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Drug-inducible remote control of gene expression by probiotic *Escherichia coli* Nissle 1917 in intestine, tumor and gall bladder of mice

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Abstract

The probiotic bacterium *Escherichia coli* Nissle 1917 (EcN) constitutes a prospective vector for delivering heterologous therapeutic molecules to treat several human disorders. To add versatility to this carrier system, bacteria should be equipped with expression modules that can be regulated deliberately in a temporal and quantitative manner. This approach is called *in vivo* remote control (IVRC) of bacterial vectors. Here, we have evaluated promoters P_{araBAD}, P_{rhaBAD} and P_{tet}, which can be induced with L-arabinose, L-rhamnose or anhydrotetracycline, respectively. EcN harbouring promoter contructs with luciferase as reporter gene were administered either orally to healthy mice or intravenously to tumor bearing animals. Subsequent to bacterial colonization of tissues, inducer substances were administered via the oral or systemic route. By use of *in vivo* bioluminescence imaging, the time course of reporter gene expression was analyzed. Each promoter displayed a specific *in vivo* induction profile depending on the niche of bacterial residence and the route of inducer administration. Importantly, we also observed colonization of gall bladders of mice when EcN was administered systemically at high doses. Bacteria in this anatomical compartment remained accessible to remote control of bacterial gene expression.

1. Introduction

Escherichia coli Nissle 1917 (EcN) is a safe probiotic bacterium [1-3]. Usage of EcN has been recommended for the prevention of intestinal infections and for the amelioration of inflammatory gastrointestinal disorders [4, 5]. Recently, EcN was also proposed as potential anticancer bacterium [6]. When applied systemically, such bacteria were shown to efficiently target solid tumors and to replicate within this tissue. Up to 10^9 bacteria per gram of tumor were found and the microorganisms remained in the tumor over a prolonged period of time. In contrast to other tumor targeting bacteria, like *Salmonella enterica* serovar Typhimurium, *E. coli* has been shown to colonize almost exclusively the tumor tissue even in immune compromised animals [6, 7].

Probiotic bacteria, such as EcN, have been equipped with expression modules to produce therapeutic molecules. This provides new options for amelioration of metabolic disorders, allergies or autoimmune diseases [2, 8-10] but also for delivery of anticancer drugs and detection of residual cancerous tissue [11-13].

When used under such conditions, it is necessary to avoid adverse effects, for instance, due to constitutive expression of therapeutic factors. Besides, EcN should remain controllable within the host as an additional safety measure. Deliberate external control of heterologous gene expression in the carrier bacteria is therefore essential.

Recently, the concept of *in vivo* remote control (IVRC) of bacterial vectors was established by us and others (reviewed in [14]). In principle, an IVRC bacterial vector contains an appropriate inducible promoter for deliberate expression of heterologous genes after the bacterial vector has colonized its target tissue.

Promoters suitable for IVRC can either be regulated by physical means, e.g. ionizing radiation [15] or by the administration of inducer substances [6, 16, 17]. The choice of a suitable genetic switch depends on the colonized niche in the body and the specific requirements of the intervention. Proof of principal studies have identified a first set of

promoters suitable for IVRC of probiotic or tumor targeted bacterial vectors [6, 16, 17]. However, the identification of new promoters and their corresponding inducers as well as their evaluation in various locations *in vivo* is necessary in order to use such systems for the rational design of bacterial vectors.

In this study, we have used promoters P_{araBAD}, P_{rhaBAD} and P_{tet}, in conjunction with their respective inducers L-arabinose, L-rhamnose or anhydrotetracycline. We compared their suitability for IVRC of EcN that colonized the intestine or tumors of mice. In the course of these experiments, we also observed colonization of gall bladders of mice when EcN was administered systemically at high doses. Nevertheless, IVRC with P_{araBAD} and L-arabinose as inducer was possible in this anatomical compartment.

2. Materials and Methods

2.1. Bacterial strains and plasmids

EcN was kindly provided by Sya Ukena [18]. Plasmid pSL1 harbouring the *luxCDABE* operon (*lux*) of *Photorhabdus luminescens* was kindly provided by Klaus Neuhaus [19]. *lux* was placed under control of inducible promoters in the background of plasmid pASK-IBA63a-plus (IBA, Göttingen, Germany, kind gift by Lothar Germeroth and Thomas Schmidt). *lux* was excised from plasmid pSL1 with NotI, blunt ended and ligated to the XbaI-linearized, blunt ended vector pASK-IBA63a-plus, yielding plasmid pHL298. Plasmid pHL298 retains promoter P_{tet} and repressor *tetR* of plasmid pASK-IBA63a-plus for tetracycline inducible expression of *lux*. L-arabinose inducible promoter P_{araBAD} in conjunction with its regulatory gene *araC* of plasmid pHL238 [16] was excised with XbaI and BsaAI. The L-rhamnose inducible promoter P_{rhaBAD} of plasmid pWA21 [20] was excised with XbaI and Eco47-III. Both promoter fragments were blunt ended and inserted into the *Ban*I site of plasmid pSL1, yielding plasmid pHL287 and pHL297, respectively. To transfer the *lux* cassettes with P_{araBAD} and P_{rhaBAD} into plasmid pASK-IBA63a-plus, pHL287 and pHL297 where digested with XhoI, blunt ended and digested further with NcoI. Appropriate fragments where inserted into BalI and NcoI digested plasmid pHL298, yielding plasmids pHL302 and pHL303. A constitutive *lux* expression cassette was obtained from plasmid pLite201 [21] by digestion with EcoRI. This fragment was subcloned into the EcoRI site of plasmid pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany). The intermediate construct pHL300 was cut with BspLUII, blunt ended and again digested with BsrGI. The *lux* fragment was then inserted into BalI and BsrGI digested plasmid pHL298, yielding plasmid pHL304. Plasmids were transformed into EcN by electroporation. Bacteria were grown in LB medium at 37°C supplemented with 100 µg ml^{-1} ampicillin or 30 µg ml^{-1} kanamycin..

2.2. Induction of bacterial bioluminescence in culture

Bacteria harboring reporter plasmids were grown to OD_{600} 0.4. 100 mg L-arabinose, 100 mg L-rhamnose or 0.08 mg anhydrotetracycline, respectively, were added and bacterial bioluminescence determined. For measurements, 10 µl of cultures were transferred into wells of a V-shaped 96-microwell plate and light emission was detected with the IVIS-200 Xenogen system (Caliper Life Sciences, Rüsselsheim, Germany).

2.3. Animals and cells

Six-week-old female BALB/c mice were purchased from Harlan (Germany). All animal experiments were performed in accordance with institute and government regulations and were approved by the local authority LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit). Cells of the colon adenocarcinoma cell line CT26 (ATCC CRL-2638) were grown in IMDM medium (Gibco, Karslruhe, Germany) supplemented with 10 % (v/v) fetal calf serum, 2 mM L-glutamine and 10 mM HEPES. For injection into mice, CT26 cells were trypsinized, washed and resuspended in phosphate-buffered saline (PBS). 10^6 cells were injected subcutaneously at the abdomen. Mice bearing tumors of approximately 4 - 6 mm in diameter were used for experiments. Uninfected animals received standard complete diet R/M-H (SSNIFF, Soest, Germany) containing various plant components. Two days before administration of bacteria to mice and throughout the experiment, standard diet was replaced by purified experimental feed EF R/M Control (SSNIFF, Soest, Germany), if not stated otherwise. EF R/M Control feed contains D-glucose as the sole sugar component.

2.4. Administration of bacteria to mice and IVRC

Colonization of the gut with EcN was performed as described before [2]. 10^{10} bacteria in a volume of 200 µl PBS were administered to mice intragastrically with a gavage. To ensure

stable intestinal colonization with plasmid bearing EcN throughout the experiment, 0.3 mg ml^{-1} ampicillin was added to the drinking water of mice. IVRC induction experiments with orally colonized animals were started at day 3 post infection (p.i.). For systemic administration, freshly grown bacteria were suspended in PBS and injected at indicated doses intravenously into mice. These mice were subjected to IVRC induction experiments 2 days p.i.. For oral IVRC, the drinking water of mice was supplemented with either 0.1 mg ml^{-1} anhydrotetracycline, 100 mg ml^{-1} L-arabinose or 100 mg ml^{-1} L-rhamnose, respectively. For systemic IVRC, mice received intraperitoneally (i.p.) either 0.08 mg anhydrotetracycline, 100 mg L-arabinose or 100 mg L-rhamnose, respectively, dissolved in PBS. In case of intravenous (i.v.) infections, mice received a quarter of the dose used for i.p. administrations.

2.5. Recovery of EcN from infected tissues and feces

At selected time points, tissue samples were obtained. Samples were transferred into 3 ml of sterile ice-cold PBS containing 0.1 % (v/v) Triton X-100 and subsequently disrupted with a Polytron PT3000 homogenizer (Kinematica, Littau, Switzerland). For enumeration of bacteria, homogenates were serially diluted in PBS and streaked onto LB agar plates containing 100 µg ml-1 ampicillin.

2.6. Non-invasive *in vivo* imaging of bioluminescence

Bioluminescence emitted from EcN within living mice was detected by the use of the IVIS-200 Xenogen system. Prior to analysis, mice were anaesthetized with 2 % isoflurane using the XGI-8 gas anesthesia system (Caliper Life Sciences, Rüsselsheim, Germany). Pseudocolored images of photon counts and photographic images were obtained according to instructions of the manufacturer. The software Living Image 2.5 (Xenogen) was used for

image analysis and quantification of emission intensities which are expressed as radiance (p s-1 cm-2 sr-1).

2.7. Determination of L-arabinose and L-rhamnose in serum and intestinal content

L-arabinose and L-rhamnose was administred to mice either orally with drinking water or i.p. as described above. Before and at consecutive time points after application of inductors blood was taken by retro-orbital bleeding and serum was derived by centrifugation using microvettes (Sarstedt, Nuembrecht, Germany). Intestinal content of small intestine, large intestine and cecum was obtained, weighted and suspended in 100 µl PBS. The suspension was centrifuged at 13000 rpm for 5 min and the supernatant recovered for analysis. To determine the concentration of respective sugars, samples were analyzed by gas chromatography-mass spectrometry (GC-MS). After methanolysis (0.625 M HCl in methanol, 12 h 70°C) the constituents (monosaccharide methylglycosides) were pertrimethylsilylated and analyzed by GC-MS on a 30-m DB5 capillary column connected to a Finnigan GCQ ion trap mass spectrometer (Finnigan MAT Corp., San Jose, USA) running in the electronic impact mode. The components were identified by their retention time on the GC column and their mass spectra. Exact quantification was achieved by electronic peak integration and use of an internal standard (1 µg inositol).

3. Results

3.1. Colonization of mice by constitutive bioluminescent *E. coli* Nissle 1917

Non-invasive bioluminescence imaging was used to follow the time course of EcN colonization in mice. Bacteria were equipped with a construct mediating constitutive bioluminescence emission. Such bacteria were administered orally to healthy mice using a gavage. To ensure stable plasmid propagation, mice were supplied with drinking water containing ampicillin [2]. With such infected mice, a series of live bioluminescence images was obtained (Fig. 1A) and light intensities from regions of the gastrointestinal tract of the animals were quantified (Fig. 1B). Strong light emission at the abdomen of these mice was already detected shortly after oral administration of the bacteria. A gradual decrease of light intensity at subsequent time points was observed, most likely due to shedding of the bacteria from the intestine. Plating of intestinal sections of orally inoculated mice was used to determine the number and location of bacteria within the intestinal tract (Fig. 1C). EcN colonized the cecum and the lower intestinal tract, the upper intestine was almost completely free of EcN after three days.

Similar experiments were carried out with mice bearing the CT26 tumor subcutaneously. EcN constitutively expressing *lux* were administered i.v. and monitored during colonization. In tumors, bacteria became detectable one day after application (Fig. 1D and E). At two days post infection, the bioluminescence intensity from colonized tumors peaked and decreased thereafter (Fig. 1D and E).

Plating tissue homogenates three hours after administration, bacteria were recovered from tumors and livers at similar numbers. Numbers in spleen were slightly higher at this time point (Fig. 1F). Within the first 24 hrs, apparently exponential bacterial growth took place in tumors since a 10^5-fold increase in bacterial numbers was found at that time point. Subsequently, bacterial numbers in tumors remained constant at high levels. In contrast, bacterial numbers decreased steadily in spleen and liver, although low numbers could be

recovered until termination of the experiment. Under these conditions, the bacteria gradually lost the constitutive reporter plasmid. At day 4, only 12-17 % of bacteria had retained the plasmid. The decrease of bioluminescence emission between day 2 to 4 after administration of bacteria most likely reflects this instability of plasmid maintenance.

3.2. IVRC of EcN colonizing the intestine of mice

Inducible promoters P_{araBAD}, P_{rhaBAD} or P_{tet} were chosen to evaluate their suitability for IVRC of EcN in the intestinal tract and tumors. First, we tested *in vitro* induction of bioluminescence by bacteria harboring reporter constructs. For all three promoters bioluminescence emission peaked one hour after addition of inducer substrates to the culture medium and decreased thereafter, most likely due to degradation of inducers (Fig. 2A). Importantly, inducers only activated their corresponding promoter and not the others (Fig. 2B).

Recombinant EcN with such reporter constructs were then administered orally to healthy mice. Subsequent to colonization, inducer substances L-arabinose, L-rhamnose or anhydrotetracycline were administered orally via the drinking water. By use of *in vivo* bioluminescence imaging, the time course of reporter gene expression was analyzed.

Interestingly, intestinal EcN harboring a reporter construct driven either by P_{araBAD} or P_{rhaBAD} displayed basal levels of bioluminescence when mice received standard mouse food. Feeding a diet devoid of plant material and containing D-glucose as sole sugar source abolished this basal level of reporter expression. Return to standard food induced reporter expression again (Suppl. Fig. 1). Indeed, 264 µg of L-arabinose and 12.5 µg L-rhamnose could be determined per gram of normal mouse chow. To reduce background, mice were kept on food free of plant components in all subsequent experiments.

IVRC of intestinal EcN was induced three days after oral administration of bacteria by supplementing the drinking water with inducer substances for 24 hrs. Reporter expression

mediated by promoter P_{araBAD} was induced to its highest level after 24 hrs and ceased thereafter when L-arabinose was removed from drinking water (Fig. 3A). Induction of promoter P_{rhaBAD} by L-rhamnose followed the same kinetics albeit at a lower level (Fig. 3B). This coincided well with the concentration of inducers in the intestine at this time point (Suppl. Fig. 2B and C). Promoter P_{tet} exhibited its highest level of gene expression by 36 hrs, 12 hrs after removal of anhydotetracycline from drinking water (Fig. 3C).

We also tested systemic application of the inducers for IVRC of gut residing EcN by administering a single pulse of inducer substances intraperitoneally. Here, high amounts of L-arabinose and L-rhamnose inducers could be determined in serum with a rapid decline (Suppl. Fig. 2A). Interestingly, despite i.p. administration reporter induction of gut residing EcN could readily be observed (Fig. 3D-E). Highest expression for promoters P_{araBAD} and P_{rhaBAD} was observed between 6 to 12 hrs. In two mice promotor P_{araBAD} lead to a level of expression similar to the level observed when the inducer was given via drinking water (Fig. 3D). Again, P_{araBAD} was superior compared to the two other promoters, which lead to 3-4 fold less bioluminescence. Thus, the inducer substances can reach the intestine efficiently even when applied systemically.

3.3. IVRC of EcN colonizing tumors

The recombinant EcN described above were administered i.v. to mice bearing a subcutaneous CT26 tumor. IVRC of EcN that had colonized the tumors was induced two days after administration. Oral administration of P_{tet} inducer via drinking water mediated highest reporter gene expression in tumor residing EcN (Fig. 4C). In contrast, orally administered L-arabinose or L-rhamnose mediated IVRC only to a lower degree (Fig. 4A and B).

When inducers were applied i.p. to mice, reporter gene expression in tumor colonizing EcN reached its maximum for all three promoters 6 to 12 hrs thereafter (Fig. 4D-F). In this

situation, P_{araBAD} appeared to mediate the highest gene expression compared to the other two promoters. By 6 hrs post activation, promoter P_{rhaBAD} and P_{tet} mediated reporter expression at a similar level (Fig. 4E and F). However, gene expression mediated by P_{tet} surpassed P_{rhaBAD} at 12 hrs and at 24 hrs even P_{araBAD}. In case of P_{araBAD} a 7-fold higher mean peak of bioluminescence was observed compared to oral application of L-arabinose.

3.4. IVRC of EcN inside the gall bladder

Throughout IVRC experiments with tumor bearing mice, that had received approximately $5x10^6$ recombinant EcN i.v., we observed focused bioluminescence in the upper abdominal region in some of the animals. It resembled signals previously observed in mice, where gall bladders were colonized with bioluminescent *Listeria monocytogenes* [22]. Therefore, we tested with healthy mice whether intravenous administration of EcN indeed gives rise to colonization of gall bladders and if so whether bacteria would be shed into the intestine. Three groups of mice received different doses of EcN i.v.. Three days after injection, bacteria were quantified in gall bladders and feces of mice (Fig. 5A and B). In animals, which had received the highest dose nearly all gall bladders were colonized. In addition, this extensive colonization corresponded with recovery of bacteria from feces of most of the mice. The one mouse with no detectable EcN in feces, displayed also low numbers of EcN in the gall bladder. When lower doses of bacteria were applied, gall bladders were colonized in 60 % or 20 % of mice, respectively. Again, bacteria were recovered from feces of mice, in which gall bladders exhibited a high bacterial load.

Importantly, despite the efficient colonization of gall bladder at high doses the colonization did not become chronically. To this end, we followed mice that were infected with a high dose of constitutively bioluminescent EcN. This allowed to confirm colonization of the gall bladder by *in vivo* imaging. Quantification of EcN in gall bladder of such mice at different

time points revealed a steady decline of bacterial numbers in this organ (Suppl. Fig. 3). By four weeks hardly any bacteria could be found.

To test, whether bacteria within gall bladders are also accessible to IVRC, mice were infected intravenously with EcN harboring the P_{araBAD} reporter construct. Three days later, we administered L-arabinose i.p.. Reporter expression by bacteria in gall bladder but also in the intestine was detected (Fig. 6A). In gall bladder, induction occurred quickly with its peak three hours after inducer injection (Fig. 6B). For bacteria shed into the intestine, activation of P_{araBAD} was detected almost in parallel (Fig. 6A).

The focused bioluminescence signal of the upper abdominal region in mice was proven to originate from the gall bladder by imaging of tissue from sacrificed animals (Fig. 6C). Strong bioluminescence was detected from gall bladder in contrast to absence of bioluminescence in spleen and liver of the mice. Only in liver, bioluminescence was observed restricted to the point from were the gall bladder had been removed. This point probably represents the opening of the disrupted bile duct, which connects the gall bladder with the small intestine (Fig. 6C).

4. Discussion

The *in vivo* remote control of bacterial gene expression is an essential feature of bacteria-mediated therapy. This concerns the deliberate expression of heterologous genes that encode therapeutic molecules but also might provide control of the bacterial vector when linked with inducible suicide genes [16]. The later aspect is an essential safety feature since suicide should be effective almost immediately upon induction. Administration of antibiotics should be inferior for control. Antibiotics are known to require an extended period of time to exert their effects [23].

In vivo bioluminescence imaging has established itself as a versatile tool for the kinetic analysis of bacterial colonization in living mice. Here, we first followed the course of colonization of bioluminescent EcN in intestine and tumors. Quantitative bioluminescence data correlated with conventional quantification of bacterial numbers by plating of tissue homogenates. However, only plating of intestinal sections revealed, that the small intestine is quickly cleared from EcN after oral administration.

To tailor bacteria for IVRC requires appropriate inducible promoters. Depending on the target tissue of the carrier bacteria and the therapeutic aims, promoters with defined properties have to be carefully selected. Here, we compared three of such potential control elements - P_{araBAD}, P_{rhaBAD} and P_{tet}, and their respective inducers L-arabinose, L-rhamnose or anhydrotetracycline - in living mice. Their induction pattern does not overlap i.e. each inducer substance is specific for its particular control element. This is a key prerequisite for the establishment of multiplex IVRC of bacterial vectors in which several bacterial functions should be independently regulated *in vivo* [24].

Another important feature of an inducible promoter is the tight control in uninduced state. Interestingly, initially we observed background activity with promoters P_{araBAD} and P_{rhaBAD} in the absence of inducer. However, this activity was not due to leaky control of the promoter. Rather, it was due to the presence of inducer substances in the food. Such

complications should be avoidable by appropriate diets or selection of systems like P_{tet} and anhydrotetracyclin, as we have demonstrated. On the other hand, inducible promoters that do not fulfill the criteria of tight control might be modifiable [25]. Moreover, advancing insights into natural promoter systems might allow rational design of artificial IVRC gene switches [26].

We could establish that IVRC is feasible for EcN colonizing intestine and tumors as target locations for therapeutic intervention. Two interesting aspects were observed during these experiments. IVRC is possible by oral application of the inducer substances. This was independent whether the bacteria were residing in the gut where the inducer would be expected to reach the bacteria efficiently or whether they colonized other sites in the body. In tissue, it was *a priori* not clear whether sugars or antibiotics would reach EcN to allow IVRC. They obviously did at sufficient concentrations.

A very interesting result was the rapid induction of the promoters in gut-associated bacteria by systemically applied inducer substances. The onset of gene expression was similar to orally applied inducers, only the duration of expression was more sustained when oral inducers were supplied. This however can be explained by the continuous presence of inducer in the drinking water while systemic application consisted of a single pulse.

The quick appearance of inducers in the gut after systemic application might have several reasons, like the substances might quickly diffuse through the gut wall transport of inducers into the gall bladder and subsequent release into the intestine via gall liquid. However, most likely is the transport of such substances from circulation into the gut via nutrient transporters that normally transport substances from intestine into the body [27]. Since in our case the concentration of inducers is higher in blood than in the gut, transport might be reversed. Similarly, efficient IVRC was possible with EcN that resided in the gall bladder. The kinetics of induction also suggested an active transport into this compartment.

Transition of EcN from blood into gall bladder where bacteria thrived at high numbers was not predictable. Thus far, such findings had only been reported for *Listeria monocytogenes*, an invasive Gram positive bacterium [22]. However, the presence of various bacteria in gall bladders has been observed in animals and humans, and their role in the formation of gall stones has been discussed [28]. The ability of EcN to access the gall bladder clearly depended on the dose of the systemic inoculum. Only at high doses efficient colonization was observed. This might indicate that only rarely EcN can escape from blood stream into bile ducts of the liver and reach the gall bladder. However, when the bacteria thrive in this protected compartment, they finally are shed into the gut via the gall duct.

Long-term colonization of tumors might generate high numbers of EcN in the body [6]. These numbers might be equivalent to the high doses introduced in the present work and colonization of gall bladder might occur. The risks, which gall bladder colonization represents for patients is not predictable at the moment. The mice apparently did not suffer from the colonization in our short-term assays at least no change in macroscopic appearance or behavior was observed. In addition, colonization of gall bladder was apparently not chronically.

Independent of such concerns, we could demonstrate IVRC of EcN with three independent promoters in different body niches of mice. Moreover, inducers could be either administered systemically, or simply via the oral route as additive to the drinking water or as food component. The careful kinetic analysis of additional promoters inducible with a variety of substances is an essential prerequisite to establish a panel of suitable regulatory modules. Such promoters would represent valuable sources for the rational design of bacterial IVRC vectors. Controllable bacterial vector systems should possibly help to overcome present safety concerns and enable a highly flexible and individualized treatment of various human disorders and malignancies.

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Figure legends

Figure 1. Colonization of murine intestine and solid tumors by bioluminescent *Escherichia coli* Nissle 1917. **(A)** 10^{10} constitutive bioluminescent EcN harboring plasmid pHL304 were orally administered to mice and at consecutive time points emission of bioluminescence was recorded using the IVIS 200 system. False color images of one representative animal are displayed. **(B)** Quantification of bioluminescence intensities at the abdominal region of mice colonized by bioluminescent EcN (thin lines represent individual anminals, the thick line represents the arithmetic mean). **(C)** Colonization of the intestinal tract by EcN. At consecutive time points after oral administration EcN was enumerated in tissue homogenates by plating (n=3 each time point). SI: small intestine, LI: large intestine. **(D)** 5 x 10^6 CFU of constitutive bioluminescent EcN harboring plasmid pHL304 were intravenously (i.v.) injected into mice bearing the colon adenocarcinoma CT26. At consecutive time points emission of bioluminescence was recorded using the IVIS 200. False color images of one representative animal are displayed. **(E)** Quantification of bioluminescence intensities from tumors of mice colonized by bioluminescent EcN (thin lines represent individual anminals, the thick line represents the arithmetic mean). **(F)** Colonization of tumor, spleen and liver by bioluminescent EcN. Bacterial numbers were determined in tumor, spleen and liver of animals at various time points post infection by plating (n=3 each time point). Experiments were repeated several times with similar results.

Figure 2. Induction of bacterial bioluminescence *in vitro*. **(A)** EcN harboring reporter plasmids with inducible promoters pHL302 (P_{araBAD}), pHL303 (P_{rhaBAD}), pHL298 (P_{tet}) and the constitutive promoter pHL304 (P_{lux}) were grown up to OD_{600} ~0.4. Then, 100 mg L-arabinose, 100 mg L-rhamnose or 0.08 mg anhydrotetracycline were added to cultures with the corresponding reporter construct. At subsequent time points 10 µl of each culture were transferred into a microwell plate for measurement of light emission. Bacterial numbers

were determined by plating. Bacterial bioluminescence is expressed as radiance per CFU (p s^{-1} cm^{-2} sr^{-1} CFU^{-1}). **(B)** Specificity of promoters for corresponding inducers was tested. Inducers L-arabinose (L-ara), L-rhamnose (L-rha) and anhydrotetracycline (a-tet) were added into cultures of either inducible EcN reporter strain or the constitutive bioluminescent strain as before in (A). 1 hr after inducer addition, 10 µl of each culture were subjected to measurement with the IVIS system. Pseudocolor indicates substrate specific induction of promoters. Data from representative experiments of several are displayed.

Figure 3. IVRC of intestinal EcN. 10^{10} CFU of EcN harboring plasmids pHL302 (P_{araBAD}), pHL303 (P_{rhaBAD}) or pHL298 (P_{tet}), respectively, were orally applied to mice using a gavage. (**A-C**) 3 days after administration of bacteria, the drinking water was supplemented with inducer substrates for 24 hrs and mice were subjected to non-invasive imaging using the IVIS 200 system. Emission signals from the abdominal region were quantified. (**D-E**) Colonized animals as in (A-C) received inducer substances i.p. and bioluminescence emission was quantified. Thin lines represent individual animals and the arithmetic mean of each group is represented by a thick line. Similar experiments were repeated at least three times.

Figure 4. IVRC of tumor colonizing EcN. 5 x 10^6 CFU of EcN harboring plasmids pHL302 (P_{araBAD}), pHL303 (P_{rhaBAD}) or pHL298 (P_{tet}), respectively, were intravenously administered to mice bearing CT26 tumors. Two days p.i. animals received inducer substances and mice were subjected to non-invasive imaging. Emission signals derived from tumors were quantified as in Fig 2. (**A-C**) Drinking water of mice was supplemented with inducer substrates for 24 hrs. (**D-E**) Mice received inducer substrates intraperitoneally. Thin lines represent individual animals and the arithmetic mean of each group is represented by a thick line. Experiments were repeated at least three times with similar results.

Figure 5. Colonization of gall bladders by EcN and fecal shedding. Groups of healthy mice (n=15) received intravenous doses of either 2 x 10^7, 4 x 10^6 or 8 x 10^5 CFU EcN. Bacterial counts of gall bladders (**A**) and feces (**B**) of individual animals are displayed. Horizontal lines indicate the geometric mean of bacterial numbers within each group of animals.

Figure 6. L-arabinose mediated IVRC of EcN residing in gall bladder. Mice received i.v. 2x10^7 CFU of EcN harboring plasmid pHL302. Three days post infection mice received a dose of 100 mg L-arabinose i.p. (**A**) Non-invasive imaging at consecutive time points revealed bioluminescence signals in gall bladders and in the abdominal region of mice. False color images of one representative animal are displayed. The white arrow points at one signal from the gall bladder. (**B**) Quantification of bioluminescence derived from gall bladders of mice (thin lines represent individual anminals, the thick line represents the arithmetic mean). (**C**) Six hrs post induction, liver (L), gall bladder (G) and spleen (S) of one infected mouse were separated and subjected to bioluminescence imaging. Bioluminescence of EcN is demonstrated by the pseudocolor images. The black arrow is directed to the point were the gall bladder was removed. Scale bar represents 1 cm.

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