SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
TUMOUR SUPPRESSORS
MOLECULAR ENGINEERING
GENE REGULATION
GENETIC RECOMBINATION

Received
1 July 2011

Accepted
11 May 2012

Published
1 June 2012

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Explicit hypoxia targeting with tumor suppression by creating an "obligate" anaerobic *Salmonella Typhimurium* strain

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Using bacteria as therapeutic agents against solid tumors is emerging as an area of great potential in the treatment of cancer. Obligate and facultative anaerobic bacteria have been shown to infiltrate the hypoxic regions of solid tumors, thereby reducing their growth rate or causing regression. However, a major challenge for bacterial therapy of cancer with facultative anaerobes is avoiding damage to normal tissues. Consequently the virulence of bacteria must be adequately attenuated for therapeutic use. By placing an essential gene under a hypoxia conditioned promoter, *Salmonella Typhimurium* strain SL7207 was engineered to survive only in anaerobic conditions (strain YB1) without otherwise affecting its functions. In breast tumor bearing nude mice, YB1 grew within the tumor, retarding its growth, while being rapidly eliminated from normal tissues. YB1 provides a safe bacterial vector for anti-tumor therapies without compromising the other functions or tumor fitness of the bacterium as attenuation methods normally do.

Effective tumor targeting and the toxicity of treatments are major problems in current cancer therapy. In solid tumors, hypoxic regions create a further problem as they are resistant to many treatments[1] and are linked to more malignant phenotypes[2]. A potential solution is to employ anaerobic bacteria to target the hypoxic regions to cause tumor regression[3,4]. Not only have anaerobic bacteria been shown to use chemotaxis to locate tumors and then cause regression[5] but they can also be used as anti-tumor treatment vectors[6,7] or as complements to other therapies[8,9].

Intentional use of bacteria in cancer treatment can be dated to the late 19th century with even earlier anecdotal reports of bacterial efficacy in treating cancer[3,10,11]. The first reported deliberate attempt at using bacteria (*Streptococcus pyrogenes*) to treat an inoperable sarcoma also demonstrated the inherent danger of the technique. Whilst the tumor and lymph nodes reduced appreciably, the patient died of infection within 9 days of treatment[3,10,11].

Consequently, much recent work on bacterial therapies for cancer has focused on non-pathogenic strains or the need to attenuate bacteria for use in model systems and humans. *Bifidobacteria* are non-pathogenic obligate anaerobes and have been successfully used to target tumors and as a therapeutic vectors but do not appear to have a direct oncolytic effect[8,12–14]. The spore forming *Clostridia* are obligate anaerobes with some oncolytic ability and non-pathogenic and attenuated forms have been used directly and as gene-therapy vectors[9,15,16]. Tumor colonization by *Clostridia* is strain dependent[17,18] and more effective strains such as *C. sporogenes* have been transformed to act as improved gene therapy vectors[17–19]. Clostridial spores will not germinate in aerobic tissues and so are generally safe for systemic administration[17] but may be less effective on small tumors or smaller metastases[3,11,15].

Facultative anaerobes, such as *Salmonella* enterica serovar Typhimurium, can target both small and large tumors and reduce tumor size[8]. While wild type strains target tumors, their virulence may result in the death of the host[20]. Attenuation of strains reduces the stress on the host and preferential bacterial colonization of tumors at ratios of 1,000 to 10,000:1 compared to normal tissues has been reported[20,21]. Apart from reducing tumor size or

retarding growth[20,22,24], *Salmonella* strains have also proved effective against metastasis[25–27]. When coupled with gene or conventional therapy, the anti-tumor effects of *Salmonella* can be enhanced[7,20,23].

Several attenuated *Salmonella* strains have been developed for tumor targeting studies. SL7207, which has a defect in the *aroA* gene and is a derivative of similar attenuated strains[28], has been used by several groups[29–33], although it can affect the health of immunocompromised mice[29,33]. Deletions in *purI* and *msbB* gave rise to VNP20009[21,34] which has been used for gene-targeted pro-drug therapy[35] and tested for oral delivery[36] and in clinical trials[37,38]. Strain A1[39] and its derivative A1-R[24] are leucine-arginine auxotrophs and A1-R targeted a metastases model[26]. Defects in guanosine 5′-diphosphate-3′-diphosphate synthesis attenuated *Salmonella* (strain ΔppGpp)[40] which has been shown to be effective as an inducible vector against CT-26 tumors and metastases[23]. The different nutritional environment in a tumor may compensate for the metabolic defects in these bacteria, thereby allowing effective growth in a tumor but not in normal tissues[20,39].

However, attenuation to reduce virulence in normal tissues might compromise the function of the bacteria in tumors. A large-scale study used a transposon library and a custom microarray to identify a group of *Salmonella* mutants that had reduced fitness or attenuation in normal tissues[41]. Their aim was to identify attenuated strains that retain their fitness inside tumors. Two classes of attenuated strains, those with minor or with moderate reductions in tumor fitness, were identified. STM3120, a severely attenuated SPI-3 mutant, had a minor reduction in tumor fitness and was effective in PC-3 tumors and somewhat effective in oral administration[41]. An *aroA* mutant, similar to SL7207, had moderately reduced tumor fitness. However, this study examined bacterial fitness in tumors, not tumor killing ability.

In this work, we developed a novel synthetic biology approach to engineering *Salmonella* to enhance its effectiveness in anti-tumor therapy. An essential gene (*asd*) is engineered so that it is under the control of a hypoxia-conditioned promoter. The normal functions of the bacterium are not compromised by the deletion or mutation of any of its genes. In normal tissues under aerobic conditions, *asd* is not expressed, diaminopimelic acid (DAP) is not synthesized and the bacterium will lyse during growth unless DAP is supplied by the environment. In tumor bearing nude mice the engineered strain inhibited tumor growth while not affecting the mice. In contrast, the original *Salmonella* strain was lethal to the mice.

Results

Creation of a hypoxia targeted *Salmonella* strain (YB1). Replacement of the essential gene *asd* from parental *Salmonella typhimurium* strain SL7207 with a construct where this gene is under the control of hypoxia targeted promoters was achieved by recombineering technology. In the resulting YB1 strain, the FNR related anaerobic capable promoter PpepT controls *asd* transcription while an aerobic promoter, PsodA, facilitates transcription of antisense *asd* that blocks any leakage of *asd* expression under aerobic conditions (Fig. 1a). If *asd* is not transcribed and DAP is not supplied in the environment, lysis of the YB1 bacteria will occur during bacterial growth.

Several other strain variants were constructed (YB-asd – SL7207 with no *asd* gene; YB1-pw – as YB1 but with no antisense promoter for *asd*; YB1-ew – as YB1 but with the *pepT* promoter replaced with a weaker *ansB* promoter) (Supplementary Fig. S1). Regulation of Asd expression under high and low oxygen levels was tested. Changes in Asd protein levels were demonstrated by immunoblotting of myc tagged Asd. This showed that *asd* expression in the YB1 (YB1-myc) strain was controlled by oxygen as expected (Fig. 1b and Supplementary Fig. S2). Strong Asd expression was detected under anaerobic conditions (YB1-O_2) while no expression was observed under aerobic conditions (YB1+O_2). In the YB1-pw (YB-myc-pw) strain without the antisense promoter, leaky Asd expression was observed under aerobic conditions (PW+O_2). No expression of Asd was observed under either aerobic or anaerobic conditions (EW+O_2 and EW-O_2) in strain YB1-ew (YB-myc-ew) with the weak EW promoter.

All of the strains were tested for growth in LB broth (Fig. 1c-f). Of the engineered strains in the absence of DAP, only YB1 showed the combination of growth under anaerobic culture conditions and repression in the aerobic environment. SL7207 and YB-pw showed growth in all conditions. YB-asd and YB-ew showed growth only with the addition of DAP.

Serial reductions in the oxygen level and bacterial concentration were used to establish the range of conditions under which YB1 and the other strains could survive in the presence or absence of DAP. On LB agar plates without DAP, YB1 grew only when oxygen levels decreased to below 0.5%. Strains YB-asd and YB-ew did not grow in the absence of DAP, while SL7207 and YB-pw grew in all conditions (Fig. 1g).

Ability of YB1 to invade cancer cells. Breast cancer cell line MDA-MB-231 samples were incubated with YB1 or SL7207 under oxygen concentrations below 0.5% or aerobic conditions. After removal of extra-cellular bacteria and further culturing, confocal microscopy showed that both SL7207 and YB1 had invaded the breast cancer cells under anaerobic conditions (Fig. 2a, YB1-O_2, SL7207-O_2). In comparison, under aerobic conditions (Fig. 2a, YB1+O_2, SL7207+O_2), YB1 could not survive and only SL7207 was observed in breast cancer cells (quantification of infection rates is shown in Supplementary Fig S3). In anaerobic conditions, using an annexin V/PI assay, MDA-MB-231 samples treated with each of the bacteria showed an increase in the number of dying or apoptotic cells relative to a blank control (Fig. 2b), with YB1 being somewhat more effective in causing cell death or apoptosis ($P<0.05$) (Fig. 2c).

Accumulation of YB1, VNP20009 and SL7207 in tumor and normal tissues *in vivo*. Three groups of four-week-old nude mice were inoculated with breast cancer cells and, when tumor volumes reached 500–550 mm^3, a single dose of SL7207, YB1 or VNP20009 was injected via the tail vein. At varying time points, mice were euthanized and most organs and tumor were collected, homogenized and cultured on LB agar plates with antibiotics and DAP. CFU/gram was used as a relative measure of the degree of colonization of the tissues with bacteria.

For SL7207 inoculated mice, 1E+03 to 1E+07 CFU/gram of bacteria were found in all tissues at 6 hours (Fig. 3a). Bacterial levels increased subsequently with an uncontrolled infection by day 3 (Fig. 3a). The tumor to liver ratio of SL7207 was 2.78:1 at day 3. Mice started to die on day 7. On day 11, SL7207 levels in liver reached 3.8E+09 CFU/gram (Fig. 3a) and after that all mice died.

For YB1 injected mice 6 hours after inoculation, bacterial levels were approximately the same in all tissues as for the SL7207 inoculated mice (Fig. 3b) though bacteria were eliminated from the blood of 70% of the mice. After 1 day YB1 was eliminated from the blood and subsequently the levels in all normal tissues rapidly declined. In tumor, YB1 levels increased to a plateau of ~1E+08 CFU/gram by day 3 (Fig. 3b). The tumor to liver ratio of YB1 CFU/gram was ~7,000:1 on day 3 and ~20,000:1 on day 7 (Fig. 3b) ($P<0.05$ on day 5 and day11; $P<0.01$ on day 7 and day 26). By day 26, YB1 was totally eliminated from heart, kidney, lung, lymph node, and spleen. YB1 was also eliminated from liver in five of the six mice tested, remaining in one mouse with a CFU/gram of 1.3E+03. No YB1 was detected inside bone marrow within the whole process of experiments.

Like YB1, VNP20009 preferentially accumulated in tumor tissue ($P<0.05$) as has been reported[21,34]. Bacterial levels in tumor reached a plateau of ~3E+08 CFU/gram by day 5 (Fig 3c). The best tumor to liver ratio was ~3,900:1 on day 5 (Fig 3c). When compared with the SL7207 strain, VNP20009 demonstrated quicker clearance from

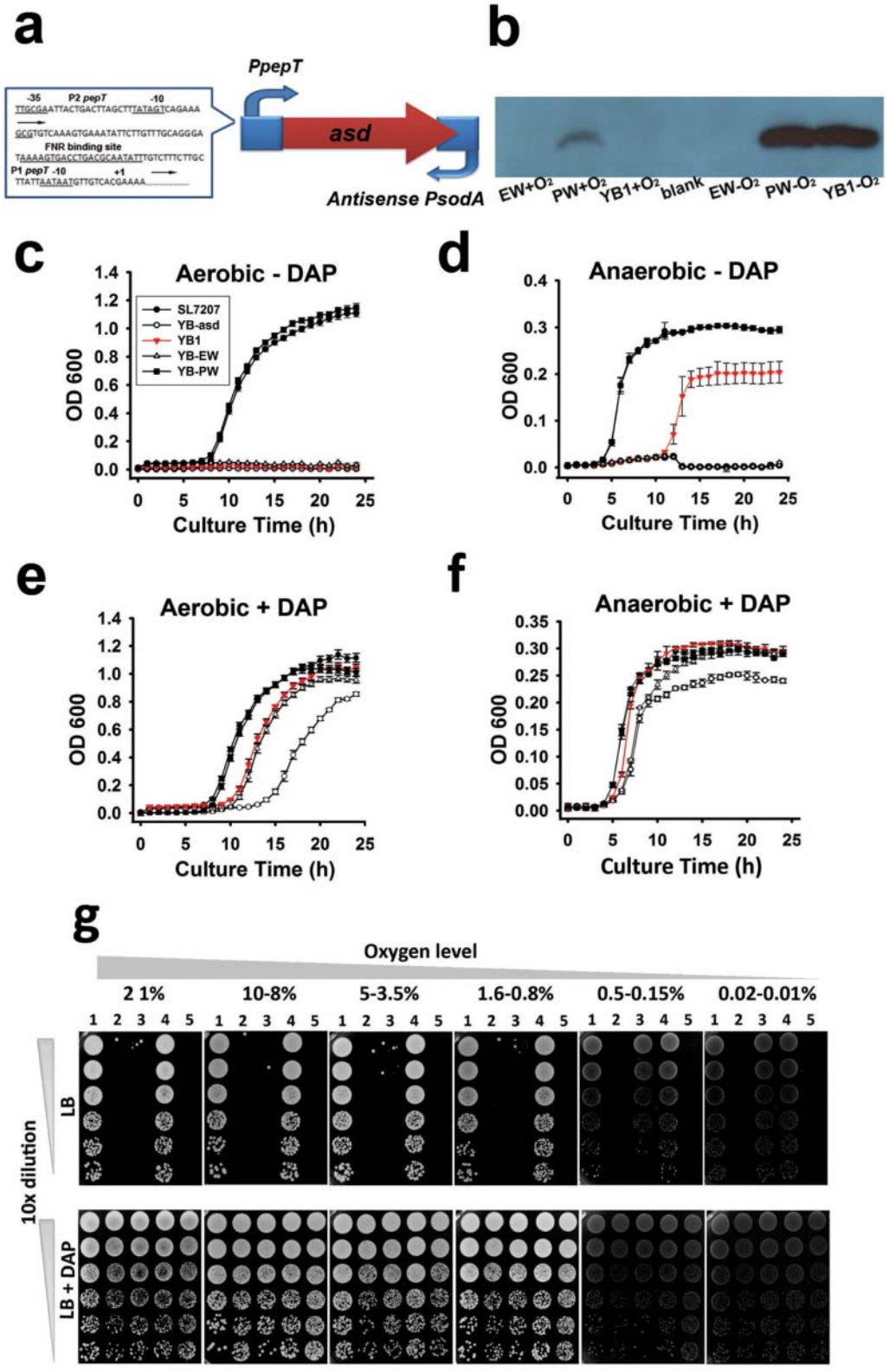

Figure 1 | Construction and testing of tumor-targeting *Salmonella* strain YB1. (a) Construction of the hypoxia conditioned *asd* gene expression cassette. The sense promoter *PpepT* contains the constitutive promoter P2 and the FNR regulated promoter P1 and *PsodA* is the antisense promoter. (b) To test *asd* expression in response to oxygen, strains YB-myc-EW, YB-myc-PW, and YB1 were cultured under aerobic ($+O_2$) or anaerobic ($-O_2$) conditions for 24 hours at 37°C. DAP was added to prevent cell lysis under aerobic conditions. Bacterial cell number was quantified by OD600 measurement and total protein was extracted from those bacteria respectively. Asd-myc expression was detected by Western blotting with loading control (Supplementary Fig. S2) (c, d) Growth rate of various strains (10^4 bacteria/ml) under aerobic or anaerobic conditions in LB broth without DAP (mean ± sd, each time point represents three individual experiments). (e, f) as in (c, d) but with DAP. (g) Different mutant strains at serial dilutions under decreasing oxygen levels were cultured for 24 hours and bacterial growth was observed. Columns: 1 SL7207; 2 YB-asd; 3 YB1; 4 YB-PW; 5 YB-EW. (Three independent experiments were performed.)

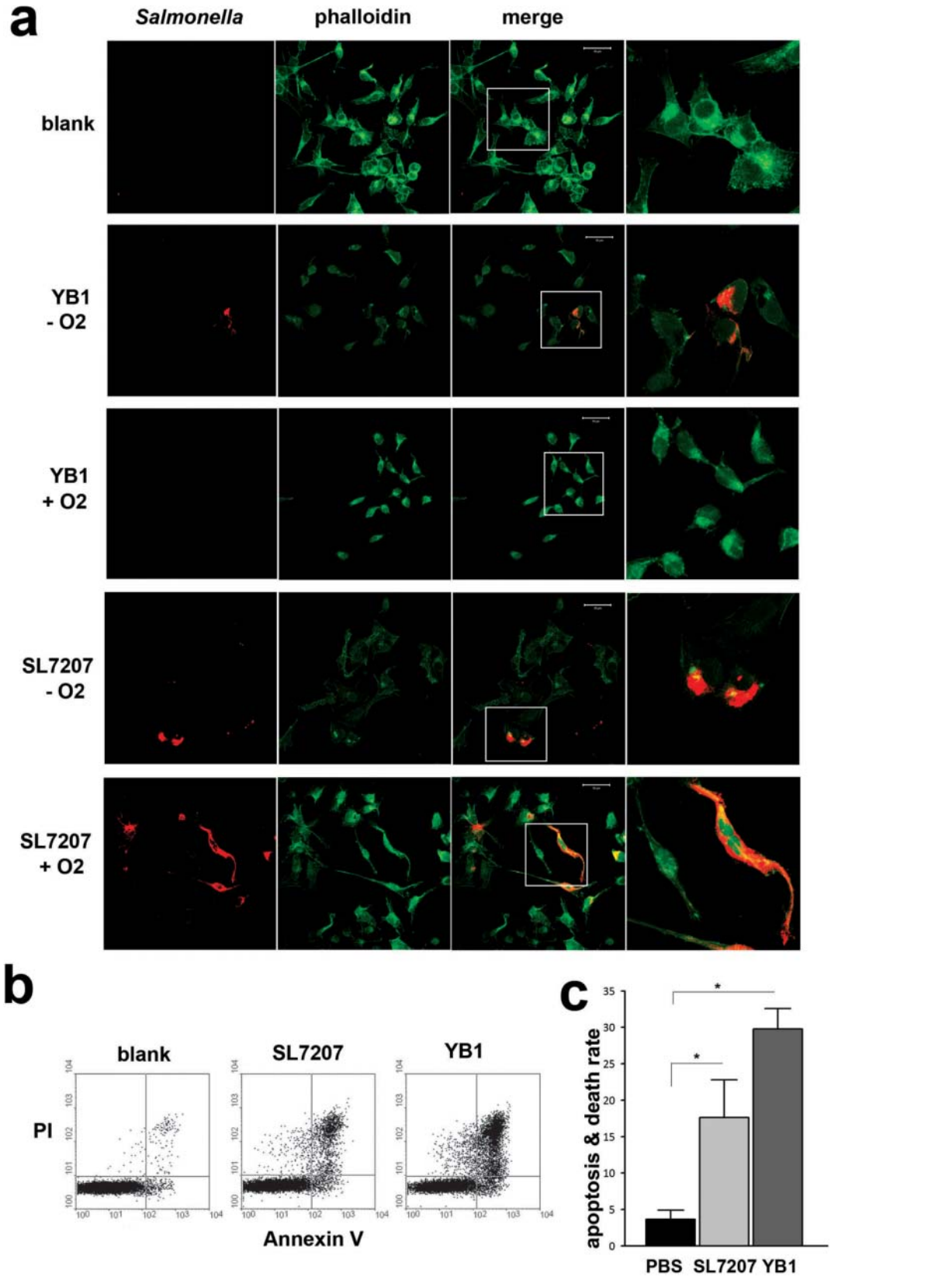

Figure 2 | ***Salmonella* YB1 and SL7207 in breast cancer cells.** (a) *In vitro* cultured breast cancer cells (MBA-MB-231) were exposed to *Salmonella* YB1 and SL7207 (1: 500∼1000) separately under anaerobic (O_2<0.5%: YB1 - O_2, SL7207 - O_2) or aerobic (O_2=21%: YB1 + O_2, SL7207 + O_2) conditions. Two hours post-incubation, breast cancer cells were washed and fresh medium containing gentamycin (50 μg/ml) was added to remove extracellular bacteria. 24∼48 hours later, breast cancer cells were collected, stained using an anti-*Salmonella* antibody (red) and phalloidin to indicate cancer cells (green) and observed by confocal microscopy. Merged and enlarged images are given. (b,c) Apoptosis and death rate of cancer cells induced by *Salmonella* under anaerobic conditions were detected by annexin-V/PI staining and measured by flow cytometry. *, P<0.05.

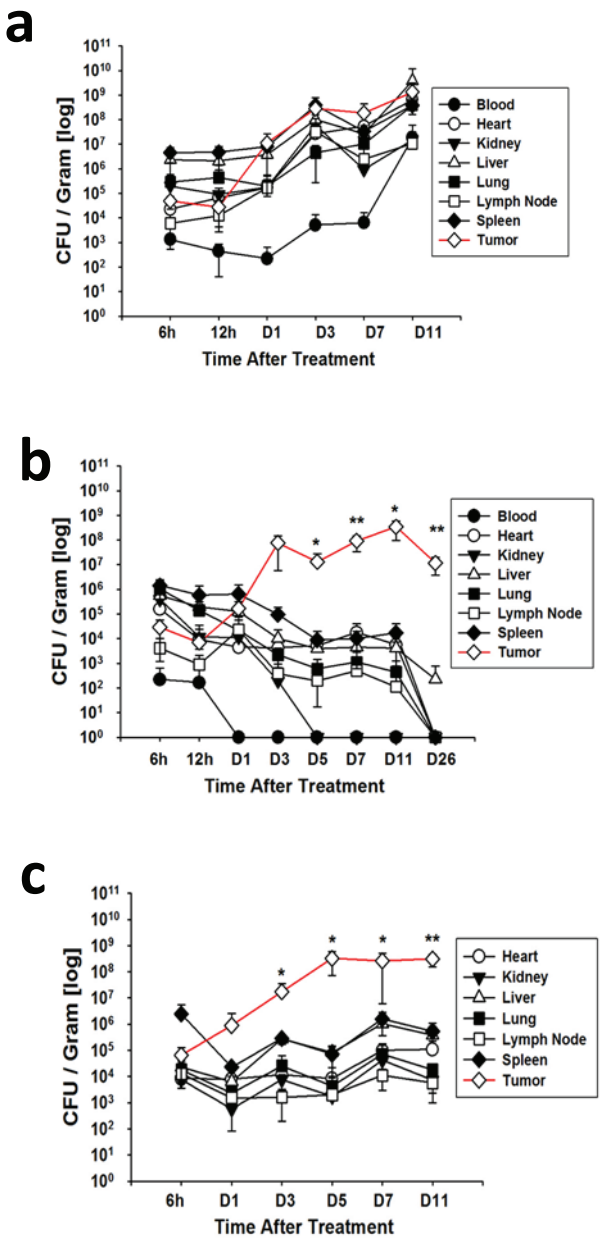

Figure 3 | CFU test of YB1, SL7207, and VNP20009 in breast tumor bearing nude mice. Nude mice with an MBA-MB-231 tumor received temporal vein injections of YB1, SL7207 or VNP20009. Mice were euthanized at the indicated time points and blood, heart, kidney, liver, lung, lymph node, spleen and tumor tissues were collected and homogenized and bacterial accumulation evaluated. In SL7207 (a) YB1 (b) or VNP20009 (c) treated mice, CFU counts per gram of most normal organs and tumor (red line) are shown over time (mean±sd, each time point represents three individual experiments with 2 mice for each experiment). Statistical significance of tumor group vs. all other groups: *. * $P<0.05$; ** $P<0.01$.

normal organs, but was more slowly eliminated than YB1 from liver ($P<0.05$), kidney ($P<0.05$), spleen ($P<0.05$), lung, lymph node, and heart (Fig. 3).

Immuno-staining of sections of tumor and liver confirmed the distribution of *Salmonella* bacteria in these tissues. Both YB1 and SL7207 targeted the tumor, with large amounts of bacteria being present from day 3 onwards (Fig. 4a). In liver, YB1 decreased and was almost eradicated by day 7 with little effect on liver structure (Fig. 4b). For SL7207 treated mice, continuing bacterial accumulation and liver damage were obvious (Fig. 4b). Enlarged images are shown in Supplementary Fig. S4 (tumor section) and S5 (liver section).

YB1 targeting of hypoxic and necrotic regions in tumors. Hypoxyprobe™-1 (pimonidazole hydrochloride) was used as a hypoxia marker to demonstrate the distribution of *Salmonella* in tumors. Immunostaining of breast cancer tumor sections revealed hypoxic and necrotic areas (Supplementary Fig. S6), which is consistent with previous reports. After the injection of *Salmonella* into tumor-bearing mice, most bacteria accumulated in the Hypoxyprobe™-1 marked region (Fig. 5a, Supplementary Fig. S6). Formation of hypoxic regions in a tumor might be due to disorganization of blood vessel development. The area colonized by YB1 had little or no blood vessels as indicated by CD31 staining (Fig. 5b), which suggested colonization by bacteria of the hypoxic region in the tumor. Staining with a GR-1 antibody to examine the immune response to bacterial invasion revealed infiltration of Gr-1^+ host neutrophils into the breast tumor where they appeared to form a barrier around YB1 (Fig. 5c, 5d).

YB1 inhibited tumor growth *in vivo*. As YB1 invaded MDA-MB-231 breast cancer cells *in vitro*, causing cell apoptosis, its effect *in vivo* was measured. Tumor growth (from a volume of ~500–550 mm^3 at bacterial inoculation) in YB1 treated mice was initially inhibited and then delayed relative to PBS treated mice ($P<0.05$ on day 3, $P<0.001$ from day 5 to day 21) (Fig. 6a). Little further tumor growth was seen in SL7207 treated mice as bacterial toxicity caused death between days 7 and 11 (Fig. 6a). Mice treated with YB1 (with or without a tumor) and YB-asd treated tumor free mice survived more than 25 days, as did mice (with or without a tumor) treated with PBS (Fig. 6b). SL7207 treated mice started to die on days 5 and 7 with all mice dying by days 8 and 11 (without or with a tumor, respectively). SL7207 treated mice with a tumor had a slightly better survival rate (Fig. 6b).

While the reduction in tumor growth in YB1 treated mice was marked compared with PBS treated mice, the tumor was still growing. Treatment of tumor bearing mice with the therapeutic agent 5-FU showed only a small reduction ($P>0.05$) in tumor growth relative to PBS treatment (Fig 6c) although 5-FU was toxic to breast cancer cells (Supplementary Fig. S7). However, when 5-FU was given to YB1 infected tumor bearing mice, much greater, and statistically significant, reductions in tumor size were observed than with the individual treatments (Fig 6c).

Comparison of strains YB1 and VNP20009 in tumor targeting and regression. A single dose of VNP20009 or YB1 was injected via the tail vein to mice bearing a breast tumor of ~360 mm^3 and tumor size was measured every two days. Both YB1 ($P<0.01$) and VNP20009 ($P<0.05$) could delay tumor growth when compared with a PBS treatment group. However, YB1 showed greater tumor inhibition than VNP20009 ($P<0.05$) (Fig 6d).

The health of these mice was monitored by measuring body weight. In the first two days after innoculation, both YB1 and VNP20009 treatment groups showed significant body weight loss compared with the control group ($P<0.01$) but not to each other (Supplementary Fig. S8). The body weight of both groups started recovering after 3 to 5 days, and no mouse died due to the bacterial treatment.

Discussion

Anaerobic bacteria provide an important treatment opportunity in cancer therapy due to their ability to target the hypoxic region of solid tumors that is resistant to conventional treatment[1,3]. If *Salmonella*, a facultative anaerobic bacterium, is to be a successful treatment agent in anti-cancer therapy, bacterial virulence in the host needs to be addressed[11]. In most cases attenuated forms are created and used as

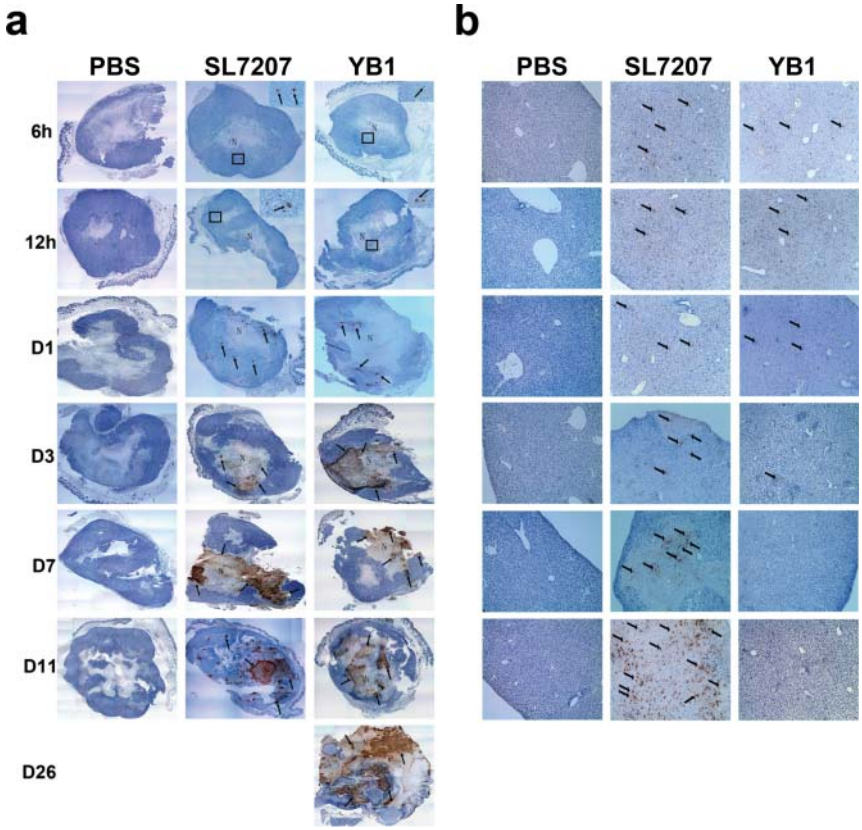

Figure 4 | Paraffin section test of YB1 and SL7207 in tumor and liver. The distribution of *Salmonella* in tumor. (a) and liver (b) of breast tumor bearing mice over time was demonstrated in tissue paraffin sections by immuno-staining. (Arrows: *Salmonella*).

test therapeutic agents[24,29,34,40]. However the mutations required to attenuate a bacterium might also compromise its tumor targeting and killing ability. This was suggested as a possible reason for the poor performance of VNP20009 in clinical trials[11]. Recently, a systematic study of *Salmonella* mutants[41] partially addressed this issue by identifying several attenuated mutant bacteria with either mild or moderate reductions in tumor fitness. Tumor killing by these mutants could not be examined[41].

An alternative approach taken here is to use recombineering to make *Salmonella* not viable in normal tissues by placing an essential gene, *asd*, under the control of a hypoxia-induced promoter. The *asd* gene of *Salmonella* encodes an enzyme essential for the synthesis of DAP, which is itself an essential component of the bacterial cell wall and not present in mammalian systems[7]. With *asd* expressed only in hypoxic conditions the bacterium is able to grow readily under hypoxia, but will lyse under normal growth conditions. Thus *Salmonella* can be converted from a facultative to an "obligate" anaerobe, rendering it safe in normal tissues. Previously, programmed bacterial lysis by conditioning *asd* expression on a supplied nutrient (arabinose) was demonstrated as a vaccine system[7].

Regulation of the fumarate and nitrate reduction gene (*fnr*) is involved in the switch between aerobic and anaerobic growth[42]. Promoters containing FNR binding sites are activated under hypoxia[43]. Such a case was demonstrated with the *pepT* promoter to create a potential gene therapy vector only expressed in hypoxic regions[43]. Here, the *pepT* promoter (PpepT) was used to drive expression of *asd*, conditional on hypoxia, in a recombineered version of *Salmonella* SL7207 (YB-pw) with the aim of limiting bacterial viability to hypoxic regions. In the engineered strain, the *asd* gene was replaced with a *PpepT-asd* construct. However, this strain was still able to grow under normal oxygen levels. To prevent leakage from the *pepT* promoter, an antisense promoter of the *sodA* gene, which is negatively regulated by FNR[44], was added to the *PpepT-asd* construct to make the strain YB1. This effectively inhibited the growth of *Salmonella*, as shown in Fig. 1c-f where YB1 could only grow in the absence of DAP under anaerobic but not aerobic conditions. Another *asd* based cell lysis system also required anti-sense transcription for efficacy[7]. An alternate construct using the *ansB* promoter (YB-EW) proved ineffective under anaerobic conditions, perhaps due to the construct used being a less efficient promoter.

In the absence of DAP, YB1 was the only strain that had the combination of growth under anaerobic but not aerobic conditions. A detailed titration of oxygen level and bacterial concentration showed that, in the absence of DAP, YB1 was only viable at oxygen levels below 0.5% (Fig. 1g). Unlike SL7207, YB1 only infiltrated the MDA-MB-231 breast cancer cells under anaerobic conditions. However, it was more effective at inducing apoptosis or cell death, possibly due to the anaerobic expression of *asd* being stronger under the hypoxia conditioned promoter as compared to the wild type one.

SL7207, YB1 and an attenuated *Salmonella* strain VNP20009 were able to infiltrate MDA-MB-231 tumors induced in nude mice, as evidenced by the considerable number of bacteria found in the tumor and the considerable tumor damage observed. Although quiescent YB1 cells appear to persist briefly in aerobic tissues in the absence of DAP[45], YB1 was effectively cleared from normal tissues. By 3 days post infection, bacteria were barely detectable in liver. VNP20009 was somewhat less effectively cleared from normal tissues than YB1 and less effective at reducing tumor size. SL7207, despite being an attenuated vaccine strain, had a similar effect on normal and tumor cells and killed all mice by 11 days post infection with substantial bacterial induced liver destruction apparent. While SL7207 might not affect immuno-competent mice, in the system studied here the conversion of SL7207 to the "obligate" anaerobic YB1 prevented bacterial killing of the mice while maintaining, or enhancing, tumor killing ability. YB1 appeared to be more effective on smaller tumors (Fig. 3a and d).

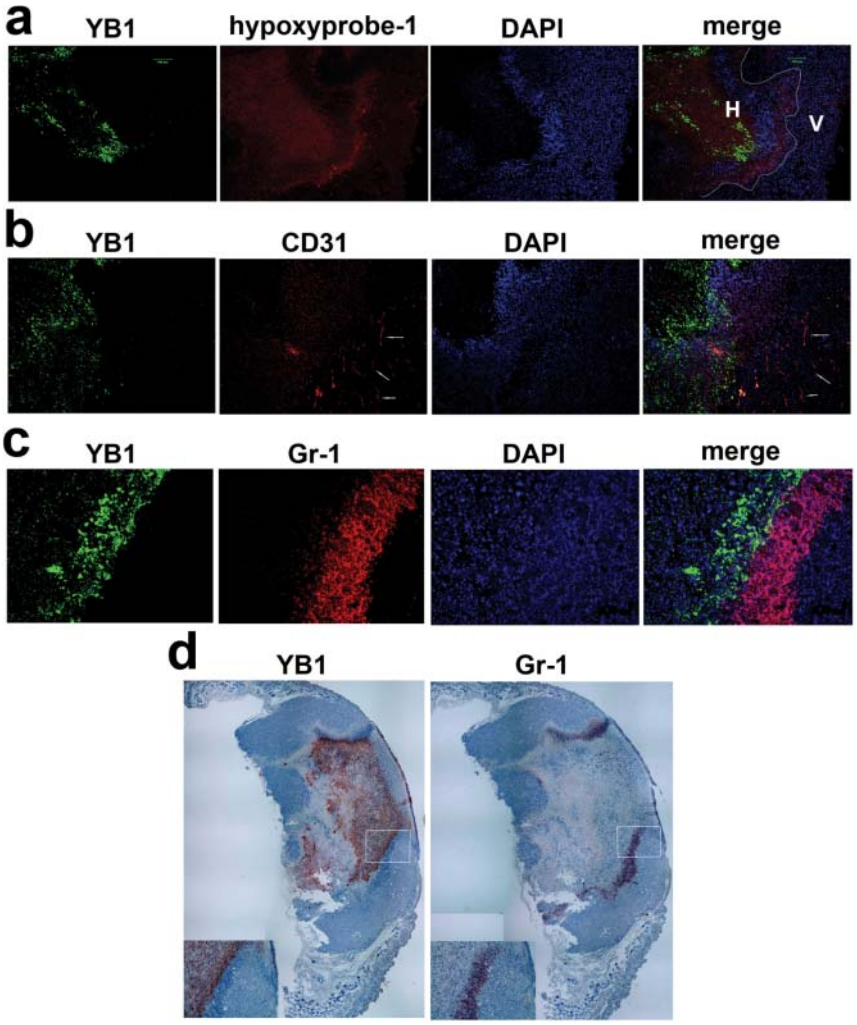

Figure 5 | Confinement of YB1 in the tumor. (a) YB1 and the hypoxic region are indicated by staining with anti-Salmonella (green) and anti-hypoxyprobe (red) antibodies, respectively. DNA is indicated by DAPI staining (purple). H: hypoxic area. V: viable area. (b) Blood vessels in the tumor are shown by an anti-CD31 antibody (red, arrows). (c) Immunocytes were detected with an anti-Gr-1 antibody (red). (d) An overview of YB1 and immunocyte distribution in tumors.

A more detailed examination of the effect of YB1 in tumors showed that its design as an "obligate" anaerobe was effective in that it was tightly confined to the hypoxic regions of tumors and kept distant from blood vessels. As bacteria are expected to induce a host immune response, neutrophils were found in the YB1 infected tumors. YB1 and neutrophils aligned against each other with neutrophils possibly acting as a barrier against further bacterial spread. YB1 may enhance tumor killing by strongly attracting neutrophils to the tumor.

However, YB1 did not totally inhibit breast tumor growth. It is common for bacterial treatment of tumors to be used in conjunction with drugs or as a gene therapy agent to deliver a drug or pro-drug to the tumor environment[8,9,18,19]. Consequently, the anti-cancer drug 5-FU was also administered to the tumor bearing mice. When compared with untreated mice, YB1 retarded tumor growth with an effectiveness greater than that of the drug 5-FU alone. In combination, YB1 and 5-FU were even more effective. SL7207 was too toxic and was lethal to the mice before effects on tumor growth could be observed.

The precise modification of *Salmonella* strain SL7207, by placing an essential gene under a hypoxia conditioned promoter, as performed here has successfully converted the bacterium to an "obligate" anaerobe, thereby removing the lethal toxicity of the host strain while maintaining its tumor targeting and possibly enhancing its tumor killing abilities. YB1 has shown comparable or better tumor colonization, to other bacterial anti-tumor agents such as *Chlostridia*[17,18] and other *Salmonella* strains[5,24,34,43]. While the ease of modifying *Chlostridia* to produce gene therapy vectors[17,18] has improved[46], *Salmonella* can be readily transformed using long-established techniques and YB1 could be developed similarly. YB1-like bacteria could have the advantages of an obligate anaerobic bacterium while maintaining the chemotaxic properties[5,22] and ability to target metastasis[25–27] of *Salmonella*.

Conditioning *Salmonella* growth on hypoxia provides an alternative to conventional attenuation techniques, which require a mutation of the bacteria to compromise some normal function. The recombineered "obligate" anaerobe YB1 represents a new direction in producing bacterial therapeutic agents for cancer.

Methods

Bacterial strains, animals, cell lines, enzymes and chemicals. *S. typhimurium* strain SL7207 was kindly provided by Dr. B. A. D. Stocker[28]. *S. typhimurium* strain VNP20009 was bought from the American Type Culture Collection (ATCC) (202165). Four-week-old female Nude-Mice were purchased from the Laboratory Animal Unit of The University of Hong Kong. The research protocols were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR 1685-08). The breast cancer cell line MDA-MB-231 was from the ATTC (HTB-26™) and was maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin. Enzymes were from New England Biolabs and chemicals were from Sigma. Antibiotic working solutions were prepared as

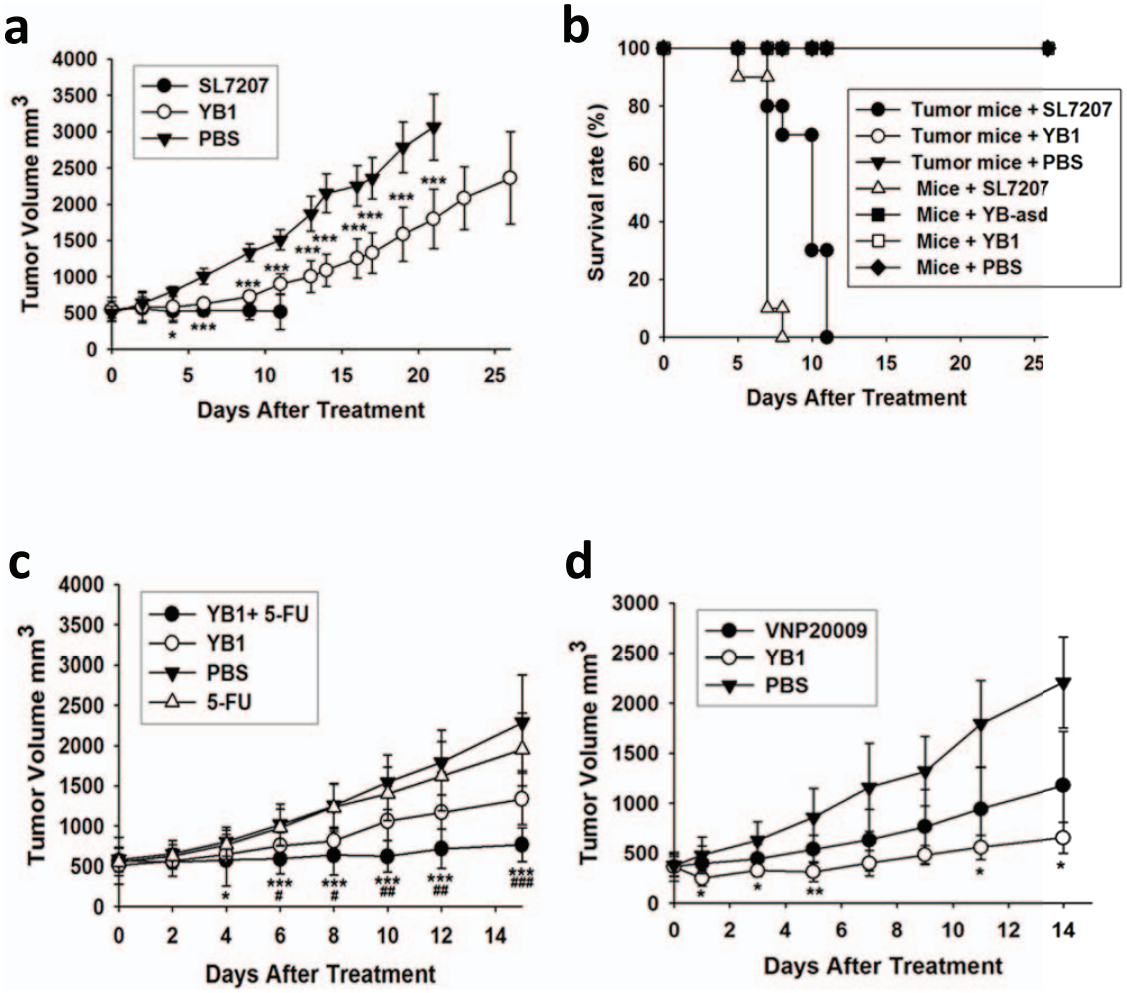

Figure 6 | Repression of tumor growth by *Salmonella* strains. (a) Tumor volume (initial size ~500–550 mm^3) in mice injected with YB1, SL7207 or PBS (n=10, mean±sd). SL7207 treated mice died by day 11. Statistical significance of YB1 group vs. PBS group*. (b) Survival chart for tumor free and tumor bearing mice treated with YB1, SL7207, YB-asd or PBS, respectively (n=10 each). (c) Tumor bearing mice were treated with YB1 or PBS (n=24 each). After three days, 5-FU was injected i.p. (60 mg/kg) to half the mice of each group (n=12) and repeated every three to four days for 2 weeks. Statistical significance of YB1+5-FU group vs. PBS group*; YB1+5-FU group vs. YB1 group#. (d) Tumor volume (starting size ~360 mm^3) in mice after treatment with VNP20009, YB1, or PBS, respectively (n=6, mean±sd). Statistical significance of YB1 group vs. VNP20009 group*. Significance symbols *, # $P<0.05$; **, ## $P<0.01$; ***, ### $P<0.001$.

follows: Chloramphenicol, 25 μg/ml in methanol; Ampicillin, 100 μg/ml; Kanamycin, 50 μg/ml; Gentamycin, 50 μg/ml. Strains were supplied with 50 μg/ml DAP where noted.

Gene cloning and Plasmid construction. Bacteria and plasmids used or created here are given in Supplementary Table S1 and primers used are in Supplementary Table S2. The *asd* gene and the promoter region of the *pepT* gene were cloned from the chromosome of SL7207 by PCR with primer pairs asd-C-F and asd-C-R, pepT-F and pepT-R (preheating at 95°C for 5 mins, followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 1 min, with final extension at 72°C for 10 minutes, and then cooling to room temperature) whilst *asd-myc* was generated with the asd-C-F and asd-C-myc-R primer pair. PansB and PsodA (promoters of *ansB* and *sodA*) constructs were generated by an annealing process with oligonucleotide pairs ansB-F and ansB-R, sodA-F and sodA-R (10 μM forward and reverse primers were mixed and heated at 95°C for 5 mins, and placed at room temperature for 30 mins). The chloramphenicol resistance gene (*cm*) was amplified by PCR with primers cm-F and cm-R from a *p*loxp-cm-loxp template[47]. The plasmids for the *asd* expression vectors were built on the backbone of *p*Bluescript II SK (*p*BSK) which was digested by HindIII, XhoI, NotI and PstI. After ligation by T4 ligase, plasmids *p*YB1 (*p*BSK-cm-PpepT-*asd*-PsodA), *p*YB1-myc (*p*BSK-cm-PpepT-*asd*-myc-PsodA), *p*YB-pw (*p*BSK-cm-PpepT-*asd*), *p*YB-myc-pw (*p*BSK-cm-PpepT-*asd-myc*), *p*YB-ew (*p*BSK-cm-PansB-*asd*-PsodA), and *p*YB-myc-ew (*p*BSK-cm-PansB-*asd-myc*-PsodA) were generated.

Construction of oxygen sensitive *Salmonella* mutant (YB1). The λ-Red recombination system (plasmid *p*Sim6; a gift from Dr. Donald Court)[48] was used to replace the *asd* gene with the cm-PpepT-asd-sodA genetic circuit in SL7207. As a first step the target *asd* gene was generated with a *p*loxp-cm-loxp template in a PCR reaction, electroporated into recombination-competent cells and selected on chloramphenicol Luria-Bertani (LB) plates. Antibiotic resistance genes were removed by site-specific Cre/loxP mediated recombination by transformation of plasmid *p*705cre-Km, generating the strain YB-asd. Next, the cm-PpepT-asd-sodA genetic circuit was amplified from plasmid *p*YB1 and, after recombineering, the correct colony was selected and confirmed by PCR giving strain YB1. Strains YB1-his, YB-pw, and YB-ew were constructed similarly with the plasmids *p*YB1-myc, *p*YB-pw, *p*YB-pw-myc, *p*YB-ew, and *p*YB-ew-myc as templates, respectively.

Growth of *Salmonella* strains and mutants under aerobic and anaerobic conditions. Bacterial strains were grown in LB medium at 37°C, with shaking at 220 rpm over night. Aerobic conditions were achieved by shaking in broth, and anaerobic cultures were either grown in anaerobic tubes or an anaerobic jar (Mitsubishi Gas Chemical Company). Overnight cultures of *Salmonella* strains SL7207, YB-asd, YB1, YB-pw, and YB-ew were counted and diluted into samples at 5E+04 colony forming units (CFU)/ml, with each strain divided into two groups (with or without DAP) in LB broth. OD600 was measured every 30 minutes for aerobic cultures, and each hour for anaerobic cultures from 0 hours to 24 hours. For LB agar plate assays, an anaerobic jar was applied to generate different oxygen concentrations by combinations of AnaeroPacks and monitored by an oxygen meter. Ten serial dilutions of individual drops from a high concentration of 5E+06 CFU/ml to 5E+01 CFU/ml, where each drop contained 10 μl of bacterial culture, were added to plates that were cultured in an anaerobic jar at 37°C for 2 days.

Immunoblotting. 24-hour cultures of YB1-myc, YB-myc-pw, and YB-myc-ew under aerobic or anaerobic conditions in LB with DAP were collected and lysed in RIPA

buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1%SDS, 1% Triton X-100, 1% Na-Deoxycholate). Protein extracts in the amount equivalent to 5E+08 CFU of bacteria were used for each sample. 20 μg of total protein was subjected to SDS-PAGE, followed by incubation with a c-myc-tag antibody (Invitrogen) overnight at 4°C then with a secondary antibody at room temperature for 1 hour. Chemiluminescence was detected using an ECL kit (Amersham Life Science) according to the manufacturer's instructions.

***Salmonella* invasion of breast cancer cells *in vitro*.** *Salmonella* and MDA-MB-231 cells were prepared and co-cultured at a ratio of 1000~500:1 for 2 hours under anaerobic (O_2<0.5%) or aerobic conditions. The cells were then washed with PBS and cultured in gentamycin supplemented medium to remove extracellular bacteria. 24 hours later, cells were fixed in paraformaldehyde (4%) and stained with an anti-*Salmonella* antibody (1:500, Abcam) overnight at 4°C. A Cy3 conjugated secondary antibody was added and incubated for 1 hour at room temperature. Then FITC conjugated Phalloidin (1:1000) was applied to indicate cell boundaries. Images were observed under a confocal microscope. Cancer cell apoptosis and death induced by bacteria under anaerobic conditions were detected by an annexin V-PI kit (Biovision) according to manufacturer's instructions. As shown by flow cytometry, annexin V+/PI- cells are apoptotic and annexin V+/PI+ cells are dead.

MTT cytotoxicity assay. MDA-MB-231 breast cancer cells were seeded in a 96-well plate (3,000 cells/well) and allowed to adhere to the plates overnight. Various concentrations of 5-FU (5-fluorouracil) (1 μg/ml, 10 μg/ml, 20 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 500 μg/ml and 1000 μg/ml) were added to the cells and further cultured for 24, 48, and 72 h. After each time period, media was removed and 100 μl of MTT (methylthiazole tetrazolium, 0.5 mg/ml in DMEM without phenol red) was added to each well and incubated for 4 h at 37°C. Formazan crystals thus formed were solubilized in 200 μl of DMSO (dimethyl sulphoxide) by incubating with shaking for 10 min at room temperature. Absorbance was measured at 570 nm. The cytotoxicity of 5-FU on breast cancer cell was calculated by ([*A*]control - [*A*]test)/[*A*]control, where [*A*]test is the absorbance of the test sample and [*A*]control is the absorbance of the control sample containing medium but without 5-FU treatment.

Tumor-bearing nude-mice. 5E+05 MDA-MB-231 cells were inoculated at the fat pad of four-week-old nude mice. The tumor volumes were calculated by the following formula: $4/3 \times \pi \times (h \times w^2) / 8$, h = height and w = width. When the tumors grew to about 500–550 mm^3 (15–19 days), mice were divided into groups for experiments. If tumors reached 20 mm in any dimension[49] (or ~4000 mm^3), mice were euthanized.

To measure the effect of bacterial inoculation on mouse survival and tumor growth, two groups of 10 mice were treated with either YB1 (5E+07 CFU) or SL7207 (5E+07 CFU), and 6 mice were treated with PBS, with a volume of 100 μl injected through the tail vein (i.v.). Tumor size (with an initial volume of ~500–550 mm^3) was measured by caliper every 2 to 3 days. Mouse survival rate was recorded. For a comparison of VNP20009 and YB1, an additional two groups of 6 mice with smaller tumors (~360 mm^3) were administrated with same dose (5E+07 CFU) of VNP20009 or YB1.

To measure the bacterial distribution after inoculation, mice treated by the same method as above were sacrificed at several time points (a total of 6 mice each for the YB1 and SL7207 treated groups for each time point and 5 mice for the VNP20009 treated group for each time point). Tissues were weighed, homogenized, serially diluted in PBS and plated with the required antibiotics and DAP. CFU were counted after two days growth. The experiments involving YB1 and SL7207 treatments were repeated three times with two mice per time point per experiment and the experiments with VNP20009 were repeated two times with 2–3 mice per time point per experiment.

A possible synergistic effect of YB1 and 5-FU, was tested in 48 tumor-bearing mice that were divided into four groups with 12 mice each and treated with PBS, PBS with 5-FU (60 mg/Kg), a single dose of YB1 (5E+07 CFU) or a single dose of YB1 (5E+07 CFU) plus 5-FU. For the 5-FU-treatmenmt groups, 5-FU was intra-peritoneal (i.p) injected every four days starting from day 3 after bacterial injection.

Four groups of 10 healthy nude mice were i.v. injected with 5E+07 CFU of YB1, SL7207, YB-asd or PBS respectively, and observed for survival.

Histology. Bacteria and PBS treated tumor bearing mice were injected with a hypoxyprobe-1 solution (60 mg/kg) by i.p. 10~40 min before euthanasia. Tissues were removed from these mice and immediately fixed in 4% paraformaldehyde, paraffin embedded and sectioned into 5 μM slices. Hypoxic regions and blood vessels were visualized by mouse anti-hypoxyprobe-1 (hpi) or polyclonal goat anti PECAM1 (CD31) (Santa Cruz) antibodies, respectively. *Salmonella* and immunocytes were separately detected by rabbit anti-*Salmonella* (Abcam) or rat anti-mouse GR-1 (Bioscience) antibodies. Bound primary antibodies were detected using fluorescence conjugated secondary antibodies or horseradish peroxidase conjugated secondary antibodies which then developed in DAB solution (Daco). Pictures were taken under a fluorescence microscope or a light microscope.

Statistical analysis. Statistical analysis was calculated with the Student's *t* test, with $P<0.05$ considered as significant.

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Acknowledgments

This work was supported by a grant from NSFC and RGC (N_HKU 719/08) to JDH and ES, a grant from CRCG Seed Funding Programme for Applied Research and a CRF Grant from the RGC (HKU1/CRF/10) to JDH.

Author contributions

BY, EWS and JDH designed the experiment; BY, MY, LS, YY, QJ, XL performed the experiments; BY, MY, DKS and JDH wrote the manuscript. LHT, BJZ, and KYY provided essential reagents and critical comments.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yu, B. *et al.* Explicit hypoxia targeting with tumor suppression by creating an "obligate" anaerobic *Salmonella Typhimurium* strain. *Sci. Rep.* **2**, 436; DOI:10.1038/srep00436 (2012).