plasmid or a chromosome of the recombinant virulence attenuated Gram-negative bacterial strain at the 3' end of a delivery signal from a bacterial effector protein encoded by the chromosome or the endogenous virulence plasmid, wherein the heterologous protein fused to the delivery signal is expressed and secreted.

In case the recombinant virulence attenuated Gram-negative bacterial strain is a *Yersinia* strain the endogenous virulence plasmid for insertion is pYV (plasmid of *Yersinia* Virulence).

In case the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain, the endogenous location for insertion is one of the gene clusters called *Spil* or *SpilI* (for *Salmonella* pathogenicity island), a position where an effector protein is elsewhere encoded or alternatively one of the *Salmonella* virulence plasmids (SVPs).

Preferably the first and the second DNA sequence or the nucleotide molecule are inserted on an endogenous virulence plasmid at the native site of a bacterial effector protein e.g. at the native site of a virulence factor, preferably in case the recombinant virulence attenuated Gram-negative bacterial strain is a *Yersinia* strain, at the native site of YopE or another Yop (YopH, YopO, YopP, YopM, YopT), preferably at the native site of YopE or in case the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain at the native site of an effector protein encoded within *Spil*, *SpilI* or encoded elsewhere, preferably at the native site of an effector protein encoded within *Spil* or *SpilI*, more preferably at the native site of SopE or SteA. Preferably the first and the second DNA sequence or the

nucleotide molecule are operably linked to a native promoter of a bacterial effector protein present on an endogenous virulence plasmid e.g. in case the recombinant virulence attenuated Gram-negative bacterial strain is a *Yersinia* strain to a native promoter from a *Yersinia* virulon gene as outlined below, more preferably to the native YopE promoter or another Yop (YopH, YopO, YopP, YopM, YopT) promoter, preferably to the native YopE promoter or in case the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain to a native promoter from *SpiI* or *SpiII* pathogenicity island or from an effector protein elsewhere encoded as outlined below, more preferably to the native SopE, InvB or SteA promoter.

In one embodiment of the present invention the recombinant virulence attenuated Gram-negative bacterial strain is selected from the group consisting of the genera *Yersinia*, *Escherichia*, *Salmonella* and *Pseudomonas*. In one embodiment the recombinant virulence attenuated Gram-negative bacterial strain is selected from the group consisting of the genera *Yersinia* and *Salmonella*. Preferably the recombinant virulence attenuated Gram-negative bacterial strain is a *Yersinia* strain, more preferably a *Yersinia enterocolitica* strain. Most preferred is *Yersinia enterocolitica* E40 (O:9, biotype 2) [16] or Ampicilline sensitive derivatives thereof as *Y. enterocolitica* MRS40 (also named *Y. enterocolitica* subsp. *paleartica* MRS40) as described in [17]. *Y. enterocolitica* E40 and its derivative *Y. enterocolitica* MRS40 as described in [17] is identical to *Y. enterocolitica* subsp. *paleartica* E40 and its derivative *Y. enterocolitica* subsp. *paleartica* MRS40 as described in [12,14,18]. Also preferably the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain, more preferably a *Salmonella enterica* strain. Most preferred is *Salmonella enterica* Serovar Typhimurium SL1344 as described by the Public health England culture collection (NCTC 13347).

In some embodiments of the present invention the recombinant virulence attenuated Gram-negative bacterial strain is a strain which does not produce a siderophore e.g. is deficient in the production of a siderophore, preferably does not produce siderophores e.g. is deficient in the production of any siderophore. Such a strain is for example *Y. enterocolitica* subsp. *paleartica* MRS40 as described in [14,17] which does not produce yersiniabactin and which is preferred.

In one embodiment of the present invention the delivery signal from a bacterial effector protein comprises a bacterial effector protein or a N-terminal fragment thereof.

In one embodiment of the present invention the delivery signal from a bacterial effector protein is a bacterial T3SS effector protein comprising a bacterial T3SS effector protein or a

5 N-terminal fragment thereof wherein the T3SS effector protein or a N-terminal fragment thereof may comprise a chaperone binding site. A T3SS effector protein or a N-terminal fragment thereof which comprises a chaperone binding site is particularly useful as delivery signal in the present invention. Preferred T3SS effector proteins or N-terminal fragments thereof are selected from the group consisting of SopE, SopE2, SptP, YopE, ExoS, SipA, SipB, SipD, SopA, SopB, SopD, IpgB1, IpgD, SipC, SifA, SseJ, Sse, SrfH, YopJ, AvrA, AvrBsT, YopT, YopH, YpkA, Tir, EspF, TccP2, IpgB2, OspF, Map, OspG, OspI, IpaH, SspH1, VopF, ExoS, ExoT, HopAB2, XopD, AvrRpt2, HopAO1, HopPtoD2, HopU1, GALA family of proteins, AvrBs2, AvrD1, AvrBS3, YopO, YopP, YopE, YopM, YopT, EspG, EspH, EspZ, IpaA, IpaB, IpaC, VirA, IcsB, OspC1, OspE2, IpaH9.8, IpaH7.8, AvrB, AvrD, 15 AvrPphB, AvrPphC, AvrPphEpto, AvrPpiBpto, AvrPto, AvrPtoB, VirPphA, AvrRpm1, HopPtoE, HopPtoF, HopPtoN, PopB, PopP2, AvrBs3, XopD, and AvrXv3. More preferred T3SS effector proteins or N-terminal fragments thereof are selected from the group consisting of SopE, SptP, YopE, ExoS, SopB, IpgB1, IpgD, YopJ, YopH, EspF, OspF, ExoS, YopO, YopP, YopE, YopM, YopT, whereof most preferred T3SS effector proteins or N-terminal 20 fragments thereof are selected from the group consisting of IpgB1, SopE, SopB, SptP, OspF, IpgD, YopH, YopO, YopP, YopE, YopM, YopT, in particular YopE or an N-terminal fragment thereof.

Equally preferred T3SS effector proteins or N-terminal fragments thereof are selected from the group consisting of SopE, SopE2, SptP, SteA, SipA, SipB, SipD, SopA, SopB, SopD, 25 IpgB1, IpgD, SipC, SifA, SifB, SseJ, Sse, SrfH, YopJ, AvrA, AvrBsT, YopH, YpkA, Tir, EspF, TccP2, IpgB2, OspF, Map, OspG, OspI, IpaH, VopF, ExoS, ExoT, HopAB2, AvrRpt2, HopAO1, HopU1, GALA family of proteins, AvrBs2, AvrD1, YopO, YopP, YopE, YopT, EspG, EspH, EspZ, IpaA, IpaB, IpaC, VirA, IcsB, OspC1, OspE2, IpaH9.8, IpaH7.8, AvrB, AvrD, AvrPphB, AvrPphC, AvrPphEpto, AvrPpiBpto, AvrPto, AvrPtoB, VirPphA, 30 AvrRpm1, HopPtoD2, HopPtoE, HopPtoF, HopPtoN, PopB, PopP2, AvrBs3, XopD, and AvrXv3. Equally more preferred T3SS effector proteins or N-terminal fragments thereof are selected from the group consisting of SopE, SptP, SteA, SifB, SopB, IpgB1, IpgD, YopJ, YopH, EspF, OspF, ExoS, YopO, YopP, YopE, YopT, whereof equally most preferred T3SS

effector proteins or N-terminal fragments thereof are selected from the group consisting of IpgB1, SopE, SopB, SptP, SteA, SifB, OspF, IpgD, YopH, YopO, YopP, YopE, and YopT, in particular SopE, SteA, or YopE or an N-terminal fragment thereof, more particular SteA or YopE or an N-terminal fragment thereof, most particular YopE or an N-terminal fragment thereof.

In some embodiments the delivery signal from the bacterial T3SS effector protein encoded by the first DNA sequence comprises the bacterial T3SS effector protein or an N-terminal fragment thereof, wherein the N-terminal fragment thereof includes at least the first 10, preferably at least the first 20, more preferably at least the first 100 amino acids of the bacterial T3SS effector protein.

In some embodiments the delivery signal from the bacterial T3SS effector protein encoded by the first DNA sequence comprises the bacterial T3SS effector protein or an N-terminal fragment thereof, wherein the bacterial T3SS effector protein or the N-terminal fragment thereof comprises a chaperone binding site.

Preferred T3SS effector proteins or a N-terminal fragment thereof, which comprise a chaperone binding site comprise the following combinations of chaperone binding site and T3SS effector protein or N-terminal fragment thereof: SycE-YopE, InvB-SopE, SicP-SptP, SycT-YopT, SycO-YopO, SycN/YscB-YopN, SycH-YopH, SpsS-ExoS, CesF-EspF, SycD-YopB, SycD-YopD. More preferred are SycE-YopE, InvB-SopE, SycT-YopT, SycO-YopO, SycN/YscB-YopN, SycH-YopH, SpsS-ExoS, CesF-EspF. Most preferred is a YopE or an N-terminal fragment thereof comprising the SycE chaperone binding site such as an N-terminal fragment of a YopE effector protein containing the N-terminal 138 amino acids of the YopE effector protein designated herein as YopE₁₋₁₃₈ and as shown in SEQ ID NO. 2 or a SopE or an N-terminal fragment thereof comprising the InvB chaperone binding site such as an N-terminal fragment of a SopE effector protein containing the N-terminal 81 or 105 amino acids of the SopE effector protein designated herein as SopE₁₋₈₁ or SopE₁₋₁₀₅ respectively, and as shown in SEQ ID NO.: 142 or 143.

In one embodiment of the present invention the recombinant virulence attenuated Gram-negative bacterial strain is a *Yersinia* strain and the delivery signal from the bacterial T3SS effector protein encoded by the first DNA sequence comprises a YopE effector protein or an N-terminal part, preferably the *Y. enterocolitica* YopE effector protein or an N-terminal part thereof. Preferably the SycE binding site is comprised within the N-terminal part of the YopE

effector protein. In this connection an N-terminal fragment of a YopE effector protein may comprise the N-terminal 12, 16, 18, 52, 53, 80 or 138 amino acids [19-21]. Most preferred is an N-terminal fragment of a YopE effector protein containing the N-terminal 138 amino acids of the YopE effector protein e.g. as described in Forsberg and Wolf-Watz [22] designated
5 herein as YopE₁₋₁₃₈ and as shown in SEQ ID NO.: 2.

In one embodiment of the present invention the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain and the delivery signal from the bacterial T3SS effector protein encoded by the first DNA sequence comprises a SopE or SteA effector
10 protein or an N-terminal part thereof, preferably the *Salmonella enterica* SopE or SteA effector protein or an N-terminal part thereof. Preferably the chaperon binding site is comprised within the N-terminal part of the SopE effector protein. In this connection an N-terminal fragment of a SopE effector protein may comprise the N-terminal 81 or 105 amino acids. Most preferred is the full length SteA and an N-terminal fragment of a SopE
15 effector protein containing the N-terminal 105 amino acids of the effector protein e.g. as described in SEQ ID NO. 142 or 143.

One skilled in the art is familiar with methods for identifying the polypeptide sequences of an effector protein that are capable of delivering a protein. For example, one such method is
20 described by Sory et al. [16]. Briefly, polypeptide sequences from e.g. various portions of the Yop proteins can be fused in-frame to a reporter enzyme such as the calmodulin-activated adenylate cyclase domain (or Cya) of the *Bordetella pertussis* cyclolysin. Delivery of a Yop-Cya hybrid protein into the cytosol of eukaryotic cells is indicated by the appearance of cyclase activity in the infected eukaryotic cells that leads to the accumulation of cAMP. By
25 employing such an approach, one skilled in the art can determine, if desired, the minimal sequence requirement, i.e., a contiguous amino acid sequence of the shortest length, that is capable of delivering a protein, see, e.g. [16]. Accordingly, preferred delivery signals of the present invention consists of at least the minimal sequence of amino acids of a T3SS effector protein that is capable of delivering a protein.

30 The present invention provides recombinant virulence attenuated Gram-negative bacterial strains for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain, which is deficient in

producing at least one bacterial effector protein which is virulent toward eukaryotic cells, accumulates in the malignant solid tumor. In some embodiments the recombinant virulence attenuated Gram-negative bacterial strains are deficient in producing at least one, preferably at least two, more preferably at least three, even more preferably at least four, in particular at least five, more particularly at least six, most particularly all bacterial effector proteins which are virulent toward eukaryotic cells i.e. recombinant virulence attenuated Gram-negative bacterial strains which are deficient in producing at least one preferably at least two, more preferably at least three, even more preferably at least four, in particular at least five, more particularly at least six, most particularly all functional bacterial effector proteins which are virulent toward eukaryotic cells such that the resulting recombinant virulence attenuated Gram-negative bacterial strain produces less bacterial effector proteins or produces bacterial effector proteins to a lesser extent compared to the non virulence attenuated Gram-negative bacterial wild type strain i.e. compared to the Gram-negative bacterial wild type strain which normally produces bacterial effector proteins or such that the resulting recombinant virulence attenuated Gram-negative bacterial strain no longer produce any functional bacterial effector proteins which are virulent toward eukaryotic cells.

In some embodiments the recombinant virulence attenuated Gram-negative bacterial strains for use in a method of treating a malignant solid tumor in a subject is deficient in the production of all effector proteins which are virulent toward eukaryotic cells and the delivery signal from a bacterial effector protein is the delivery signal from a bacterial T3SS effector protein and the heterologous protein is selected from the group consisting of proteins involved in apoptosis or apoptosis regulation.

According to the present invention, such a mutant Gram-negative bacterial strain i.e. such a recombinant virulence attenuated Gram-negative bacterial strain which is deficient in producing at least one bacterial effector protein which is virulent toward eukaryotic cells e.g. such a mutant *Yersinia* strain can be generated by introducing at least one mutation into at least one effector-encoding gene. Preferably, such effector-encoding genes include YopE, YopH, YopO/YpkA, YopM, YopP/YopJ and YopT as far as a *Yersinia* strain is concerned. Preferably, such effector-encoding genes include AvrA, CigR, GogB, GtgA, GtgE, PipB, SifB, SipA/SspA, SipB, SipC/SspC, SipD/SspD, SlrP, SopB/SigD, SopA, SpiC/SsaB, SseB, SseC, SseD, SseF, SseG, SseI/SrfH, SopD, SopE, SopE2, SspH1, SspH2, PipB2, SifA, SopD2, SseJ, SseK1, SseK2, SseK3, SseL, SteC, SteA, SteB, SteD, SteE, SpvB, SpvC, SpvD,

SrfJ, SptP, as far as a *Salmonella* strain is concerned. Most preferably, all effector-encoding genes are deleted. The skilled artisan may employ any number of standard techniques to generate mutations in these T3SS effector genes. Sambrook et al. describe in general such techniques. See Sambrook et al. [23].

- 5 In accordance with the present invention, the mutation can be generated in the promoter region of an effector-encoding gene so that the expression of such effector gene is abolished. The mutation can also be generated in the coding region of an effector-encoding gene such that the catalytic activity of the encoded effector protein is abolished. The "catalytic activity" of an effector protein refers normally to the anti-target cell function of an effector protein, i.e.,
10 toxicity. Such activity is governed by the catalytic motifs in the catalytic domain of an effector protein. The approaches for identifying the catalytic domain and/or the catalytic motifs of an effector protein are well known by those skilled in the art. See, for example, [24,25].

- Accordingly, one preferred mutation of the present invention is a deletion of the entire
15 catalytic domain. Another preferred mutation is a frameshift mutation in an effector-encoding gene such that the catalytic domain is not present in the protein product expressed from such "frameshifted" gene. A most preferred mutation is a mutation with the deletion of the entire coding region of the effector protein. Other mutations are also contemplated by the present invention, such as small deletions or base pair substitutions, which are generated in the
20 catalytic motifs of an effector protein leading to destruction of the catalytic activity of a given effector protein.

- The mutations that are generated in the genes of the functional bacterial effector proteins may be introduced into the particular strain by a number of methods. One such method involves cloning a mutated gene into a "suicide" vector which is capable of introducing the mutated
25 sequence into the strain via allelic exchange. An example of such a "suicide" vector is described by [26].

- In this manner, mutations generated in multiple genes may be introduced successively into a Gram-negative bacterial strain giving rise to polymutant, e.g. a sextuple mutant recombinant strain. The order in which these mutated sequences are introduced is not important. Under
30 some circumstances, it may be desired to mutate only some but not all of the effector genes. Accordingly, the present invention further contemplates polymutant *Yersinia* other than sextuple-mutant *Yersinia*, e.g., double-mutant, triple-mutant, quadruple-mutant and quintuple-

mutant strains. For the purpose of delivering proteins, the secretion and translocation system of the instant mutant strain needs to be intact.

A preferred recombinant virulence attenuated Gram-negative bacterial strain of the present invention is a sextuple-mutant *Yersinia* strain in which all the effector-encoding genes are mutated such that the resulting *Yersinia* no longer produce any functional effector proteins. Such sextuple-mutant *Yersinia* strain is designated as $\Delta yopH,O,P,E,M,T$ for *Y. enterocolitica*. As an example such a sextuple-mutant can be produced from the *Y. enterocolitica* MRS40 strain giving rise to *Y. enterocolitica* MRS40 $\Delta yopH,O,P,E,M,T$, (also named *Y. enterocolitica* subsp. *paleartica* MRS40 $\Delta yopH,O,P,E,M,T$ herein) which is preferred. Equally preferred is *Y. enterocolitica* MRS40 $\Delta yopH,O,P,E,M,T \Delta asd$ (also named *Y. enterocolitica* subsp. *paleartica* MRS40 $\Delta yopH,O,P,E,M,T \Delta asd$ herein).

Vectors which can be used according to the invention to transform a Gram-negative bacterial strain depend on the Gram-negative bacterial strains used as known to the skilled person.

Promoter, heterologous protein and protease cleavage site as described herein can be used for the vector of the recombinant virulence attenuated Gram-negative bacterial strain. Vectors which can be used according to the invention include expression vectors (including synthetic or otherwise generated modified versions of endogenous virulence plasmids), vectors for chromosomal or virulence plasmid insertion and DNA fragments for chromosomal or virulence plasmid insertion. Expression vectors which are useful in e.g. *Yersinia*, *Escherichia*, *Salmonella* or *Pseudomonas* strain are e.g. pUC, pBad, pACYC, pUCP20 and pET plasmids. Vectors for chromosomal or virulence plasmid insertion which are useful in e.g. *Yersinia*, *Escherichia*, *Salmonella* or *Pseudomonas* strain are e.g. pKNG101. DNA fragments for chromosomal or virulence plasmid insertion refer to methods used in e.g. *Yersinia*,

Escherichia, *Salmonella* or *Pseudomonas* strain as e.g. lambda-red genetic engineering. Vectors for chromosomal or virulence plasmid insertion or DNA fragments for chromosomal or virulence plasmid insertion may insert the first, second and/or third DNA sequence of the present invention so that the first, second and/or third DNA sequence is operably linked to an endogenous promoter of the recombinant virulence attenuated Gram-negative bacterial strain.

Thus if a vector for chromosomal or virulence plasmid insertion or a DNA fragment for chromosomal or virulence plasmid insertion is used, an endogenous promoter can be encoded on the endogenous bacterial DNA (chromosomal or plasmid DNA) and only the first and second DNA sequence will be provided by the engineered vector for chromosomal or

virulence plasmid insertion or DNA fragment for chromosomal or virulence plasmid insertion. Alternatively, if a vector for chromosomal or virulence plasmid insertion or a nucleotide molecule such as e.g. a DNA sequence for chromosomal or virulence plasmid insertion is used, an endogenous promoter and the delivery signal from a bacterial effector protein can be encoded on the endogenous bacterial DNA (chromosomal or plasmid DNA) and only the nucleotide molecule such as e.g. a DNA sequence encoding the heterologous protein will be provided by a vector for chromosomal or virulence plasmid insertion or by a nucleotide molecule such as e.g. a DNA sequence for chromosomal or virulence plasmid insertion. Thus a promoter is not necessarily needed to be comprised by the vector used for transformation of the recombinant virulence attenuated Gram-negative bacterial strains i.e. the recombinant virulence attenuated Gram-negative bacterial strains of the present invention may be transformed with a vector which does not comprise a promoter.

In a preferred embodiment the vector of the present invention comprises in the 5' to 3' direction:

a first DNA sequence encoding a delivery signal or a fragment thereof from a bacterial effector protein;

a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence.

A preferred vector e.g. a preferred expression vector for *Yersinia* is selected from the group consisting of pBad_Si_1 and pBad_Si_2. pBad_Si2 was constructed by cloning of the SycE-YopE₁₋₁₃₈ fragment containing endogenous promoters for YopE and SycE from purified pYV40 into KpnI/HindIII site of pBad-MycHisA (Invitrogen). Additional modifications include removal of the NcoI/BglII fragment of pBad-MycHisA by digest, Klenow fragment treatment and religation. Further at the 3' end of YopE₁₋₁₃₈ the following cleavage sites were added: XbaI-XhoI-BstBI-(HindIII). pBad_Si1 is equal to pBad_Si2 but encodes EGFP amplified from pEGFP-C1 (Clontech) in the NcoI/BglII site under the Arabinose inducible promoter. Equally preferred is the use of modified versions of the endogenous *Yersinia* virulence plasmid pYV encoding heterologous proteins as fusions to a T3SS signal sequence.

A preferred vector e.g. a preferred expression vector for *Salmonella* is selected from the group consisting of pSi_266, pSi_267, pSi_268 and pSi_269. Plasmids pSi_266, pSi_267, pSi_268 and pSi_269 containing the corresponding endogenous promoter and the SteA₁₋₂₀ fragment (pSi_266), the full length SteA sequence (pSi_267), the SopE₁₋₈₁ fragment (pSi_268) or the

SopE₁₋₁₀₅ fragment (pSi_269) were amplified from *S. enterica* SL1344 genomic DNA and cloned into NcoI/KpnI site of pBad-MycHisA (Invitrogen).

The vectors of the instant invention may include other sequence elements such as a 3' termination sequence (including a stop codon and a poly A sequence), or a gene conferring a drug resistance which allows the selection of transformants having received the instant vector. The vectors of the present invention may be transformed by a number of known methods into the recombinant virulence attenuated Gram-negative bacterial strains. For the purpose of the present invention, the methods of transformation for introducing a vector include, but are not limited to, electroporation, calcium phosphate mediated transformation, conjugation, or combinations thereof. For example, a vector can be transformed into a first bacteria strain by a standard electroporation procedure. Subsequently, such a vector can be transferred from the first bacteria strain into the desired strain by conjugation, a process also called "mobilization". Transformant (i.e., Gram-negative bacterial strains having taken up the vector) may be selected, e.g., with antibiotics. These techniques are well known in the art. See, for example, [16].

In accordance with the present invention, the promoter of the vector of the recombinant virulence attenuated Gram-negative bacterial strain of the invention can be a native promoter of a T3SS effector protein of the respective strain or a compatible bacterial strain or a promoter used in expression vectors which are useful in e.g. *Yersinia*, *Escherichia*, *Salmonella* or *Pseudomonas* strain e.g pUC and pBad. Such promoters are the T7 promoter, Plac promoter or the arabinose inducible Ara-bad promoter.

If the recombinant virulence attenuated Gram-negative bacterial strain is a *Yersinia* strain the promoter can be from a *Yersinia* virulon gene. A "Yersinia virulon gene" refers to genes on the *Yersinia* pYV plasmid, the expression of which is controlled both by temperature and by contact with a target cell. Such genes include genes coding for elements of the secretion machinery (the Ysc genes), genes coding for translocators (YopB, YopD, and LcrV), genes coding for the control elements (YopN, TyeA and LcrG), genes coding for T3SS effector chaperones (SycD, SycE, SycH, SycN, SycO and SycT), and genes coding for effectors (YopE, YopH, YopO/YpkA, YopM, YopT and YopP/YopJ) as well as other pYV encoded proteins as VirF and YadA.

In a preferred embodiment of the present invention, the promoter is the native promoter of a T3SS functional effector encoding gene. If the recombinant virulence attenuated Gram-

negative bacterial strain is a *Yersinia* strain the promoter is selected from any one of YopE, YopH, YopO/YpkA, YopM and YopP/YopJ. More preferably, the promoter is from YopE or SycE.

If the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain the promoter can be from SpiI or SpiII pathogenicity island or from an effector protein elsewhere encoded. Such genes include genes coding for elements of the secretion machinery, genes coding for translocators, genes coding for the control elements, genes coding for T3SS effector chaperones, and genes coding for effectors as well as other proteins encoded by SPI-1 or SPI-2. In a preferred embodiment of the present invention, the promoter is the native promoter of a T3SS functional effector encoding gene. If the recombinant virulence attenuated Gram-negative bacterial strain is a *Salmonella* strain the promoter is selected from any one of the effector proteins. More preferably, the promoter is from SopE, InvB or SteA.

In one embodiment of the present invention the vector e.g the expression vector comprises a DNA sequence encoding a protease cleavage site. Generation of a functional and generally applicable cleavage site allows cleaving off the delivery signal after translocation. As the delivery signal can interfere with correct localization and/or function of the translocated protein within the target cells the introduction of a protease cleavage site between the delivery signal and the protein of interest provides for the first time delivery of almost native proteins into eukaryotic cells. Preferably the protease cleavage site is an amino acid motif which is cleaved by a protease or the catalytic domains thereof selected from the group consisting of enterokinase (light chain), enteropeptidase, prescission protease, human rhinovirus protease 3C, TEV protease, TVMV protease, FactorXa protease and thrombin, more preferably an amino acid motif which is cleaved by TEV protease. Equally preferable the protease cleavage site is an amino acid motif which is cleaved by a protease or the catalytic domains thereof selected from the group consisting of enterokinase (light chain), enteropeptidase, prescission protease, human rhinovirus protease 3C, TEV protease, TVMV protease, FactorXa protease, ubiquitin processing protease, called Deubiquitinating enzymes, and thrombin. Most preferred is an amino acid motif which is cleaved by TEV protease or by an ubiquitin processing protease.

Thus in a further embodiment of the present invention, the heterologous protein is cleaved from the delivery signal from a bacterial T3SS effector protein by a protease. Preferred methods of cleavage are methods wherein:

a) the protease is translocated into the eukaryotic cell by a recombinant virulence attenuated Gram-negative bacterial strain as described herein which expresses a fusion protein which comprises the delivery signal from the bacterial T3SS effector protein and the protease as heterologous protein; or

5 b) the protease is expressed constitutively or transiently in the eukaryotic cell.

Usually the recombinant virulence attenuated Gram-negative bacterial strain used to deliver a desired protein into a eukaryotic cell and the recombinant virulence attenuated Gram-negative bacterial strain translocating the protease into the eukaryotic cell are different.

10 In one embodiment of the present invention the vector comprises a further DNA sequence encoding a labelling molecule or an acceptor site for a labelling molecule. The further DNA sequence encoding a labelling molecule or an acceptor site for a labelling molecule is usually fused to the 5' end or to the 3' end of the second DNA sequence. A preferred labelling molecule or an acceptor site for a labelling molecule is selected from the group consisting of
15 enhanced green fluorescent protein (EGFP), coumarin, coumarin ligase acceptor site, resorufin, resorufin ligase acceptor site, the tetra-Cysteine motif in use with FLAsH/ReAsH dye (life technologies). Most preferred is resorufin and a resorufin ligase acceptor site or EGFP. The use of a labelling molecule or an acceptor site for a labelling molecule will lead to the attachment of a labelling molecule to the heterologous protein of interest, which will then
20 be delivered as such into the eukaryotic cell and enables tracking of the protein by e.g. live cell microscopy.

In one embodiment of the present invention the vector comprises a further DNA sequence encoding a peptide tag. The further DNA sequence encoding a peptide tag is usually fused to
25 the 5' end or to the 3' end of the second DNA sequence. A preferred peptide tag is selected from the group consisting of Myc-tag, His-tag, Flag-tag, HA tag, Strep tag or V5 tag or a combination of two or more tags out of these groups. Most preferred is Myc-tag, Flag-tag, His-tag and combined Myc- and His-tags. The use of a peptide tag will lead to traceability of the tagged protein e.g. by immunofluorescence or Western blotting using anti-tag antibodies.
30 Further, the use of a peptide tag allows affinity purification of the desired protein either after secretion into the culture supernatant or after translocation into eukaryotic cells, in both cases using a purification method suiting the corresponding tag (e.g. metal-chelate affinity purification in use with a His-tag or anti-Flag antibody based purification in use with the Flag-

tag).

In one embodiment of the present invention the vector comprises a further DNA sequence encoding a nuclear localization signal (NLS). The further DNA sequence encoding a nuclear localization signal (NLS) is usually fused to the 5'end or to the 3'end of the second DNA sequence wherein said further DNA sequence encodes a nuclear localization signal (NLS). A preferred NLS is selected from the group consisting of SV40 large T-antigen NLS and derivatives thereof [27] as well as other viral NLS. Most preferred is SV40 large T-antigen NLS and derivatives thereof.

In one embodiment of the present invention the vector comprises a multiple cloning site. The multiple cloning site is usually located at the 3'end of the first DNA sequence and/or at the 5'end or 3'end of the second DNA sequence. One or more than one multiple cloning sites can be comprised by the vector. A preferred multiple cloning site is selected from the group of restriction enzymes consisting of XhoI, XbaI, HindIII, NcoI, NotI, EcoRI, EcoRV, BamHI, NheI, SacI, SalI, BstBI. Most preferred is XbaI, XhoI, BstBI and HindIII.

The fused protein expressed from the first and second and optional third DNA sequences of the vector is also termed as a "fusion protein" or a "hybrid protein", i.e., a fused protein or hybrid of delivery signal and a heterologous protein. The fusion protein can also comprise e.g. a delivery signal and two or more different heterologous proteins.

The present invention contemplates methods for delivering heterologous proteins as hereinabove described into cells of a malignant solid tumor. The proteins may be delivered i.e. translocated into the cell of a malignant solid tumor at the time of administering the recombinant virulence attenuated Gram-negative bacterial strain to a subject or may be delivered i.e. translocated into the cell of a malignant solid tumor at a later time e.g. after the the recombinant virulence attenuated Gram-negative bacterial strain has reached the site of the malignant solid tumor and/or has reached the site of the malignant solid tumor and has replicated as described above. The time of delivery can be regulated e.g by the promoter used to express the heterologous proteins in the recombinant virulence attenuated Gram-negative bacterial strain. In the first case, either a constitutive promoter or, more preferred, an endogenous promoter of a bacterial effector protein might drive the heterologous protein. In

the case of delayed protein delivery, an artificially inducible promoter, as the arabinose inducible promoter, might drive the heterologous protein. In this case, arabinose will be administered to a subject once bacteria have reached and accumulated at the desired site. Arabinose will then induce the bacterial expression of the protein to be delivered.

5

Thus in one embodiment the method for delivering heterologous proteins into cells of a malignant solid tumor comprises

- i) culturing the recombinant virulence attenuated Gram-negative bacterial strain as described herein;
- 10 ii) contacting a cell of a malignant solid tumor with the recombinant virulence attenuated Gram-negative bacterial strain of i) wherein a fusion protein which comprises a delivery signal from a bacterial effector protein and the heterologous protein is expressed by the recombinant virulence attenuated Gram-negative bacterial strain and is translocated into the cell of a malignant solid tumor; and optionally
- 15 iii) cleaving the fusion protein so that the heterologous protein is cleaved from the delivery signal from the bacterial effector protein inside of the cell of a malignant solid tumor.

In some embodiments at least two fusion proteins which comprise each a delivery signal from a bacterial effector protein and a heterologous protein are expressed by the recombinant
20 virulence attenuated Gram-negative bacterial strain and are translocated into the eukaryotic cell by the methods of the present inventions.

The recombinant virulence attenuated Gram-negative bacterial strain can be cultured so that a fusion protein is expressed which comprises the delivery signal from the bacterial effector
25 protein and the heterologous protein according to methods known in the art (e.g. FDA, Bacteriological Analytical Manual (BAM), chapter 8: *Yersinia enterocolitica*). Preferably the recombinant virulence attenuated Gram-negative bacterial strain can be cultured in Brain Heart infusion broth e.g. at 28°C. For induction of expression of T3SS and e.g. YopE/SycE promoter dependent genes, bacteria can be grown at 37°C.

30

In one embodiment, the cell of a malignant solid tumor is contacted with two recombinant virulence attenuated Gram-negative bacterial strains of i), wherein the first recombinant virulence attenuated Gram-negative bacterial strain expresses a first fusion protein which

comprises the delivery signal from the bacterial T3SS effector protein and a first heterologous protein and the second recombinant virulence attenuated Gram-negative bacterial strain expresses a second fusion protein which comprises the delivery signal from the bacterial effector protein and a second heterologous protein, so that the first and the second fusion protein are translocated into the cell of a malignant solid tumor. This embodiment provided for co-infection of e.g. a cell of a malignant solid tumor with two bacterial strains as a valid method to deliver e.g. two different hybrid proteins into single cells to address their functional interaction.

Those skilled in the art can also use a number of assays to determine whether the delivery of a fusion protein is successful. For example, the fusion protein may be detected via immunofluorescence using antibodies recognizing a fused tag (like Myc-tag). The determination can also be based on the enzymatic activity of the protein being delivered, e.g., the assay described by [16].

The present invention also provides a pharmaceutical composition comprising a recombinant virulence attenuated Gram-negative bacterial strain for use in a method of treating a malignant solid tumor in a subject, wherein the recombinant virulence attenuated Gram-negative bacterial strain accumulates in the malignant solid tumor.

The recombinant virulence attenuated Gram-negative bacteria can be compounded for convenient and effective administration in an amount that is sufficient to treat the subject as pharmaceutical composition with a suitable pharmaceutically acceptable carrier. A unit dosage form of the recombinant virulence attenuated Gram-negative bacteria or of the pharmaceutical composition to be administered can, for example, contain the recombinant virulence attenuated Gram-negative bacteria in an amount from about 10^5 to about 10^9 bacteria per ml, preferably about 10^6 to about 10^8 bacteria per ml, more preferably about 10^7 to about 10^8 bacteria per ml, most preferably about 10^8 bacteria per ml.

By "amount that is sufficient to treat the subject " or "effective amount" which are used herein interchangeably is meant to be an amount of a bacterium or bacteria, high enough to significantly positively modify the condition to be treated but low enough to avoid serious side effects (at a reasonable benefit/risk ratio), within the scope of sound medical judgment.

An effective amount of a bacterium will vary with the particular goal to be achieved, the age and physical condition of the subject being treated, the duration of treatment, the nature of concurrent therapy and the specific bacterium employed. The effective amount of a bacterium will thus be the minimum amount, which will provide the desired effect. Usually an amount from about 10^5 to about 10^9 bacteria e.g. from about 10^5 to about 10^9 bacteria/m² body surface, preferably from about 10^6 to about 10^8 bacteria e.g. from about 10^6 to about 10^8 bacteria/m² body surface, more preferably from about 10^7 to about 10^8 bacteria e.g. from about 10^7 to about 10^8 bacteria/m² body surface, most preferably 10^8 bacteria e.g. 10^8 bacteria/m² body surface are administered to the subject.

A single dose of the recombinant virulence attenuated Gram-negative bacterial strain to administer to a subject, e.g. to a human to treat a malignant solid tumor is usually from about 10^4 to about 10^{10} bacteria e.g. from about 10^4 bacteria/m² body surface to about 10^{10} bacteria/m² body surface, preferably from about 10^5 to about 10^9 bacteria e.g. from about 10^5 to about 10^9 bacteria/m² body surface, more preferably from about 10^6 to about 10^8 bacteria e.g. from about 10^6 to about 10^8 bacteria/m² body surface, even more preferably from about 10^7 to about 10^8 bacteria e.g. from about 10^7 to about 10^8 bacteria/m² body surface, most preferably 10^8 bacteria e.g. 10^8 bacteria/m² body surface of total recombinant virulence attenuated Gram-negative bacteria.

Examples of substances which can serve as pharmaceutical carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethylcellulose, ethylcellulose and cellulose acetates; powdered tragacanth; malt; gelatin; talc; stearic acids; magnesium stearate; calcium sulfate; calcium carbonate; vegetable oils, such as peanut oils, cotton seed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, manitol, and polyethylene glycol; agar; alginic acids; pyrogen-free water; isotonic saline; cranberry extracts and phosphate buffer solution; skim milk powder; as well as other non-toxic compatible substances used in pharmaceutical formulations such as Vitamin C, estrogen and echinacea, for example. Wetting agents and lubricants such as sodium lauryl sulfate, as well as coloring agents, flavoring agents, lubricants, excipients, tableting agents, stabilizers, anti-oxidants and preservatives, can also be present.

Modes of administration of the recombinant virulence attenuated Gram-negative bacteria to a subject may be selected from the group consisting of intravenous, intratumoral, intraperitoneal and per-oral administration. Although this invention is not intended to be limited to any particular mode of application, intravenous or intratumoral administration of the bacteria or the pharmaceutical compositions is preferred.

Depending on the route of administration, the active ingredients which comprise bacteria may be required to be coated in a material to protect said organisms from the action of enzymes, acids and other natural conditions which may inactivate said organisms. In order to administer bacteria by other than parenteral administration, they should be coated by, or administered with, a material to prevent inactivation. For example, bacteria may be co-administered with enzyme inhibitors or in liposomes. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DFP) and trasylol. Liposomes include water-in-oil-in-water P40 emulsions as well as conventional and specifically designed liposomes which transport bacteria, such as *Lactobacillus*, or their by-products to an internal target of a host subject.

One bacterium may be administered alone or in conjunction with a second, different bacterium. Any number of different bacteria may be used in conjunction. By "in conjunction with" is meant together, substantially simultaneously or sequentially. The compositions may be also administered in the form of tablet, pill or capsule, for example, such as a freeze-dried capsule comprising the bacteria or the pharmaceutical compositions of the present invention or as frozen solution of bacteria or the pharmaceutical compositions of the present invention containing DMSO or glycerol. Another preferred form of application involves the preparation of a lyophilized capsule of the bacteria or the pharmaceutical compositions of the present invention. Still another preferred form of application involves the preparation of a heat dried capsule of the bacteria or the pharmaceutical compositions of the present invention.

The recombinant virulence attenuated Gram-negative bacteria or the pharmaceutical composition to be administered can be administered by injection. Forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof and

vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

In some embodiments of the present invention the recombinant virulence attenuated Gram-negative bacterial strain is co-administered with a siderophore to the subject. These embodiments are preferred. Siderophores which can be co-administered are siderophores including hydroxamate, catecholate and mixed ligand siderophores. Preferred siderophores are Deferoxamine (also known as desferrioxamine B, desferoxamine B, DFO-B, DFOA, DFB or desferal), Desferrioxamine E, Deferasirox (Exjade, Desirox, Defrijet, Desifer) and Deferiprone (Ferriprox), more preferred is Deferoxamine. Deferoxamine is a bacterial siderophore produced by the Actinobacteria *Streptomyces pilosus* and is commercially available from e.g. Novartis Pharma Schweiz AG (Switzerland).

Co-administration with a siderophore can be before, simultaneous to or after administration of the recombinant virulence attenuated Gram-negative bacterial strain. Preferably a siderophore is administered before the administration of recombinant virulence attenuated Gram-negative bacterial strain, more preferably is administered at least 1 hour, preferably at least 6 hours, more preferably at least 12, hours, in particular at least 24 hours before the administration of the recombinant virulence attenuated Gram-negative bacterial strain to the subject. In a particular embodiment the subject is pretreated with desferoxamine 24h prior to infection with the recombinant virulence attenuated Gram-negative bacterial strain in order to allow bacterial growth. Usually a siderophore is co-administered at a single dose from about 0.5×10^{-5} Mol to about 1×10^{-3} Mol, more preferably from about 1×10^{-5} Mol to about 1×10^{-4} Mol preferably from about 3.5×10^{-5} Mol to about 1.1×10^{-4} Mol per kg of body weight. Usually desferoxamine is co-administered at single dose from about 20 mg to about 60 mg preferably from about 20 mg to about 60 mg per kg of body weight.

Dosis regimens of the administration of the recombinant virulence attenuated Gram-negative bacterial strain or the pharmaceutical composition described herein will vary with the particular goal to be achieved, the age and physical condition of the subject being treated, the

duration of treatment, the nature of concurrent therapy and the specific bacterium employed, as known to the skilled person. The recombinant virulence attenuated Gram-negative bacterial strain is usually administered to the subject according to a dosing regimen consisting of a single dose every 2-20 days, preferably every 6-10 days, more preferably every 7-9 days, preferably according to a dosing regimen consisting of a single dose every 2-8 weeks, preferably every 2-6 weeks, more preferably every 3-4 weeks. The period of administration is usually about 20 to about 60 days, preferably about 30-40 days. Alternatively the period of administration is usually about 8 to about 32 weeks, preferably about 8 to about 24 weeks, more preferably about 12 to about 16 weeks.

In a further embodiment the present invention provides a kit for treating malignant solid tumors, preferably in human. Such kits generally will comprise the recombinant virulence attenuated Gram-negative bacterial strain or the pharmaceutical composition described herein, and instructions for using the kit. In some embodiments, kits include a carrier, package, or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) including one of the separate elements to be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, and test tubes. In other embodiments, the containers are formed from a variety of materials such as glass or plastic.

Examples

Example 1:

A) Materials and Methods

5 Bacterial strains and growth conditions. The strains used in this study are listed in Figs. 14A to N. *E. coli* Top10, used for plasmid purification and cloning, and *E. coli* Sm10 λ pir, used for conjugation, as well as *E. coli* BW19610 [28], used to propagate pKNG101, were routinely grown on LB agar plates and in LB broth at 37°C. Ampicillin was used at a concentration of 200 μ g/ml (*Yersinia*) or 100 μ g/ml (*E. coli*) to select for expression vectors. 10 Streptomycin was used at a concentration of 100 μ g/ml to select for suicide vectors. *Y. enterocolitica* MRS40 (O:9, biotype 2) [17] a non Ampicillin resistant E40-derivate [16] and strains derived thereof were routinely grown on Brain Heart Infusion (BHI; Difco) at RT. To all *Y. enterocolitica* strains Nalidixic acid was added (35 μ g/ml) and all *Y. enterocolitica* *asd* strains were additionally supplemented with 100 μ g/ml *meso*-2,6-Diaminopimelic acid 15 (*mDAP*, Sigma Aldrich). *S. enterica* SL1344 were routinely grown on LB agar plates and in LB broth at 37°C. Ampicillin was used at a concentration of 100 μ g/ml to select for expression vectors in *S. enterica*.

Genetic manipulations of *Y. enterocolitica*. Genetic manipulations of *Y. enterocolitica* has 20 been described [29,30]. Briefly, mutators for modification or deletion of genes in the pYV plasmids or on the chromosome were constructed by 2-fragment overlapping PCR using purified pYV40 plasmid or genomic DNA as template, leading to 200–250 bp of flanking sequences on both sides of the deleted or modified part of the respective gene. Resulting fragments were cloned in pKNG101 [26] in *E. coli* BW19610 [28]. Sequence verified 25 plasmids were transformed into *E. coli* Sm10 λ pir, from where plasmids were mobilized into the corresponding *Y. enterocolitica* strain. Mutants carrying the integrated vector were propagated for several generations without selection pressure. Then sucrose was used to select for clones that have lost the vector. Finally mutants were identified by colony PCR. Specific mutators (pSi_408, pSi_419) are listed in Table III.

30

Construction of plasmids. Plasmid pBad_Si2 or pBad_Si1 (Fig. 9) were used for cloning of fusion proteins with the N-terminal 138 amino acids of YopE (SEQ ID No. 2). pBad_Si2 was constructed by cloning of the SycE-YopE₁₋₁₃₈ fragment containing endogenous promoters for

YopE and SycE from purified pYV40 into KpnI/HindIII site of pBad-MycHisA (Invitrogen). Additional modifications include removal of the NcoI/BglII fragment of pBad-MycHisA by digestion, Klenow fragment treatment and religation. A bidirectional transcriptional terminator (BBa_B1006; iGEM foundation) was cloned into KpnI cut and Klenow treated (pBad_Si2) or BglII cut site (pBad_Si1). Further at the 3' end of YopE₁₋₁₃₈ the following cleavage sites were added: XbaI-XhoI-BstBI-(HindIII) (Fig. 9 B). pBad_Si1 is equal to pBad_Si2 but encodes EGFP amplified from pEGFP-C1 (Clontech) in the NcoI/BglII site under the Arabinose inducible promoter. Plasmids pSi_266, pSi_267, pSi_268 and pSi_269 containing the corresponding endogenous promoter and the SteA₁₋₂₀ fragment (pSi_266), the full length SteA sequence (pSi_267), the SopE₁₋₈₁ fragment (pSi_268) or the SopE₁₋₁₀₅ fragment (pSi_269) were amplified from *S. enterica* SL1344 genomic DNA and cloned into NcoI/KpnI site of pBad-MycHisA (Invitrogen).

Full length genes or fragments thereof were amplified with the specific primers listed in Table I below and cloned as fusions to YopE₁₋₁₃₈ into plasmid pBad_Si2 or in case of z-BIM (SEQ ID No. 21) into pBad_Si1 (see Table II below). For fusion to SteA or SopE, synthetic DNA constructs were cleaved by KpnI/HindII and cloned into pSi_266, pSi_267, pSi_268 or pSi_269 respectively. In case of genes of bacterial species, purified genomic DNA was used as template (*S. flexneri* M90T, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* SL1344, *Bartonella henselae* ATCC 49882). For human genes a universal cDNA library (Clontech) was used if not otherwise stated (Figs. 14A to N), zebrafish genes were amplified from a cDNA library (a kind gift of M. Affolter). Ligated plasmids were cloned in *E. coli* Top10. Sequenced plasmids were electroporated into the desired *Y. enterocolitica* or *S. enterica* strain using settings as for standard *E. coli* electroporation.

Table I (Primer Nr. Si_:Sequence)

285:	CATACCATGGGAGTGAGCAAGGGCGAG
286:	GGAAGATCTtACTTGTACAGCTCGTCCAT
287:	CGGGGTACCTCAACTAAATGACCGTGGTG
288:	GTTAAAGCTTtctgaatctagactcgagCGTGGCGAACTGGTC
292:	CAGTctcgagCAAATTCTAAACAAAATACTTCCAC
293:	cagfTTCGAATTAATTTGTATTGCTTTGACGG
296:	CAGTctcgagACTAACATAACACTATCCACCCAG

- 297: GTTAAAGCTTTCAGGAGGCATTCTGAAG
299: CAGTctcgagCAGGCCATCAAGTGTGTG
300: cagtTTCGAATCATTTTCTCTTCCTCTTCTTCA
301: CAGTctcgagGCTGCCATCCGGAA
5 302: cagtTTCGAATCACAAGACAAGGCACCC
306: GTTAAAGCTTGGAGGCATTCTGAA Gatacttatt
307: CAGTctcgagCAAATACAGAGCTTCTATCACTCAG
308: GTTAAAGCTTTCAAGATGTGATTAATGAAGAAATG
317: cagtTTCGAACCCATAAAAAAGCCCTGTC
10 318: GTTAAAGCTTCTACTCTATCATCAAACGATAAAATGg
324: CAGTctcgagTTCACTCAAGAAACGCAAA
339: cagtTTCGAATTTTCTCTTCCTCTTCTTCAcg
341: cgtaTCTAGAAAAATGATGAAAATGGAGACTG
342: GTTAAAGCTTttaGCTGGAGACGGTGAC
15 346: CAGTctcgagTTCCAGATCCCAGAGTTTG
347: GTTAAAGCTTTCACTGGGAGGGGG
351: CAGTctcgagctcgagTTATCTACTCATAGAACTACTTTTGCAG
352: cgcGGATCCtcagtgctctgcggcatta
353: CATTTATTCCTCCTAGTTAGTCAcagcaactgctgctccttc
20 354: gaaaggagcagcagttgctgTGA CTA ACTAGGAGGAATAAATG
355: cgattcacggattgctttctCATTATTCCCTCCAGGTACTA
356: TAGTACCTGGAGGGAATAATGagaaagcaatccgtgaatcg
357: cgtaTCTAGAcggctttaagtgcgacattc
364: cgtaTCTAGACTAAAGTATGAGGAGAGAAAATTGAA
25 365: GTTAAAGCTTTCAGCTTGCCGTCGT
367: CGTAtctagaGACCCGTTTCCTGGTGC
369: cgtaTCTAGAccccccaagaagaagc
373: GTTAAAGCTTGCTGGAGACGGTGACC
386: CGTAtctagaTCAGGACGCTTCGGAGGTAG
30 387: CGTAtctagaATGGACTGTGAGGTCAACAA
389: CGTAtctagaGGCAACCGCAGCA
391: GTTAAAGCTTTCAGTCCATCCCATTCTg
403: CGTAtctagatctggaatatccctggaca

- 406: GTTAAAGCTTgtctgtctcaatgccacagt
410: CAGTctcgagATGTCCGGGGTGGTg
413: cagtTTCGAATCACTGCAGCATGATGTC
417: CAGTctcgagAGTGGTGTGATGATGACATG
5 420: cagtTTCGAATTAGTGATAAAAATAGAGTTCTTTTGTGAG
423: CAGTctcgagATGCACATAACTAATTTGGGATT
424: cagtTTCGAATTATACAAATGACGAATACCCTTT
425: GTTAAAGCTTttacaccttgcgcttcttcttggcgGCTGGAGACGGTGAC
428: CGTAAtctagaATGGACTTCAACAGGAACTTT
10 429: CGTAAtctagaGGACATAGTCCACCAGCG
430: GTTAAAGCTTTCAGTTGGATCCGAAAAAC
433: CGTAAtctagaGAATTAAAAAAAACACTCATCCCA
434: CGTAAtctagaCCAAAGGCAAAAGCAAAAA
435: GTTAAAGCTTTTAGCTAGCCATGGCAAGC
15 436: CGTAAtctagaATGCCCCGCCCC
437: GTTAAAGCTTCTACCCACCGTACTCGTCAAT
438: CGTAAtctagaATGTCTGACACGTCCAGAGAG
439: GTTAAAGCTTTCATCTTCTTCGCAGGAAAAAG
445: cgcGGATCCttatgggttctcacagcaaaa
20 446: CATTATTCCTCCTAGTTAGTCAaggcaacagccaatcaagag
447: ctcttgattggctgttgccfTGACTAACTAGGAGGAATAAATG
448: ttgattgcagtgacatggtgCATTATTCCTCCAGGTACTA
449: TAGTACCTGGAGGGAATAATGcaccatgtcactgcaatcaa
450: cgtaTCTAGAtagccgcagatgttggtatg
25 451: CGTAAtctagaGATCAAGTCCAACCTGGTGG
463: CAGTctcgaggaaagcttgtttaaggggc
464: cagtTTCGAAtttagcgacggcgacg
476: GTTAAAGCTTtACTTGTACAGCTCGTCCAT
477: CGTAAtctagaGTGAGCAAGGGCGAG
30 478: CAGTctcgagATGGAAGATTATACCAAATAGAGAAA
479: GTTAAAGCTTCTACATCTTCTTAATCTGATTGTCCa
482: CGTAAtctagaATGGCGCTGCAGCt
483: GTTAAAGCTTTCAGTCATTGACAGGAATTTTg

- 486: CGTAtctagaATGGAGCCGGCGGCG
487: GTTAAAGCTTTCAATCGGGGATGTCTg
492: CGTAtctagaATGCGCGAGGAGAACAAGGG
493: GTTAAAGCTTTCAGTCCCCTGTGGCTGTGc
5 494: CGTAtctagaATGGCCGAGCCTTG
495: GTTAAAGCTTttaTTGAAGATTTGTGGCTCC
504:CGTAtctagaGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTATG
CCCCGCCCC
505: GTTAAAGCTTCCCACCGTACTCGTCAATtc
10 508:CGTAtctagaGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTATG
GCCGAGCCTTG
509: GTTAAAGCTTTTGAAGATTTGTGGCTCCc
511:CGTAtctagaGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTGTG
AGCAAGGGCGAG
15 512:CGTAtctagaGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTCCG
CCGAAAAAAAAACGTAAAGTTGTGAGCAAGGGCGAG
513:GTTAAAGCTTtAAACTTTACGTTTTTTTTTCGGCGGCTTGTACAGCTCGTCCAT
515:CGTAtctagaGAAAATCTGTATTTTCAAAGTGAAAATCTGTATTTTCAAAGTGAT
TATAAAGATGATGATGATAAAATGGCCGAGCCTTG
20 558: CGTATCTAGAATGACCAGTTTTGAAGATGC
559:GTTAAAGCTTTCATGACTCATTTCATCCAT
561:CGTATCTAGAATGAGTCTCTTAAACTGTGAGAACAG
562:GTTAAAGCTTCTACACCCCCGCATCA
580: catgccatggATTTATGGTCATAGATATGACCTC
25 585: CAGTctcgagATGCAGATCTTCGTCAAGAC
586: GTTAAAGCTTgctagcttcgaaACCACCACGTAGACGTAAGAC
588: cagtTTCGAAGATTATAAAGATGATGATGATAAAATGGCCGAGCCTTG
612: CGGGGTACCatgaggtagcttatttcctgataaag
613: CGGGGTACCataattgtccaaatagttatggtagc
30 614: catgccatggCGGCAAGGCTCCTC
615: cggggtaccTTTATTTGTCAACACTGCCC
616: cggggtaccTGCGGGGTCTTTACTCG

677:TTACTATTCGAAGAAATTATTCATAATATTGCCCCGCCATCTGGCCCAAATTGG
 TGATGAAATGGATCATTAAGCTTGGAGTA
 678:TACTCCAAGCTTAATGATCCATTTTCATCACCAATTTGGGCCAGATGGCGGGCA
 ATATTATGAATAATTTCTTCGAATAGTAA
 5 682:TTACTACTCGAGAAAAAACTGAGCGAATGTCTGCGCCGCATTGGTGATGAAC
 TGGATAGCTAAGCTTGGAGTA
 683:TACTCCAAGCTTAGCTATCCAGTTCATCACCAATGCGGGCGCAGACATTCGCTC
 AGTTTTTTCTCGAGTAGTAA
 725: TTACTATTCGAAGAAATTATTCATAATATTGCC
 10 726: TACTCCAAGCTTACGGTTGAATATTATGATCCATTTTCATCACCAATTTGG
 727: TTACTATTCGAAGCCGGTGGTGCCGAAGAAATTATTCATAATATTGCC
 728: TACTCCAAGCTTAATGATCCATTTTCATCA
 733:TTACTACTCGAGGGTGCCATCGATGCCGAAGAAATTATTCATAATATTGCCCCG
 734:TACTCCTTCGAAGGCACCATGATCCATTTTCATCACCAATTTGG
 15 735:TACTCCTTCGAATTAATGATCCATTTTCATCACCAATTTG
 736:TTACTACTCGAGGGTGCCATCGATGCCAAAAAACTGAGCGAATGTCTGCG
 737: TACTCCTTCGAAGGCACCGCTATCCAGTTCATCACCAATG
 738:TACTCCTTCGAATTAGCTATCCAGTTCATCACCAATG

20

Table II: Cloned fusion proteins

Protein to be delivered by T3SS	Protein Seq. ID. No.	Backbone plasmid	Resulting plasmid name	Primers. Si_Nr.:	Primer Seq. ID No.
YopE1-138-MycHis	3	pBad-MycHisA (Invitrogen)	pBad_Si_1	285/286 (EGFP), 287/288 (sycE-YopE1-138)	44/45 and 46/47
YopE1-138-MycHis	3	pBad-MycHisA (Invitrogen)	pBad_Si_2	287/288 (sycE-YopE1-138)	46/47
YopE1-138-IpgB1	4	pBad_Si_2	pSi_16	292/293	48/49
YopE1-138-SopE	5	pBad_Si_2	pSi_20	296/297	50/51
YopE1-138-Rac1 Q61L	26	pBad_Si_2	pSi_22	299/300	52/53

YopE1-138-RhoA Q61E	27	pBad_Si_2	pSi_24	301/302	54/55
YopE1-138-SopE-MycHis	135	pBad_Si_2	pSi_28	296/306	50/56
YopE1-138-SopB	6	pBad_Si_2	pSi_30	307/308	57/58
YopE1-138-FADD	28	pBad_Si_2	pSi_37	367/386	76/79
YopE1-138-OspF	7	pBad_Si_2	pSi_38	317/318	59/60
YopE1-138-BepG 715-end	136	pBad_Si_2	pSi_43	324/351	61/67
YopE1-138-Rac1 Q61L-MycHis	137	pBad_Si_2	pSi_51	299/339	52/62
YopE1-138-Slmb1-VhH4	32	pBad_Si_2	pSi_53	341/342	63/64
YopE1-138-Bad	29	pBad_Si_2	pSi_57	346/347	65/66
YopE1-138-SptP	8	pBad_Si_2	pSi_64	364/365	74/75
YopE1-138-NLS-Slmb1-VhH4	33	pBad_Si_2	pSi_70	369/342	77/64
YopE1-138-Bid	24	pBad_Si_2	pSi_85	387/391	80/82
YopE1-138-t-Bid	25	pBad_Si_2	pSi_87	389/391	81/82
YopE1-138-Caspase3 p17	22	pBad_Si_2	pSi_97	403/406	83/84
YopE1-138-GPCR GNA12	30	pBad_Si_2	pSi_103	410/413	85/86
YopE1-138-Caspase3 p10/12	23	pBad_Si_2	pSi_106	417/420	87/88
YopE1-138-lpgD	9	pBad_Si_2	pSi_111	423/424	89/90
YopE1-138-Slmb1-VhH4-NLS	34	pBad_Si_2	pSi_112	341/425	63/91
YopE1-138-z-Bid	19	pBad_Si_2	pSi_116	428/430	92/94
YopE1-138-z-t-Bid	20	pBad_Si_2	pSi_117	429/430	93/94
YopE1-138-BepA E305-end	11	pBad_Si_2	pSi_118	433/435	95/97
YopE1-138-BepA	10	pBad_Si_2	pSi_119	434/435	96/97
YopE1-138-ET1	36	pBad_Si_2	pSi_120	436/437	98/99
YopE1-138-z-BIM	21	pbad_Si_1	pSi_121	438/439	100/101
YopE1-138-VhH4 nanobody recognizing EGFP	31	pBad_Si_2	pSi_124	451/373	108/78
YopE1-138-TEV protease S219V	42	pBad_Si_2	pSi_132	463/464	109/110
YopE1-138-EGFP	37	pBad_Si_2	pSi_140	477/476	112/111
YopE1-138-Cdk1	14	pBad_Si_2	pSi_143	478/479	113/114
YopE1-138-Mad2	15	pBad_Si_2	pSi_145	482/483	115/116
YopE1-138-Ink4A	16	pBad_Si_2	pSi_147	486/487	117/118
YopE1-138-Ink4B	17	pBad_Si_2	pSi_150	492/493	119/120
YopE1-138-Ink4C	18	pBad_Si_2	pSi_151	494/495	121/122

YopE1-138-TIFA	13	pBad_Si_2	pSi_153	558/559	131/132
YopE1-138-2x TEVsite - ET1	41	pBad_Si_2	pSi_156	504/505	123/124
YopE1-138- 2xTEVsite - EGFP - NLS	39	pBad_Si_2	pSi_159	511/513	127/129
YopE1-138- 2xTEVsite - NLS - EGFP	38	pBad_Si_2	pSi_160	512/476	128/111
YopE1-138-2x TEVsite - INK4C	40	pBad_Si_2	pSi_161	508/509	125/126
YopE1-138-2x TEVsite - Flag - INK4C	43	pBad_Si_2	pSi_164	515/509	130/126
YopE1-138-murine Traf6	12	pBad_Si_2	pSi_166	561/562	133/134
YopE1-138-Y. enterocolitica codon optimized murine tBid BH3 part	138	pBad_Si_2	pSi_318	677/678	148/149
YopE1-138-Y. enterocolitica codon optimized murine Bax BH3 part	139	pBad_Si_2	pSi_322	682/683	150/151
SteA1-20	140	pBad- MycHisA (Invitrogen)	pSi_266	580/612	152/153
SteA	141	pBad- MycHisA (Invitrogen)	pSi_267	580/613	152/154
SopE1-81	142	pBad- MycHisA (Invitrogen)	pSi_268	614/615	155/156
SopE1-105	143	pBad- MycHisA (Invitrogen)	pSi_269	614/616	155/157
SteA1-20-S. enterica codon optimized murine tBid	144	pSi_266	pSi_270	synthetic construct	/
SteA-S. enterica codon optimized murine tBid	145	pSi_267	pSi_271	synthetic construct	/
SopE1-81-S. enterica codon optimized	146	pSi_268	pSi_272	synthetic construct	/

murine tBid					
SopE1-105-S. enterica codon optimized murine tBid	147	pSi_269	pSi_273	synthetic construct	/
YopE1-138-Y. enterocolitica codon optimized Ink4A 84- 103	158	pBad_Si_2	pSi_362	745/746	172/173
YopE1-138-Y. enterocolitica codon optimized p107/RBL1 657-662 (AAA02489.1)	159	pBad_Si_2	pSi_363	747/748	174/175
YopE1-138-Y. enterocolitica codon optimized p21 141- 160 (AAH13967.1)	160	pBad_Si_2	pSi_364	749/750	176/177
YopE1-138-Y. enterocolitica codon optimized p21 145- 160 (AAH13967.1)	161	pBad_Si_2	pSi_366	753/754	178/179
YopE1-138-Y. enterocolitica codon optimized p21 17-33 (AAH13967.1)	162	pBad_Si_2	pSi_367	755/756	180/181
YopE1-138-Y. enterocolitica codon optimized cyclin D2 139-147 (CAA48493.1)	163	pBad_Si_2	pSi_368	757/758	182/183
SteA-Ink4a-MycHis	164	pSi_267	pSi_333	703/704	184/185
SopE1-105-Ink4a- MycHis	165	pSi_269	pSi_334	703/704	184/185
SteA-Ink4c-MycHis	166	pSi_267	pSi_335	PCR1: 705/706; PCR2: 707/708; overlappin g PCR: 705/708	186/187, 188/189
SopE1-105-Ink4c- MycHis	167	pSi_269	pSi_336	PCR1: 705/706; PCR2: 707/708; overlappin g PCR: 705/708	186/187, 188/189

SteA-Mad2-MycHis	168	pSi_267	pSi_337	709/710	190/191
SopE1-105-Mad2-MycHis	169	pSi_269	pSi_338	709/710	190/191
SteA-Cdk1-MycHis	170	pSi_267	pSi_339	711/712	192/193
SopE1-105-Cdk1-MycHis	171	pSi_269	pSi_340	711/712	192/193
YopE1-138-Y. enterocolitica codon optimized murine tBid	194	pBad_Si_2	pSi_315	synthetic construct	/
YopE1-138-Ubiquitin	195	pBad_Si_2	pSi_236	585/586	197/198
YopE1-138- Ubiquitin-Flag- INK4C-MycHis	196	pSi_236	pSi_237_II	588/509	199/126
YopE1-138-(Y. enterocolitica codon optimized murine tBid BH3 part) ready for insertion of further domains	200	pBad_Si_2	pSi_357	733/735	204/205
YopE1-138-(Y. enterocolitica codon optimized murine BAX BH3 part) ready for insertion of further domains	201	pBad_Si_2	pSi_358	736/738	206/207
YopE1-138-(Y. enterocolitica codon optimized murine tBid BH3 part) ₂	202	pSi_357	pSi_371	733/734	204/208
YopE1-(138-Y. enterocolitica codon optimized murine tBid BH3 part- Y. enterocolitica codon optimized murine BAX BH3 part	203	pSi_358	pSi_373	733/734	204/208
YopE ₁₋₁₃₈ - codon optimized murine tBid BH3 extended part	209	pBad_Si_2	pSi_353	725/726	212/213
YopE ₁₋₁₃₈ -10 Aa linker - Y. enterocolitica codon optimized murine tBid BH3 part	210	pBad_Si_2	pSi_354	727/728	214/215
YopE1-(138-Y. enterocolitica codon	211	pSi_357	pSi_374	736/737	206/216

optimized murine Bax BH3 part- Y. enterocolitica codon optimized murine tBid BH3 part					
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Table III: Mutators for genetic modification

Mutator/ Construct	To be inserted onto:	Backbone plasmid	Resulting plasmid name	Primers Si_Nr.:	Primers Seq.Id No.	used with parent strain
YopE ₁₋₁₃₈ - tBID BH3	pYV	pKNG101	pSi_408	Synthetic gene	/	/
YopE ₁₋₁₃₈ - (tBID BH3) ₂	pYV	pKNG101	pSi_419	Synthetic gene	/	Strain mutated with pSi_408

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Yop secretion. Induction of the *yop* regulon was performed by shifting the culture to 37°C in BHI-Ox (secretion-permissive conditions) [31]. As carbon source glucose was added (4 mg/ml).

Total cell and supernatant fractions were separated by centrifugation at 20 800 g for 10 min at 4°C. The cell pellet was taken as total cell fraction. Proteins in the supernatant were precipitated with trichloroacetic acid 10% (w/v) final for 1 h at 4°C. After centrifugation (20 800 g for 15 min) and removal of the supernatant, the resulting pellet was washed in ice-cold Acetone over-night. The samples were centrifuged again, the supernatant was discarded and the pellet was air-dried and resuspended in 1x SDS loading dye.

15 Secreted proteins were analysed by SDS-PAGE; in each case, proteins secreted by 3×10^8 bacteria were loaded per lane. Detection of specific secreted proteins by immunoblotting was performed using 12.5% SDS-PAGE gels. For detection of proteins in total cells, 2×10^8 bacteria were loaded per lane, if not stated otherwise, and proteins were separated on 12.5% SDS-PAGE gels before detection by immunoblotting.

Immunoblotting was carried out using rat monoclonal antibodies against YopE (MIPA193 – 13A9 ; 1:1000, [32]). The antiserum was preabsorbed twice overnight against *Y. enterocolitica* ΔHOPEMT *asd* to reduce background staining. Detection was performed with secondary antibodies directed against rat antibodies and conjugated to horseradish peroxidase (1:5000; Southern biotech), before development with ECL chemiluminescent substrate (LumiGlo, KPM).

Cell culture and infections. HeLa Ccl2, swiss 3T3 fibroblast cells, 4T1, B16F10 and D2A1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 2mM L-Glutamine (cDMEM). HUVECs were isolated and cultivated as described [33]. Jurkat and 4T1 cells were cultured in RPMI 1640 supplemented with 10% FCS and 2mM L-Glutamine. *Y. enterocolitica* were grown in BHI with additives overnight at RT, diluted in fresh BHI to an OD₆₀₀ of 0.2 and grown for 2h at RT before a temperature shift to a 37°C waterbath shaker for further 30 min or for 1h in case of delivery of EGFP. Finally, the bacteria were collected by centrifugation (6000 rcf, 30 sec) and washed once with DMEM supplemented with 10 mM HEPES and 2 mM L-glutamine. *S. enterica* were grown in LB with additives overnight at 37°C and either diluted 1:40 in fresh LB and grown for 2.5h at 37°C (SpiI T3SS inducing conditions) or the overnight culture was further incubated at 37°C (SpiII T3SS inducing conditions). Finally, the bacteria were collected by centrifugation (6000 rcf, 30 sec) and washed once with DMEM supplemented with 10 mM HEPES and 2 mM L-glutamine. Cells seeded in 96-well (for Immunofluorescence) or 6-well (for Western blotting) plates were infected at indicated MOIs in DMEM supplemented with 10 mM HEPES and 2 mM L-glutamine. After adding bacteria, plates were centrifuged for 1 min at 1750 rpm and placed at 37°C for indicated time periods. Extracellular bacteria were killed by gentamicin (100 mg/ml) if indicated. In case of immunofluorescence analysis, infection assays were stopped by 4% PFA fixation. For Western blot analysis cells were washed twice with ice-cold PBS and Phospho-safe lysis buffer (Novagen) was added to lyse the cells. After incubation on ice, the cells were centrifuged (16 000 rcf, 25 min, 4°C). Supernatants were collected and analyzed for total protein content by Bradford BCA assay (Pierce) before SDS PAGE and Western blotting using anti-Phospho-Akt (Ser473 and T308, both Cell Signaling), anti-Actin (Millipore), Anti-Bid (Cell Signaling), anti-Myc (Santa Cruz), anti-p38 (Cell Signaling), anti-phospho-p-38 (Thr180/Tyr182; Cell Signaling), anti-Caspase-3 p17 (Cell Signaling) and anti-Ink4C (Cell Signaling) antibody.

Western blotting of T3SS translocated proteins from infected cells. HeLa cells in 6-well plates were infected at an MOI of 100 as described above. In case of coinfection with the TEV protease translocating *Y. enterocolitica* strain, the OD₆₀₀ of the strains was set and the two bacterial suspensions were mixed in a tube at a ratio of 1:1 (if not otherwise indicated) before addition to the cells. At the end of the infection, the cells were washed twice with ice-cold PBS and collected by scraping in a small volume of ice-cold PBS. After centrifugation (16 000 rcf, 5 min, 4°C) the pellet was dissolved in 0.002% digitonin supplemented with a protease inhibitor cocktail (Roche complete, Roche). The dissolved pellets were incubated for 5 minutes on ice and then centrifuged (16 000 rcf, 25 min, 4°C). Supernatants were collected and analyzed for total protein content by Bradford BCA assay (Pierce) before SDS PAGE and Western blotting using an anti-Myc (Santa Cruz, 9E11) or anti-Ink4C (Cell Signaling) antibody.

Immunofluorescence. Cell seeded in 96-well plates (Corning) were infected as described above and after fixation with 4% PFA the cells were washed three times with PBS. The wells were then blocked using 5% goat serum in PBS 0.3% Triton X-100 for 1h at RT. The primary antibody (anti-Myc, Santa Cruz, 1:100) was diluted in PBS with 1% BSA and 0.3% Triton X-100 and cells were incubated overnight at 4°C. Cells were washed 4 times with PBS before the secondary antibody (AF 488 anti-mouse, life technologies, 1:250) diluted in PBS with 1% BSA and 0.3% Triton X-100 was added. If needed Hoechst DNA staining (life technologies, 1:2500) and/or actin staining (Dy647-Phalloidin, DyeOmics) were included. In some cases only the DNA and/or actin stain was applied directly after washing the PFA off. Cells were incubated for 1h at RT, washed three times with PBS and analyzed by automated image analysis as described below.

Automated Microscopy and Image Analysis. Images were automatically acquired with an ImageXpress Micro (Molecular devices, Sunnyvale, USA). Quantification of anti-Myc staining intensities was performed using MetaXpress (Molecular devices, Sunnyvale, USA). Regions within cells excluding nuclear regions and regions containing bacteria were manually chosen (circles with an area of 40 pixels) and average intensity was recorded.

- TNF α stimulation and Western blotting of phospho-p38.** HeLa cells seeded in 6-well plates were infected with an MOI of 100 as described above. 30min p.i. Gentamicin was added and 45 min p.i. TNF α was added (10 ng/ml). 1 h 15min p.i. cells were washed twice with ice-cold PBS and Phospho-safe lysis buffer (Novagen) was added to lyse the cells. After incubation on ice, the cells were centrifuged (16 000 rcf, 25 min, 4°C). Supernatants were collected and analyzed for total protein content by Bradford BCA assay (Pierce) before SDS PAGE and Western blotting using an anti-Phospho-p38, total p38 antibodies (Cell Signaling) and anti-Actin antibody (Millipore).
- cAMP level determination of infected HeLa cells.** HeLa cells seeded in 96-well plates were infected as described above. 30 min before the infection cDMEM was changed to DMEM supplemented with 10 mM HEPES and 2 mM L-glutamine and 100 μ M 3-Isobutyl-1-methylxanthin (IBMX, Sigma Aldrich). 60 min p.i. Gentamicin was added and cells were further incubated at 37°C for another 90 min. Determination of cAMP was performed using a competitive ELISA according to the manufacturers instructions (Amersham, cAMP Biotrak, RPN225). As a positive control indicated amount of cholera toxin (C8052, Sigma Aldrich) was added for 1 h to cells in DMEM supplemented with 10 mM HEPES and 2 mM L-glutamine and 100 μ M IBMX.
- Sample Preparation for Phosphoproteomics.** For each condition, two 6-well plates of HeLa CCL-2 cells were grown to confluency. Cells were infected for 30 min as described above. At the indicated time-points, the plates were put on ice and washed twice with ice-cold PBS. Samples were then collected in urea solution [8 M Urea (AppliChem), 0.1 M Ammoniumbicarbonate (Sigma), 0.1% RapiGest (Waters), 1 $\times$ PhosSTOP (Roche)]. The samples were briefly vortexed, sonicated at 4 °C (Hielscher), shaken for 5 min on a thermomixer (Eppendorf) and centrifuged for 20 min at 4 °C and 16'000g. Supernatants were collected and stored at –80 °C for further processing. BCA Protein Assay (Pierce) was used to measure protein concentration.
- Phosphopeptide Enrichment.** Disulfide bonds were reduced with tris(2-carboxyethyl)phosphine at a final concentration of 10 mM at 37 °C for 1 h. Free thiols were alkylated with 20 mM iodoacetamide (Sigma) at room temperature for 30 min in the dark. The excess of iodoacetamide was quenched with N-acetyl cysteine at a final concentration of 25 mM for 10 min at room temperature. Lys-C endopeptidase (Wako) was added to a final

enzyme/protein ratio of 1:200 (w/w) and incubated for 4 h at 37 °C. The solution was subsequently diluted with 0.1 M ammoniumbicarbonate (Sigma) to a final concentration below 2 M urea and digested overnight at 37 °C with sequencing-grade modified trypsin (Promega) at a protein-to-enzyme ratio of 50:1. Peptides were desalted on a C18 Sep-Pak cartridge (Waters) and dried under vacuum. Phosphopeptides were isolated from 2 mg of total peptide mass with TiO₂ as described previously [34]. Briefly, dried peptides were dissolved in an 80% acetonitrile (ACN)–2.5% trifluoroacetic acid (TFA) solution saturated with phthalic acid. Peptides were added to the same amount of equilibrated TiO₂ (5-µm bead size, GL Sciences) in a blocked Mobicol spin column (MoBiTec) that was incubated for 30 min with end-over-end rotation. The column was washed twice with the saturated phthalic acid solution, twice with 80% ACN and 0.1% TFA, and finally twice with 0.1% TFA. The peptides were eluted with a 0.3 M NH₄OH solution. The pH of the eluates was adjusted to be below 2.5 with 5% TFA solution and 2 M HCl. Phosphopeptides were again desalted with microspin C18 cartridges (Harvard Apparatus).

LC-MS/MS analysis. Chromatographic separation of peptides was carried out using an EASY nano-LC system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75 µm x 45 cm) packed in-house with 1.9 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Aliquots of 1 µg total phosphopeptide sample were analyzed per LC-MS/MS run using a linear gradient ranging from 98% solvent A (0.15% formic acid) and 2% solvent B (98% acetonitrile, 2% water, 0.15% formic acid) to 30% solvent B over 120 minutes at a flow rate of 200 nl/min. Mass spectrometry analysis was performed on a dual pressure LTQ-Orbitrap mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan (acquired in the Orbitrap) was followed by collision-induced dissociation (CID, acquired in the LTQ) of the 20 most abundant precursor ions with dynamic exclusion for 30 seconds. For phosphopeptide analysis the 10 most abundant precursor ions were subjected to CID with enabled multistage activation. Total cycle time was approximately 2 s. For MS1, 10⁶ ions were accumulated in the Orbitrap cell over a maximum time of 300 ms and scanned at a resolution of 60,000 FWHM (at 400 m/z). MS2 scans were acquired using the normal scan mode, a target setting of 10⁴ ions, and accumulation time of 25 ms. Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 32%, and one microscan was acquired for each spectrum.

Label-free quantification and database searching. The acquired raw-files were imported into the Progenesis software tool (Nonlinear Dynamics, Version 4.0) for label-free quantification using the default parameters. MS2 spectra were exported directly from Progenesis in mgf format and searched using the MASCOT algorithm (Matrix Science, Version 2.4) against a decoy database [35] containing normal and reverse sequences of the predicted SwissProt entries of *Homo sapiens* (www.ebi.ac.uk, release date 16/05/2012) and commonly observed contaminants (in total 41,250 sequences) generated using the SequenceReverser tool from the MaxQuant software (Version 1.0.13.13). To identify proteins originating from *Y. enterocolitica*, non phosphopeptide enriched samples were searched against the same database above including predicted SwissProt entries of *Y. enterocolitica* (www.ebi.ac.uk, release date 15/08/2013) The precursor ion tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 Da. The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues unless followed by proline), 2 missed cleavages were allowed, carbamidomethylation (C) was set as fixed modification and phosphorylation (S,T,Y) or oxidation (M) as a variable modification for TiO2 enriched or not enriched samples, respectively. Finally, the database search results were exported as an xml-file and imported back to the Progenesis software for MS1 feature assignment. For phosphopeptide quantification, a csv-file containing the MS1 peak abundances of all detected features was exported and for not enriched samples, a csv-file containing all protein measurements based on the summed feature intensities of all identified peptides per protein was created. Importantly, the Progenesis software was set that proteins identified by similar sets of peptides are grouped together and that only non-conflicting peptides with specific sequences for single proteins in the database were employed for protein quantification. Both files were further processed using the in-house developed SafeQuant v1.0 R script (unpublished data, available at <https://github.com/eahrne/SafeQuant/>). In brief, the software sets the identification level False Discovery Rate to 1% (based on the number of decoy protein sequence database hits) and normalizes the identified MS1 peak abundances (Extracted Ion Chromatogram, XIC) across all samples, *i.e.* the summed XIC of all confidently identified peptide features is scaled to be equal for all LC-MS runs. Next, all quantified phosphopeptides/proteins are assigned an abundance ratio for each time point, based on the median XIC per time point. The statistical significance of each ratio is given by its q-value (False Discovery Rate adjusted p-values), obtained by calculating modified t-statistic p-values [36] and adjusting for multiple testing [37]. The location of the

phosphorylated residues was automatically assigned by MASCOT (score >10). All annotated spectra together with the MS raw files and search parameters employed, will be deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [38].

- 5 **Sequence alignment** was performed using EMBL-EBI web based ClustalW2 multiple sequence alignment tool at <http://www.ebi.ac.uk/Tools/msa/clustalw2/>.

Dose-escalation study

- 10 All animal experiments were approved (license 1908; Kantonales Veterinärämte Basel-Stadt) and performed according to local guidelines (Tierschutz-Verordnung, Basel-Stadt) and the Swiss animal protection law (Tierschutz-Gesetz). 6 week old C57Bl/6 and BALB/c mice were ordered from Janvier Labs. After at least one week of accommodation, mice were infected with *Y. enterocolitica* MRS40 Δ HOPEMT or *S. typhimurium* Δ aroA by injection into the tail vein. Throughout the experiment, mice were scored for behavior and physical appearance, and
15 surface temperature, as well as body weight was measured. The inoculum i.v. administered to the mice was validated by dilution plating. On respective days postinfection, mice were sacrificed by CO₂ inhalation. A blood sample was immediately isolated through aspiration from the heart. Liver, spleen, lung and the tumor were isolated and their weight determined. The organs and the tumor were homogenized. CFU in each sample was determined by
20 spotting of serial dilutions onto LB agar plates containing nalidixic acid (35 ug/ml).

Biodistribution in B16-F10 and 4T1 tumor allograft mouse models

- 25 All animal experiments were approved (license 1908; Kantonales Veterinärämte Basel-Stadt) and performed according to local guidelines (Tierschutz-Verordnung, Basel-Stadt) and the Swiss animal protection law (Tierschutz-Gesetz). 6 week old C57Bl/6 and BALB/c mice were ordered from Janvier Labs. After at least one week of accommodation, mice were anesthetized using isoflurane and 100 ul B16-F10 or 4T1 cells (1×10^5 - 1×10^6 cells) were subcutaneously injected into the flank of C57Bl/6 and BALB/c, respectively. Throughout the experiment, mice were scored for behavior and physical appearance, and surface temperature, as well as
30 body weight was measured.

Once tumors had developed, mice were administered an 8 mg/ml desferal solution (10 ml/kg) through i.p. injection. On the following day, mice were infected with *Y. enterocolitica* MRS40

or *Y. enterocolitica* MRS40 Δ HOPEMT (2×10^5 , 1×10^6 or 1×10^7 bacteria) by injection into the tail vein. The inoculum i.v. administered to the mice was validated by dilution plating. In some experiments, tumor progression was followed by daily measurements of tumor length and width with digital calipers. Tumor volume was determined as $0.523 \times \text{length} \times \text{width}^2$. On
5 respective days postinfection, mice were sacrificed by CO₂ inhalation. A blood sample was immediately isolated through aspiration from the heart. Liver, spleen, lung and the tumor were isolated and their weight determined. The organs and the tumor were homogenized. CFU in each sample was determined by spotting of serial dilutions onto LB agar plates containing nalidixic acid (35 ug/ml).

10

B) RESULTS

A protein delivery system based on type 3 secretion of YopE fusion proteins

While the very N-terminus of the *Y. enterocolitica* T3SS effector YopE (SEQ ID No. 1) contains the secretion signal sufficient to translocate heterologous proteins [19], the
15 chaperone-binding site (CBS) for its chaperone (SycE) is not included [39]. We selected the N-terminal 138 amino acids of YopE (SEQ ID No. 2) to be fused to proteins to be delivered, as this had been shown to give best results for translocation of other heterologous T3S substrates [21]. As these N-terminal 138 amino acids of YopE contain the CBS, we further decided to coexpress SycE. The SycE-YopE₁₋₁₃₈ fragment cloned from purified *Y.*
20 *enterocolitica* pYV40 virulence plasmid contains the endogenous promoters of YopE and of its chaperone SycE (Fig. 9). Therefore, SycE and any YopE₁₋₁₃₈ fusion protein are induced by a rapid temperature shift from growth at RT to 37°C. Culture time at 37°C will affect fusion protein amount present in bacteria. A multiple cloning site (MCS) was added at the 3' end of YopE₁₋₁₃₈ (Fig. 9 B) followed by a Myc and a 6xHis tag and a Stop codon.

25 The background strain was carefully selected. First, to limit the translocation of endogenous effectors, we used a *Y. enterocolitica* strain that was deleted for all known effectors, Yop H, O, P, E, M and T (named Δ HOPEMT) [40]. In addition, we occasionally used an auxotroph mutant that cannot grow in absence of exogenous *meso*-2,6-diaminopimelic acid [41]. This strain was deleted for the aspartate-beta-semialdehyde dehydrogenase gene (Δ *asd*), and
30 classified as biosafety level 1 by the Swiss safety agency (amendment to A010088/2). In addition, we deleted the adhesion proteins YadA and/or InvA to offer a larger choice of background strains. While the use of the *yadA* or *yadA/invA* strains reduce the background signalling induced [42], the delivered protein amount is affected as well [43].

Characterization of YopE fusion protein delivery into eukaryotic cells

In an *in-vitro* secretion assay (see Fig. 1 A), protein secretion into the surrounding liquid is artificially induced. After TCA based protein precipitation, Western blot analysis with anti-YopE antibody was used to determine protein amounts secreted (Fig. 1 B). While a wt strain secreted full length YopE, the $\Delta HOPEMT$ *asd* strains did not. Upon presence of YopE₁₋₁₃₈-Myc-His (further termed YopE₁₋₁₃₈-Myc; SEQ_ID_No._3) a smaller YopE band became visible (Fig. 1 B). Hence, the YopE₁₋₁₃₈ fragment is well secreted in the set up described here. To analyze homogeneity of protein translocation into eukaryotic cells, we infected HeLa cells with the YopE₁₋₁₃₈-Myc encoding strain and stained the Myc tag by IF (Fig. 2 A and B). While in the beginning only the bacteria were stained, at 30 min post infection (p.i.) cell outlines start to be visible, which is enhanced upon increased infection time (Fig. 2 B). This trend is well reflected by the Myc tag staining intensity inside HeLa cells (Fig. 2 A and B). The YopE₁₋₁₃₈-Myc can be detected everywhere in the cells (Fig. 2 A), except in the nuclei [44]. Remarkably, most if not all cells were reached by this approach in a comparable way. As *Y. enterocolitica* is known to infect many different cell types [45], we followed YopE₁₋₁₃₈-Myc delivery into various cell lines. The same homogenous anti-Myc IF staining was observed in infected murine fibroblasts, Jurkat cells and HUVECs (Fig. 11). Even more, tuning the MOI up or down allows modulating the protein amount delivered (Fig. 2 C), while still most of the cells remain targeted. A low bacterial number will not result in few cells with lots of delivered protein but rather with most cells containing a low amount of delivered protein (Fig. 2 C).

Redirection of T3SS delivered proteins to the nucleus

As YopE itself localized to the cytoplasm (Fig. 2 A), it is of special interest to test if the YopE₁₋₁₃₈ fragment hampers localization of nuclear fusion proteins. We therefore added the SV40 NLS to the C-terminus (and N-terminus, similar results) of YopE₁₋₁₃₈-EGFP (SEQ ID No. 39 and SEQ ID No. 38, respectively). While YopE₁₋₁₃₈-EGFP (SEQ ID No. 37) led to a weak cytoplasmic staining, YopE₁₋₁₃₈-EGFP-NLS gave rise to a stronger nuclear EGFP signal in HeLa cells infected (Fig. 3). This indicates that the YopE₁₋₁₃₈ fragment is compatible with the use of an NLS. While mCherry had already been used in plant pathogens [46], this represents a successful delivery of a GFP-like protein via human or animal pathogenic

bacteria encoding a T3SS. This validates the SycE and YopE₁₋₁₃₈ dependent strategy to be very promising for delivery of many proteins of choice.

Removal of the YopE₁₋₁₃₈ appendage after translocation of the fusion protein to the eukaryotic cell

While for bacterial delivery the YopE₁₋₁₃₈ fragment is of great benefit, it might hamper the fusion proteins function and/or localization. Therefore, its removal after protein delivery would be optimal. To this end, we introduced two TEV cleavage sites (ENLYFQS) [47-49] in between YopE₁₋₁₃₈ and a fusion partner (the transcriptional regulator ET1-Myc (SEQ ID No. 36 and 41) [50] and human INK4C (SEQ ID No. 40 and SEQ ID No. 43)). To keep the advantages of the presented method, we further fused the TEV protease (S219V variant; [51]) to YopE₁₋₁₃₈ (SEQ ID No. 42) in another *Y. enterocolitica* strain. HeLa cells were infected with both strains at once. To allow analysis of the translocated fraction of proteins only, infected HeLa cells were lysed at 2 h p.i. (Fig. 4) with Digitonin, which is known not to lyse the bacteria ([52]; see Fig. 11 for control). Western blot analysis revealed the presence of the YopE₁₋₁₃₈-2xTEV-cleavage-site-ET1-Myc or YopE₁₋₁₃₈-2xTEV-cleavage-site-Flag-INK4C-Myc only when cells had been infected with the corresponding strain (Fig. 4 A and C). Upon overnight digestion of this cell-lysate with purified TEV protease, a shifted band could be observed (Fig. 4 A and C). This band corresponds to ET1-Myc (Fig. 4 C) or Flag-INK4C (Fig. 4 A) with the N-terminal remnants of the TEV cleavage site, most likely only one Serine. Upon coinfection of cells with the strain delivering the TEV protease, the same cleaved ET1-Myc or Flag-INK4C fragment became visible, indicating that the TEV protease delivered via T3SS is functional and that single cells had been infected by both bacterial strains (Fig. 4 A and C). While cleavage is not complete, the majority of translocated protein is cleaved already 2h post infection and even over-night digestion with purified TEV protease did not yield better cleavage rates (Fig. 4 B). As reported, TEV protease dependent cleavage might need optimization dependent on the fusion protein [53,54]. TEV protease dependent removal of the YopE₁₋₁₃₈ appendage after translocation hence provides for the first time a T3SS protein delivery of almost native heterologous proteins, changing the amino acid composition by only one N-terminal amino acid.

An alternative approach to the TEV protease dependent cleavage of the YopE fragment consisted in incorporating Ubiquitin into the fusion protein of interest. Indeed, Ubiquitin is processed at its C-terminus by a group of endogenous Ubiquitin-specific C-terminal proteases

(Deubiquitinating enzymes, DUBs). As the cleavage is supposed to happen at the very C-terminus of Ubiquitin (after G76), the protein of interest should be free of additional amino acid sequence. This method was tested on the YopE1-138-Ubiquitin-Flag-INK4C-MycHis fusion protein. In control cells infected by YopE1-138-Flag-INK4C-MycHis-expressing bacteria, a band corresponding to YopE1-138-Flag-INK4C-MycHis was found, indicative of efficient translocation of the fusion protein (Figure 23). When cells were infected for 1h with YopE1-138-Ubiquitin-Flag-INK4C-MycHis-expressing bacteria, an additional band corresponding to the size of Flag-INK4C-MycHis was visible, indicating that part of the fusion protein was cleaved. This result shows that the introduction of Ubiquitin into the fusion protein enables to cleave off the YopE1-138 fragment without a need for an exogenous protease.

Translocation of type III and type IV bacterial effectors

SopE from *Salmonella enterica* is a well-characterized guanine nucleotide exchange factor (GEF) that interacts with Cdc42, promoting actin cytoskeletal remodeling [55]. Whereas the translocation of YopE₁₋₁₃₈-Myc into HeLa cells has no effect, translocated YopE₁₋₁₃₈-SopE (SEQ ID No. 5 and 135) induced dramatic changes in the actin network (Fig. 5 A). Similar results were obtained with another GEF effector protein, IpgB1 from *Shigella flexneri* (SEQ ID No. 4). Remarkably, first changes in the actin cytoskeleton were observed as fast as 2 min p.i. (Fig. 5 A). Therefore, one can conclude that T3SS dependent protein delivery happens immediately after infection is initiated by centrifugation. To proof strict T3SS dependent transport, one of the T3SS proteins forming the translocation pore into the eukaryotic cell membrane was deleted (YopB, see [56]) (Fig. 11).

During *Salmonella* infection, SopE translocation is followed by translocation of SptP, which functions as a GTPase activating protein (GAP) for Cdc42 [57]. Whereas the translocation of YopE₁₋₁₃₈-SopE-Myc (SEQ ID No. 135) alone triggered massive F-actin rearrangements, the co-infection with YopE₁₋₁₃₈-SptP (SEQ ID No. 8) expressing bacteria abolished this effect in a dose dependent manner (Fig. 5 B). An anti-Myc staining indicated that this inhibition was not due to a reduced level of YopE₁₋₁₃₈-SopE-Myc translocation (Fig. 5 B). Together these results showed that the co-infection of cells with two bacterial strains is a valid method to deliver two different effectors into single cells to address their functional interaction.

The *S. flexneri* type III effector OspF functions as a phosphothreonine lyase that dephosphorylates MAP kinases p38 and ERK [58]. To test the functionality of translocated YopE₁₋₁₃₈-OspF (SEQ ID No. 7), we monitored the phosphorylation of p38 after stimulation with TNF α . In uninfected cells or in cells infected with YopE₁₋₁₃₈-Myc expressing bacteria, TNF α -induced p38 phosphorylation. In contrast, after translocation of YopE₁₋₁₃₈-OspF, TNF α -induced phosphorylation was abolished, showing that the delivered OspF is active towards p38 (Fig. 6 A).

During *Salmonella* infection, the type III effector SopB protects epithelial cells from apoptosis by sustained activation of Akt [59]. Whereas the translocation of YopE₁₋₁₃₈-Myc or YopE₁₋₁₃₈-SopE had no effect on Akt, the translocation of YopE₁₋₁₃₈-SopB (SEQ ID No. 6) induced a strong phosphorylation of Akt at T308 and S473, reflecting the active form (Fig. 6 B). Similar results were obtained with the SopB-homolog from *S. flexneri* (IpgD, SEQ ID No. 9). Altogether, our results show that the YopE₁₋₁₃₈-based delivery system functions for all T3S effectors tested so far, and that it allows investigating proteins involved in the control of central cellular functions including the cytoskeleton, inflammation and cell survival.

A number of bacteria, including *Agrobacterium tumefaciens*, *Legionella pneumophila* and *Bartonella henselae*, use type IV secretion to inject effectors into cells. We tested whether the type IV effector BepA from *B. henselae* could be translocated into HeLa cells using our tool. Full length BepA (SEQ ID No. 10) and BepA_{E305-end} (SEQ ID No. 11) containing the C-terminal Bid domain, were cloned and cells were infected with the respective strains. As BepA was shown to induce the production of cyclic AMP (cAMP) [60], the level of cAMP in HeLa cells was measured after infection. Whereas the translocation of the Bid domain of the *B. henselae* effector BepG (SEQ ID No. 136) failed to induce cAMP, full length BepA and BepA_{E305-end} triggered cAMP production in expected amounts [60] (Fig. 6 C). This result shows, that type IV effectors can also be effectively delivered by the YopE₁₋₁₃₈-based delivery system into host cell targets and that they are functional.

Translocation of eukaryotic proteins into epithelial cells

To show that human proteins can translocate via type III secretion we fused human apoptosis inducers for delivery by *Y. enterocolitica* to YopE₁₋₁₃₈ or for delivery by *S. enterica* to SteA₁₋₂₀, SteA, SopE₁₋₈₁ or SopE₁₋₁₀₅. We then monitored the translocation of the human BH3 interacting-domain death agonist (BID, SEQ ID No. 24), which is a pro-apoptotic member of

the Bcl-2 protein family. It is a mediator of mitochondrial damage induced by caspase-8 (CASP8). CASP8 cleaves BID, and the truncated BID (tBID, SEQ ID No. 25) translocates to mitochondria where it triggers cytochrome c release. The latter leads to the intrinsic mode of caspase 3 (CASP3) activation during which it is cleaved into 17 and 12 kDa subunits [61].

Whereas infection for 1 h with YopE₁₋₁₃₈-Myc or YopE₁₋₁₃₈-BID expressing *Y. enterocolitica* failed to induce apoptosis, the translocation of human tBID triggered cell death in larger extend than the well-characterized apoptosis inducer staurosporin (Fig. 7 A and C). As expected, the translocation of tBID lead to the production of CASP3 p17 subunit, even in larger amounts as with staurosporin (Fig. 7 A). To be able to compare translocated protein amounts to endogenous Bid, HeLa cells were lysed with Digitonin and analyzed by Western blotting using an anti Bid antibody (Fig. 7 B). T3SS delivered YopE₁₋₁₃₈-tBID reached about endogenous Bid levels in HeLa cells, while delivered YopE₁₋₁₃₈-BID was present in even higher quantities (2.5 fold) (Fig. 7 B). A deep proteome and transcriptome mapping of HeLa cells estimated 4.4 fold 10⁵ copies of BID per single cell [62]. Therefore, one can conclude that T3SS dependent human protein delivery reaches 10⁵ to 10⁶ proteins per cell. These numbers fit the copies per cell of nanobodies translocated via *E. coli* T3SS [63]. Assuming a levelling of a factor of 10 for the MOI and for the duration of the infection, a factor of 3.2 for the time-point of antibiotic addition and for the culture time at 37°C before infection, the delivered protein copies/cell can be tuned from some 1000 copies/cell up to some 10⁶ copies/cell Altogether, these results indicated that translocated tBID was functional and delivered at relevant levels. This validated the translocation tool to study the role of proteins in the regulation of apoptosis, a central aspect of cell biology.

We further fused murine tBID (codon optimized for *Y. enterocolitica*; SEQ ID No. 194) or the BH3 domains of murine tBID or murine BAX (in both cases codon optimized for *Y. enterocolitica*; SEQ ID No. 138 and 139) to YopE₁₋₁₃₈ for delivery by *Y. enterocolitica*. Whereas infection for 2.5 h with *Y. enterocolitica* ΔHOPEMT *asd* delivering no protein or YopE₁₋₁₃₈-Myc failed to induce apoptosis, the translocation of murine tBID (codon optimized to *Y. enterocolitica*, SEQ ID No. 194) triggered cell death in B16F10 (Fig. 15), D2A1 (Fig. 16), HeLa (Fig. 17) and 4T1 (Fig. 18) cells. The translocation of the BH3 domain of murine BID codon optimized for *Y. enterocolitica* (SEQ ID 138) or murine BAX codon optimized for *Y. enterocolitica* (SEQ ID 139) were as well found to induce massive cell death in B16F10 (Fig. 15), D2A1 (Fig. 16), HeLa (Fig. 17) and 4T1 (Fig. 18) cells. Further versions include a tandem repeat of the BH3 domain of murine BID codon optimized for *Y. enterocolitica* fused

to YopE₁₋₁₃₈ (SEQ ID 202) or linking the BH3 domain of murine BID codon optimized for *Y. enterocolitica* to the BH3 domain of murine BAX codon optimized for *Y. enterocolitica*, fused to YopE₁₋₁₃₈ (SEQ ID 203).

Whereas infection for 4 h with *S. enterica aroA* bacteria failed to induce apoptosis, the translocation of murine tBID triggered apoptosis, as the translocation of murine tBID lead to the production of CASP3 p17 subunit (Figs. 19 and 20). The extent of apoptosis induction for SopE fusion proteins was larger when using SpiI T3SS inducing conditions (Fig. 19), which reflects the transport of SopE exclusively by SpiI T3SS. SteA₁₋₂₀ fused murine tBID failed to induce apoptosis, very likely because the secretion signal within the 20 N-terminal amino acids of SteA is not sufficient to allow delivery of a fusion protein (Figs. 19 and 20). Murine tBID fused to full length SteA lead to apoptosis induction in HeLa cells (Figs. 19 and 20), both in SpiI and SpiII T3SS inducing conditions, reflecting the ability of SteA to be transported by both T3SS. It has to be noted that even under SpiII T3SS inducing conditions, a partial activity of the SpiI T3SS is expected as seen by the activity of SopE fusion proteins in SpiII T3SS inducing conditions (Fig. 20).

Besides the here functionally elaborated translocated eukaryotic proteins, several other eukaryotic proteins have been secreted using the here-described tool. This includes for delivery by *Y. enterocolitica* (Figs. 12, 13 and 22) proteins from cell cycle regulation (Mad2 (SEQ ID No. 15), CDK1 (SEQ ID No. 14), INK4A (SEQ ID No. 16), INK4B (SEQ ID No. 17) and INK4C (SEQ ID No. 18)) as well as parts thereof (INK4A 84-103 (SEQ ID No. 158), p107 657-662 (SEQ ID No. 159), p21 141-160 (SEQ ID No. 160), p21 145-160 (SEQ ID No. 161), p21 17-33 (SEQ ID No. 162) and cyclin D2 139-147 (SEQ ID No. 163)), apoptosis related proteins (Bad (SEQ ID No. 29), FADD (SEQ ID No. 28), and Caspase 3 p17 (SEQ ID No. 22) and p12 (SEQ ID No. 23), zebrafish Bid (SEQ ID No. 19) and t-Bid (SEQ ID No. 20)) as well as parts thereof (tBid BH3 (SEQ ID No. 138), Bax BH3 (SEQ ID No. 139)), signalling proteins (murine TRAF6 (SEQ ID No. 12), TIFA (SEQ ID No. 13)), GPCR G α subunit (GNA12, shortest isoform, (SEQ ID No. 30)), nanobody (vhhGFP4, (SEQ ID No. 31)) and nanobody fusion constructs for targeted protein degradation (Slmb-vhhGFP4; (SEQ_ID_Nos. 32, 33, 34) [64]) (Fig. 12 and 13) as well as small GTPases (Rac1 Q61E (SEQ ID No. 26 and 137) and RhoA Q63L (SEQ ID No. 27) and Pleckstrin homology domain from human Akt (SEQ ID No. 35). Besides the functionally elaborated apoptosis related proteins (murine tBid, SEQ ID No. 144-147), this further includes for delivery by *S. enterica* (Fig. 21)

proteins from cell cycle regulation (Mad2 (SEQ ID No. 168-169), CDK1 (SEQ ID No. 170-171), INK4A (SEQ ID No. 164-165) and INK4C (SEQ ID No. 166-167)). While those proteins have not been functionally validated, the possibility of T3SS dependent secretion of diverse eukaryotic proteins in combination with the possible removal of the YopE appendage opens up new vistas on the broad applicability of T3SS in cell biology and therapeutic applications, especially for treatment of malignant solid tumors.

Phosphoproteomics reveal the global impact of translocated proteins on protein phosphorylation

Phosphorylation is a wide-spread post-translational modification which can either activate or inactivate biological processes and is therefore a suitable target to study signaling events [65]. Despite this, no systems-level analysis of phosphorylation in apoptosis is available today. To analyze the impact of human tBid delivered into HeLa cells, we used a label-free phosphoproteomic approach by LC-MS/MS. In three independent experiments, cells were either left untreated, infected with $\Delta HOPEMT\ asd + YopE_{1-138}\text{-Myc}$ or with $\Delta HOPEMT\ asd + YopE_{1-138}\text{-tBid}$ for 30 minutes. Cells were lysed, followed by enzymatic digestion, phosphopeptide enrichment and quantification and identification of individual phosphopeptides. We compared cells infected with $\Delta HOPEMT\ asd + YopE_{1-138}\text{-Myc}$ to cells infected with $\Delta HOPEMT\ asd + YopE_{1-138}\text{-tBid}$, allowing us to identify 363 tBid dependent phosphorylation events. 286 phosphopeptides showed an increase in phosphorylation whereas 77 were less phosphorylated upon tBid delivery, corresponding to 243 different proteins, which we defined as the tBid phosphoproteome. The STRING database was used to create a protein-protein interaction network of the tBid phosphoproteome [66] (Fig. 8 A). Additionally 27 proteins known to be related to mitochondrial apoptosis were added to the network, building a central cluster. Interestingly, only few proteins from the tBid phosphoproteome are connected to this central cluster indicating that many proteins undergo a change in phosphorylation that were so far not directly linked to apoptotic proteins. To characterize the biological functions covered by the tBid phosphoproteome, we performed a gene ontology analysis using the functional annotation tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov/>) [67,68]. Identified biological functions show that diverse cellular processes are affected by tBid. Many proteins involved in chromatin rearrangement and the regulation of transcription undergo a change in phosphorylation (i.e. CBX3, CBX5, TRIM28, HDAC1). HDAC1 for example is a histone deacetylase playing a

role in regulation of transcription. It has been shown that HDAC1 can modulate transcriptional activity of NF- κ B, a protein also participating in apoptosis. We additionally identified a cluster of proteins involved in RNA processing which has previously been shown to play an important role in the regulation of apoptosis [69]. HNRPK for instance mediates a p53/TP53 response to DNA damage and is necessary for the induction of apoptosis [70]. Furthermore, the phosphorylation of proteins involved in protein translation is also affected. Several eukaryotic initiation factors (i.e. EIF4E2, EIF4B, EIF3A, EIF4G2) undergo a change in phosphorylation, which is in line with the observation that overall protein synthesis is decreased in apoptotic cells. Interestingly, the phosphorylation of many proteins involved in cytoskeleton remodeling (e.g. PXN, MAP1B9 are altered upon tBid delivery. This is in concordance with the observation that the morphology of cells changes dramatically upon tBid delivery (Figure 8 B). Cells shrinkage and loss of contact is reflected by the fact that we observe phosphorylation of adhesion related proteins like ZO2 and Paxillin. Similarly, shrinkage of the nuclei is accompanied by phosphorylation of laminar proteins like LaminA/C and Lamin B1. Altogether, tBID delivery induces a rapid apoptotic response also indicated by rupture of the mitochondrial integrity (Fig. 8 B). We showed that tBid induced apoptosis affects hundreds of phosphorylation events participating in diverse cellular processes. While many identified proteins have been related to apoptosis, only few were known to be phosphorylated upon apoptosis induction. The phosphoproteomic approach thus provides a useful resource for further studies on apoptosis.

Translocation of eukaryotic heterologous fusion proteins consisting of repeated identical or variable protein domains into epithelial cells

To show that heterologous fusion proteins consisting of repeated identical or variable protein domains can translocate via type III secretion we fused murine apoptosis inducers for delivery by *Y. enterocolitica* to YopE₁₋₁₃₈. As control, we fused murine tBID (codon optimized for *Y. enterocolitica*; SEQ ID No. 194) or the BH3 domains of murine tBID or murine BAX (in both cases codon optimized for *Y. enterocolitica*; SEQ ID No. 200 and 201) to YopE₁₋₁₃₈ for delivery by *Y. enterocolitica*. The heterologous fusion protein consisted in one case of murine BH3 domain of tBID fused to itself, resulting in YopE₁₋₁₃₈-(tBID-BH3)₂ (SEQ ID No. 202). In a second case, the heterologous fusion proteins consisted of murine BH3 domain of tBID fused to murine BH3 domain of BAX, resulting in YopE₁₋₁₃₈-(tBID-BH3)-(BAX-BH3) (SEQ

ID No. 203). In the case of murine tBID and murine BAX the codon was optimized for *Y. enterocolitica*.

Whereas infection for 4 h with *Y. enterocolitica* Δ HOPEMT *asd* delivering YopE₁₋₁₃₈-Myc failed to induce apoptosis, the translocation of murine BH3 domain tBID (codon optimized to *Y. enterocolitica*, SEQ ID No. 194) triggered cell death in B16F10 and 4T1 cells, with a clear dose-response effect upon increasing multiplicity of infection (MOI). Interestingly, delivered YopE₁₋₁₃₈-(tBID-BH3)-(BAX-BH3) or YopE₁₋₁₃₈-(tBID-BH3)₂ were found more active than YopE₁₋₁₃₈-(tBID-BH3) at lower MOI. This indicates, that upon delivery of repeated identical domains or combination of different protein domains, the impact on a desired cellular pathway as apoptosis can be enlarged.

Virulence attenuation by deletion/mutation of bacterial effector proteins with virulence activity towards eukaryotic cells

In case of *Y. enterocolitica*, the virulence was reduced by deletion of the six endogenous effector proteins, called “Yersinia outer proteins” (Yops), in detail YopH, O, P, E, M, T (MRS40 pIML421 [yopH Δ 1–352, yopO Δ 65–558, yopP23, yopE21, yopM23, yopT135]) [40]. These Yops are encoded on the “*Yersinia* virulence plasmid” (pYV), a about 70kbp sized plasmid, on which the complete type 3 secretion system (T3SS) as well as other virulence players are encoded (**Fig. 24**). YopH, O, P, E, M and T are the six effector proteins, which are delivered to host cells by the bacterial type three secretion system in order to modulate and dampen the immune system. Each Yop has a specific biochemical activity in the host cell. YopT cleaves off the C-terminal Cysteine of Rho GTPases and thus removes the isoprenyl group anchoring the GTPases to the membrane. This inactivation of the Rho due to mislocalization avoids phagocytosis by immune cells as macrophages and neutrophils [71]. In the same pathway, YopE acts as GTPase activating protein (GAP) for Rho GTPases, deactivating them. This results in decreased phagocytosis and inhibition of release of IL-1 beta by immune cells [71]. Furthermore, YopO acts as guanidine nucleotide dissociation inhibitor (GDI), deactivating Rho GTPases. YopO further has a serine/threonine kinase domain acting in a not yet defined way on the actin cytoskeleton [71]. YopH is a tyrosine phosphatase acting on focal adhesion proteins as Focal adhesion kinase (Fak), paxillin and others, thus strongly preventing phagocytosis by macrophages and neutrophils [71]. YopP, termed YopJ in *Y. pseudotuberculosis* or *Y. pestis*, was found to inactivate the MAPK/NFkB

pathway in immune cells, preventing TNF α and IL-8 release from immune cells stimulated by the presence of the bacteria. Furthermore, YopP was found to induce apoptosis in immune cells, which might be related to the effect in the MAPK pathway, which in its activated state protects cells from apoptosis [71]. The role of YopM is not yet completely clear, but it was found associated with ribosomal S6 kinase 1 (RSK1) and protein kinase C-like 2 (PRK2). It seems as if YopM could stimulate phosphorylation of RSK1 and thus affects downstream targets, as e.g. cell cycle progression [71]. By deleting one or several of these Yops, the defense mechanism of the bacteria against the immune system are dramatically affected [72]. Mutation of respective yops was confirmed by PCR on the respective region, and by in vitro secretion assay (**Fig. 25**). Analysis of in vitro secretion by SDS-PAGE and Coomassie-blue staining confirmed absence of full-length YopH,O,M and YopE.

Furthermore, a *Y. enterocolitica* strain with deletions in *asd* (aspartate semialdehyde dehydrogenase) was constructed. The mutation in *asd* leads to a complete loss of growth capability without addition of meso-diamino-pimelic acid. This allows generating antibiotic free plasmid maintenance systems based on the presence of *asd* on the respective plasmid. In a similar way, other auxotroph mutants might be used.

Dose escalation study on healthy mice

In order to assess the acute toxicity, a dose-escalation study on healthy immuno-competent mice (C57BL/6) was performed. In this experiments, *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T \Delta asd$ (replication deficient due to Δasd and further virulence attenuated due to $\Delta yopH,O,P,E,M,T$) was compared to *S. typhimurium* $\Delta aroA$ (growth attenuated due to $\Delta aroA$, as used by other [73-75]). Acute toxicity post infection was assessed based on weight gain or weight loss of the mice following i.v. injection of bacteria. Furthermore, bacterial counts in various organs was determined by organ homogenization, serial dilution and plating. As acute toxicity was assessed and bacterial growth was not of main interest, no desferoxamine pretreatment was applied. Four different bacterial loads were tested for each strain (10^5 , 10^6 , 10^7 , 10^8 cfu per animal). *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T \Delta asd$ did not lead to a weight reduction at the two lower doses (10^5 , 10^6 cfu) (**Fig. 26**). At the dose of 10^7 bacteria, the weight dropped by about 6% within the first 24h, before stabilization and continuing raise. With the highest dose, weight was found dropped by 7.5% 48h post infection (**Fig. 26**). As *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T \Delta asd$ can't establish an infection, even more in the absence of

desferoxamine pretreatment, bacterial counts were expected to drop rapidly, which was found (Fig. 27 - 30). Nevertheless, a strong acute toxicity due to e.g. endotoxic shock was not observed even for the highest dose of *Y. enterocolitica* applied. This is in contrast to the growth- and infection-competent *S. typhimurium* $\Delta aroA$, which has many times been used in murine studies by others [73-75] and the *aroA* and *aroD* double mutant even in clinical trials (NCT01099631). Physical appearance and behavior scoring urged us to sacrifice all mice infected with the highest dose of *S. typhimurium* $\Delta aroA$ at day 1 post infection and day 4 for the 10^7 cfu dose. Progressive decreasing weight was found for the three highest doses (Fig. 26), while the lowest dose was found to induce only a permanent mild weight loss (Fig. 26). Even at the lowest dose tested, *S. typhimurium* $\Delta aroA$ was found present in liver, spleen and lung up to 8 days post infection (Fig. 27 - 30). This might reflect the non-optimal biodistribution of *S. typhimurium* $\Delta aroA$ seen by others [76].

In the dose-escalation experiments on acute toxicity done with immunocompetent mice *Y. enterocolitica* $\Delta yopH,O,P,E,M,T \Delta asd$ showed to be safely tolerated up to 10^8 bacteria per mouse upon i.v. administration. Such a dose might be used as well in human clinical trials [77], with a weight difference from mice to man of several thousand fold. As mice are known to sense gram-negative bacteria the same way as humans via TLR4 and tend to respond similarly to the same lipid A stimulus [78], an initial toxicity due to a septic shock caused by lipid A in human patients is unlikely at the bacterial loads used so far in clinical trials [77]. Furthermore, the lack of normal immune-stimulatory lipid A has been proposed to account for the variance observed in the clinical tests with *S. enterica* VNP20009 [79]. It might be thus more favorable to reduce the initial bacterial load administered to patients while maintaining the wild-type lipid A structure in order to reach an optimal tumor colonization, where higher loads can be obtained through bacterial replication.

At the site of bacterial presence in the body, an immune response will be launched to fight the bacteria. Upon bacterial accumulation and growth at the site of solid tumors [76,80-82]), the immune system will, after initial dissemination of the bacteria, be triggered at the tumor site. While a septic shock and acute toxicity in patients has to be avoided by reducing the initial bacterial load and/or reducing the endotoxicity of the bacteria by lipid A modifications, the immune system stimulation at the site of the tumor is highly desired, as it assists clearance of the cancerous tissue (immunotherapy, immunosensitization). The current pilot phase II

clinical trial (EudraCT No. 2005-005775-15) with *S. enterica* builds on this immunotherapeutic effect and the natural toxicity of the bacteria [83]. Immunosensitization is one of the key mechanisms of genetically non-equipped *Salmonella* acting on tumors, as e.g. *Salmonella choleraesuis* accumulates in tumors and induces neutrophil infiltration and an antitumor immune response [84]. Furthermore, the immune system activation at the tumor site has shown not to prevent a multiple application of bacteria in oncotherapy [85].

In summary, the immune response triggered by bacterial cancer therapeutics has to be subdivided into a non-desired acute phase, which has to be kept low, and a desired later-stage activation, which assists tumor eradication. This can either be reached by administration of high doses of lower endotoxic bacteria or by administering lower levels of normal endotoxic bacteria, which have increased capacity to colonize and replicate in solid tumors [79].

Biodistribution studies in a murine model of melanoma

In order to validate gram-negative bacteria with mutation(s) in key virulence determinants like the T3SS effectors as tumor specific vehicle, murine allograft tumor studies using the well-established B16F10 melanoma model ([86], ATCC No. CRL-6475) were performed. When s.c. tumors had reached a certain size (about 100-200 mm³), mice were i.v. infected with 2×10^5 cfu *Y. enterocolitica subsp. palearctica* MRS40 or *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$. In order to allow bacterial growth, mice were pretreated 24h prior to infection with desferrioxamine. Mice infected with the wt *Y. enterocolitica subsp. palearctica* MRS40 strain had increased scoring for physical appearance and behavior (**Fig. 31-32**) and exhibited significant weight loss over the first 48 of infection (**Fig. 33**), which urged us to sacrifice all of the mice in this group already at day 2 post infection. In contrast, mice infected with the virulence attenuated *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$ strain did not show significant weight loss and scored normally for physical appearance and behavior (**Fig. 31-33**) still at day 4 post infection. In mice infected with the wt strain (*Y. enterocolitica subsp. palearctica* MRS40) living bacteria were detected in all organs assessed, and furthermore in the blood (**Fig. 35**). While wt bacteria were found present in the malignant solid tumor, equally high or higher counts were found in other organs, highest in the spleen (**Fig. 35**). In sharp contrast, in mice infected with *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$ living bacteria were mainly found in the malignant solid tumor at day 1 post infection, with low bacterial counts observed in spleen, liver and lung. Notably, at day 4 post infection, the bacterial count in the malignant

solid tumor had increased by some orders of magnitude (reaching more than 10^8 cfu/g of tumor tissue), while in all other organs assessed the bacterial counts dropped below the detection limit (**Fig. 34**). *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$ thus accumulated at day 4 post infection with a ration of about (minimally) one million fold at the site of the malignant solid tumor as compared to spleen or liver (when calculating the ration against the detection limit).

These results validate this strategy for virulence attenuation by mutation of key virulence determinants to generate a bacterial vehicle specifically targeting the malignant solid tumor.

10 Biodistribution studies in a murine model of breast cancer

In order to validate gram-negative bacteria with mutation(s) in key virulence determinants like the T3SS effectors as tumor specific vehicle, murine allograft tumor studies using the well-established 4T1 model of breast cancer (ATCC No. CRL-2539) were performed. When s.c. tumors had reached a certain size (about 100-200 mm³), mice were i.v. infected with 2×10^5 cfu *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$. In order to allow bacterial growth, mice were pretreated 24h prior to infection with desfreoxamine. Mice infected with the virulence attenuated *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$ strain did not show significant weight loss and scored normally for physical appearance and behavior still at day 8 post infection. In these mice infected with *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$ living bacteria were exclusively found in the malignant solid tumor at day 8 post infection (**Fig. 36**). *Y. enterocolitica subsp. palearctica* MRS40 $\Delta yopH,O,P,E,M,T$ thus accumulated at day 8 post infection with a ration of about (minimally) several 10'000 fold at the site of the malignant solid tumor as compared to spleen or liver (when calculating the ration against the detection limit).

These results validate this strategy for virulence attenuation by mutation of key virulence determinants to generate a bacterial vehicle specifically targeting the malignant solid tumor.

Generation of enhanced pro-apoptotic bacteria

In above mentioned experiments it is shown that the T3SS-based delivery of pro-apoptotic proteins (e.g. t-BID (SEQ ID No. 25) or BIM (SEQ ID No. 21)) efficiently induces cell death in both murine and human cells, including cancerous cells, and that this effect could be increased when using murine t-BID optimized to the bacterial codon usage (SEQ ID No. 138).

This increased cell killing very likely reflects increased amount of protein production and following delivery via T3SS due to optimal codons used.

In order to optimize the delivery of pro-apoptotic proteins, strains transformed with different pro-apoptotic proteins have been generated according to Table IV.

5

Table IV: Strains transformed with different pro-apoptotic proteins

Strain Name	Background strain	Protein to be delivered by T3SS	Backbone plasmid	Resulting plasmid name	Primers. Si_Nr.:	resistances
YopE1-138-(Y. enterocolitica codon optimized murine tBid BH3 extended part)	Y. enterocolitica Δ yopH,O,P,E, M,T Δ asd	YopE1-138-Y. enterocolitica codon optimized murine tBid BH3 extended (by 4 Aa)	pBad_Si_2	pSi_353		Nal Amp
YopE1-138-10 Aa linker-(Y. enterocolitica codon optimized murine tBid BH3 part)	Y. enterocolitica Δ yopH,O,P,E, M,T Δ asd	YopE1-138-10 Aa linker-Y. enterocolitica codon optimized murine tBid BH3	pBad_Si_2	pSi_354	727/728	Nal Amp
YopE1-(138-Y. enterocolitica codon optimized murine Bax BH3 part- Y. enterocolitica codon optimized	Y. enterocolitica Δ yopH,O,P,E, M,T Δ asd	YopE1-138-Y. enterocolitica codon optimized murine Bax BH3-. enterocolitica codon optimized	pSi_357	pSi_374	736/737	Nal Amp

murine tBid BH3 part		murine tBid BH3				
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Shortening the delivered proteins to the essential domains required for signaling (e.g. the BH3 domain of t-BID (SEQ ID No. 138 or 200)) could increase the efficiency of cell killing (**Fig. 37**). Without being bound by theory, this increase in efficacy is likely to be related to increased amount of protein production and following delivery via T3SS due to smaller size of the delivered protein. Introduction of a linker between the YopE part and the BH3 domain of tBID (SEQ ID No. 210) decreased efficacy, as well as extending the BH3 domain by 4 further amino acids (SEQ ID No. 209) (**Fig. 37**).

Additionally, synthetic cargos with repeats of such essential domains (e.g. the BH3 domain of t-BID (SEQ ID No. 202)) or combinations of these essential domains (e.g. the BH3 domain of t-BID and the BH3 domain of BAX (SEQ ID No. 203 and 211)) were generated. Surprisingly, tandem repeats of the same or different BH3 domains were found to result in enhanced apoptosis induction on cancerous cell lines (including 4T1 and B16F10 cells, **Fig. 37**). The IC₅₀ (half maximal inhibitory concentration), referring to the number of bacteria per eukaryotic cell (MOI) needed in order to kill 50% of such cells, was found to be decreased upon delivery of tandem repeats of tBID BH3 domain as compared to a single tBID BH3 domain (**Fig. 37**). This finding was surprising, as the protein size is increased by fusing as second BH3 domain of t-BID. Due to this, decreased expression and delivery levels of YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) as compared to YopE₁₋₁₃₈-tBID BH3 (SEQ ID No. 138 or 200) would be expected, and might maximally reach equivalent levels. In order to reach an increase in cell killing activity, the fused tBID BH3 domains must simultaneously act side by side upon delivery by the T3SS into eukaryotic cells. In case only one tBID BH3 domain in the YopE₁₋₁₃₈-(tBID BH3)₂ construct would be functional, at best the same efficiency as with YopE₁₋₁₃₈-tBID BH3 might be expected.

In order to increase the genetic stability of YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) for in vivo studies, we cloned YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) by homologous recombination on the Yersinia virulence plasmid pYV at the native site of YopE and under the native YopE promoter (using mutator plasmids pSI₄₀₈ and pSI₄₁₉). Such mutators contain the DNA sequence coding for the desired protein, flanked by 200–250 bp of sequences on both sides corresponding to the site of the respective gene, where the integration shall take place. These plasmids are transformed into *E. coli* Sm10 λ pir, from where plasmids

were mobilized into the corresponding *Y. enterocolitica* strain. Mutants carrying the integrated vector were propagated for several generations without selection pressure. Then sucrose was used to select for clones that have lost the vector. Finally mutants were identified by colony PCR. The endogenous proteins for the transport by the T3SS (called “*Yersinia* outer proteins”, Yops) are encoded by *Y. enterocolitica* on this 70kb plasmid, named plasmid of *Yersinia* Virulence (pYV), which further encodes the T3SS apparatus.

Yersinia strains encoding YopE₁₋₁₃₈-(tBID BH3) (SEQ ID No. 138 or 200) or YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) on the *Yersinia* virulence plasmid pYV at the native site of YopE and under the native YopE promoter were assessed for their capacity of inducing apoptosis in cancerous cells (including 4T1 and B16F10 cells, **Fig. 38**). The IC₅₀ (half maximal inhibitory concentration), referring to the number of bacteria per eukaryotic cell (MOI) needed in order to kill 50% of such cells, was found to be decreased upon delivery of tandem repeats of tBID BH3 domain as compared to a single tBID BH3 domain, when both proteins are encoded on the *Yersinia* virulence plasmid pYV at the native site of YopE and under the native YopE promoter (**Fig. 38**). This is in agreement with findings from expression plasmid borne delivery of these proteins (**Fig. 37**). Again, this finding was surprising, as the protein size is increased by fusing a second BH3 domain of t-BID. Due to this, decreased expression and delivery levels of YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) as compared to YopE₁₋₁₃₈-tBID BH3 (SEQ ID No. 138 or 200) would be expected, and might maximally reach equivalent levels. In order to reach an increase in cell killing activity, the fused tBID BH3 domains must simultaneously act side by side upon delivery by the T3SS into eukaryotic cells. In case only one tBID BH3 domain in the YopE₁₋₁₃₈-(tBID BH3)₂ construct would be functional, at best the same efficiency as with YopE₁₋₁₃₈-tBID BH3 might be expected. Furthermore, *Yersinia* strains encoding YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) on the *Yersinia* virulence plasmid pYV at the native site of YopE and under the native YopE promoter were compared for their capacity of inducing apoptosis in cancerous cells to expression plasmid (pBad-MycHisA based) derived delivery of YopE₁₋₁₃₈-(tBID BH3)₂. In agreement with the higher copy number of pBad-MycHisA (20-25 copies) as compared to the pYV (1-6 copies are reported), pBad-MycHisA based delivery of YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) resulted in a slightly decreased IC₅₀ value on 4T1 and B16F10 cells (**Fig. 38**).

Validation of tumor specific growth in vivo up to day 14 post bacterial administration

The experiment of tumor colonization by genetically modified *Y. enterocolitica* was repeated in a syngeneic murine allograft model (4T1 breast cancer model) and bacterial colonization was followed over two weeks. This time, mice were infected with 1×10^6 colony forming units (CFU) of *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$. While obtaining similar results to the B16F10 model at early days post infection, we could further show that the tumor colonization is consistently found at day 8 and up to day 14 after infection (**Fig. 39**). Furthermore, the colonization remains highly specific with only low counts of bacteria detected in all other organs assessed (**Fig. 40**). These findings indicate that *Y. enterocolitica* $\Delta yopH,O,P,E,M,T$ is able to establish a persistent colonization of the tumor thereby preventing clearance by the immune system.

Efficacy of *Y. enterocolitica* $\Delta HOPEMT$ in delaying tumor progression

In order to assess the impact of YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) delivered to tumor cells in vivo, we performed studies in wildtype Balb/C mice allografted s.c. with 4T1 breast cancer cells. We aimed at assessing the *Y. enterocolitica* $\Delta HOPEMT$ strain encoding YopE₁₋₁₃₈-(tBID BH3)₂ (SEQ ID No. 202) on the Yersinia virulence plasmid pYV at the native site of YopE and under the native YopE promoter. Mice were i.v. injected with PBS or 1×10^7 *Y. enterocolitica* $\Delta HOPEMT$ pYV-YopE₁₋₁₃₈-(tBID BH3)₂, once the tumor had reached a size of 150-250 mm³. The day of the i.v. injection of bacteria was defined as day 0. Tumor volume was measured over the following days (day 0 to day 9 post i.v. injection of bacteria) with calipers. The tumor volume was normalized to the tumor volume at day 0 to compensate for any initial heterogeneity in tumor size. Treatment with *Y. enterocolitica* $\Delta HOPEMT$ pYV-YopE₁₋₁₃₈-(tBID BH3)₂ showed an impact on tumor volume progression, with statistically significant tumor reduction at day 8, 9 and 10 post bacterial administration (**Fig. 41**). Importantly, *Y. enterocolitica* $\Delta HOPEMT$ alone was found not to impact tumor progression in the 4T1 murine cancer model (**Fig. 42**). These findings highlight that such bacteria and their T3SS can be employed for interference with tumor progression.

List of references

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CLAIMS

1. A composition comprising:
a recombinant virulence attenuated Gram-negative bacterial strain that is deficient in the production of a siderophore and is transformed with a vector which comprises in the 5' to 3' direction:
a promoter;
a first DNA sequence encoding a delivery signal from a bacterial effector protein, operably linked to said promoter;
a second DNA sequence encoding a heterologous protein fused in frame to the 3' end of said first DNA sequence; and
a siderophore.
2. The composition according to claim 1, wherein the recombinant virulence attenuated Gram-negative bacterial strain is selected from the group consisting of the genera *Yersinia*, *Escherichia*, *Salmonella* and *Pseudomonas*.
3. The composition according to claim 1, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells.
4. The composition according to claim 1, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of all bacterial effector proteins, which are virulent toward eukaryotic cells.
5. The composition according to claim 1, wherein the recombinant virulence attenuated Gram-negative bacterial strain is a mutant *Yersinia* strain in which all the effector-encoding genes which encode bacterial effector proteins which are virulent toward eukaryotic cells are mutated such that the resulting *Yersinia* no longer produce any functional bacterial effector proteins which are virulent toward eukaryotic cells.
6. The composition according to claim 5, wherein the mutant *Yersinia* strain is *Y. enterocolitica* and the delivery signal from a bacterial effector protein is the delivery signal from a

bacterial T3SS effector protein wherein the delivery signal from the bacterial T3SS effector protein comprises the N-terminal 138 amino acids of the *Y. enterocolitica* YopE effector protein.

7. The composition according to claim 1, wherein the heterologous protein is selected from the group consisting of proteins involved in apoptosis or apoptosis regulation, cell cycle regulators, ankyrin repeat proteins, cell signaling proteins, reporter proteins, transcription factors, proteases, small GTPases, GPCR related proteins, nanobody fusion constructs and nanobodies, bacterial T3SS effectors, bacterial T4SS effectors and viral proteins.

8. A composition comprising:

a recombinant virulence attenuated Gram-negative bacterial strain that is deficient in the production of a siderophore, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of at least one bacterial effector protein which is virulent toward eukaryotic cells or is deficient in the production of at least one bacterial protein which is part of a secretion system machinery; and

a siderophore.

9. The composition according to claim 8, wherein the recombinant virulence attenuated Gram-negative bacterial strain is selected from the group consisting of the genera *Yersinia*, *Escherichia*, *Salmonella* and *Pseudomonas*, and wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of all bacterial T3SS effector proteins which are virulent toward eukaryotic cells.

10. The composition according to claim 8, wherein the recombinant virulence attenuated Gram-negative bacterial strain is deficient in the production of all effector proteins which are virulent toward eukaryotic cells and the recombinant virulence attenuated Gram-negative bacterial strain expresses a pro-drug converting enzyme.