Increasing numbers of cancer cases generate a great urge for new treatment options. Applying bacteria like Salmonella enterica for cancer therapy represents an intensively explored option. These bacteria have been shown not only to colonize solid tumors but also to exhibit an intrinsic antitumor effect. In addition, they could serve as tumor-targeting vectors for therapeutic molecules. However, the pathogenic S. Typhimurium strains used for tumor therapy need to be attenuated for safe application. Here, lipopolysaccharide (LPS) deletion mutants (ΔrfaL, ΔrfaG, ΔrfaH, ΔrfaD, ΔrfaP, and ΔmsbB mutants) of Salmonella were investigated for efficiency in tumor therapy. Of such variants, the ΔrfaD and ΔrfaG deep rough mutants exhibited the best tumor specificity and lowest pathogenicity. However, the intrinsic antitumor effect was found to be weak. To overcome this limitation, conditional attenuation was tested by complementing the mutants with an inducible arabinose promoter. The chromosomal integration of the respective LPS biosynthesis genes into the araBAD locus exhibited the best balance of attenuation and therapeutic benefit. Thus, the present study establishes a basis for the development of an applicably cancer therapeutic bacterium.

Cancer has become the second most frequent cause of death in industrialized countries. This and the drawbacks of routine therapies generate an urgent need for novel treatment options. Applying appropriately modified S. Typhimurium for therapy represents the major challenge of bacterium-mediated tumor therapy. In the present study, we demonstrated that Salmonella bacteria conditionally modified in their LPS phenotype exhibit a safe tumor-targeting phenotype. Moreover, they could represent a suitable vehicle to shuttle therapeutic compounds directly into cancerous tissue without harming the host.

Strong efforts have been mounted in recent years to revive the idea of bacterium-mediated tumor therapy. Many bacteria have been shown to target experimental solid tumors with high specificity and exert therapeutic effects on tumors. In this context, safety strains have been established by in vitro or in vivo selection (7). In addition, clinical trials have been initiated, although their success was ambiguous (8–10).

Besides the intrinsic antitumor effect, a second important aspect exists, namely, to use such bacteria as shuttle vectors for therapeutic molecules. Recombinant bacterial strains could be tailored to specifically target tumor tissue and produce therapeutic agents directly inside neoplastic tissue. Accordingly, Salmonella enterica serovar Typhimurium and Clostridium species have been intensively explored (9, 11, 12). Combining intrinsic immune-mediated and extrinsic vector-based bacterial therapies could be a novel and innovative treatment strategy for cancer patients in the future.

A problem that remains is that a suitable strain should exhibit an optimal balance between immune stimulation and being safe...
for human patients. Wild-type (WT) bacteria would cause severe septicemia with a fatal outcome. Too strongly attenuated bacteria will not stimulate the immune system sufficiently or might not reach the tumor at all. The latter might be the explanation for the disappointing outcome of the first clinical trial in which a safe variant of *S. Typhimurium* with a modified lipid A moiety of the LPS was used (10). This modification strongly reduced the immunostimulatory capacity of the bacteria, as judged by the induction of tumor necrosis factor alpha (TNF-α). However, this cytokine might be essential for efficient colonization of the tumor (13).

In the present work, an alternative attenuation strategy was used. The LPS structure was modified by deleting genes involved in its synthesis. LPS is known to be essential for the integrity of bacteria in the host (14–16). On the other hand, LPS is highly immunostimulatory. Lipid A, as the innermost structural part of LPS, is an important PAMP (17). Most likely, it is required for optimal invasion of solid tumors by bacteria.

To accommodate the two opposing demands, we first tested mutants with a modified LPS structure for their performance in vitro and in tumor-bearing mice. Only variants in which the LPS core structure was compromised showed acceptable safety profiles. However, their therapeutic potential was dramatically reduced. To reconcile these features with the required immunostimulatory and therapeutic potency, we complemented the LPS mutants with a construct that allowed inducible expression of the complementing gene. This recovered much of the therapeutic capacity without increasing the risk of septicemia problems.

**RESULTS**

LPS mutants and their in vitro characterization. LPS variants of *S. Typhimurium* strain ATCC 14028 were constructed by deleting the genes rfaL, rfaG, rfaH, rfaD, rfaP, and msbB as schematically shown in Fig. 1A. To confirm the mutations, SDS-PAGE was used to visualize the modified LPS (Fig. 1B). As expected, the ΔrfaL mutant exhibited a lack of O antigen, as indicated by loss of the repetitive bands. The shorter length of the main band of the ΔrfaH mutant is consistent with the loss of the additional sugars, similar to the ΔrfaG mutant with a further reduced outer core. The size of the major band of the ΔrfaD mutant indicates absence of the inner core. The slight shift of bands of the ΔmsbB variant is due to a

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**FIG 1** LPS phenotypes of *Salmonella* mutants. (A) Schematic representation of LPS structure. The genes encoding the enzymes for particular steps in the synthesis or modification of LPS that were deleted in the present work are red. (B) Visualization of the LPSs of various *Salmonella* mutant strains by 16.5% Tris-Tricine SDS-PAGE and silver staining. The ΔrfaL, ΔrfaH, ΔrfaG, and ΔrfaD LPS mutants lack the O antigens (repetitive bands). The different electrophoretic mobility of the LPS of the remaining variants confirms the expected structure. The ΔmsbB mutant exhibits a pattern comparable to that of the WT. However, the bands migrate slightly faster because of the penta-acylated lipid A structure.
penta-acylated lipid A structure. In the Δrfap variant, the normal O-antigen structure is present. However, the bands observed were weaker and the strong low-molecular-weight band indicates that a portion of the LPS is truncated at the core.

These mutants were further characterized to test their in vivo applicability. Growth in LB or minimal medium showed no defects (see Fig. S1 in the supplemental material). Smaller colonies of the Δrfap mutant were observed on LB plates, indicating a slight growth defect that is observable only after prolonged incubation.

As expected, the Δrfad, Δrfag, and Δrfal deletion mutants were resistant to P22 (Fig. 2A). Similarly, no motility was detected in the Δrfag, Δrfad, and Δrfal mutants within 4 h (Fig. 2B). However, after 24 h, limited motility was observed in the Δrfag and Δrfad mutants. Electron microscopy revealed that the majority of the rfas-deficient mutants did not express flagella, whereas a few bacteria still exhibited a reduced number of flagella in comparison to that of the WT (Fig. 2C).

**In vivo colonization profile and virulence.** CT26 tumor-bearing mice were infected with the LPS variant strains to evaluate their potency for cancer therapy. Blood, spleen, and tumor homogenates were plated at 12 and 36 h postinfection (hpi) (Fig. 3).

The colonization profile of the Δrfag and Δrfad LPS core mutants differed significantly from that of the WT and the other mutants. Apparently, these bacteria exhibit strongly reduced fitness in vivo. The bacterial burden was strongly decreased in the spleen and almost completely cleared from the blood within 12 h (Fig. 3A and B). The ΔmsbB mutant was cleared from the blood, the splenic burden remained relatively high in comparison to that of the Δrfad or Δrfag mutant. The Δrfal mutants behaved similar to an infection with the WT, consistent with their slight alterations of the LPS structure distal to the core membrane structures. Bacteria of all of the strains eventually colonized the tumors to the same degree (Fig. 3B).

The body weights of infected mice were monitored to evaluate the impact of the mutants on the general health of tumor-bearing mice (Fig. 3C). The ΔmsbB, Δrfad, and Δrfag mutants appeared to be appropriately attenuated. The mice recovered quickly after an initial weight loss, although the impact of the ΔmsbB mutant was much more severe. In contrast, the Δrfal, Δrfag, and Δrfad mutants were highly virulent and the mice succumbed to the infection. For that reason, the Δrfag and Δrfad mutants were cho-
serves as potential candidates for further experiments. These two strains exhibit the highest tumor-to-spleen ratio of $10^3:1$ to $10^4:1$ and the lowest health burden for the mice. To verify these findings, the $\Delta rfaG$ mutant was also tested in RenCa and F1A11 tumor-bearing BALB/c mice (see Fig. S2 in the supplemental material). The same tumor-to-spleen ratio was found. Thus, a tumor-specific effect can be excluded.

Sensitivity of LPS mutants to innate defense mechanisms. The attenuating effect of these mutations is already apparent after a short time (see Fig. S3 in the supplemental material). Hence, bacteria of such strains might be more sensitive to effector mechanisms of the innate immune system. Treatment with human serum revealed that WT salmonellae were not affected by complement while complement-induced lysis of the LPS mutants was stronger the more the LPS structure was shortened (Fig. 4A). Virtually all of the $\Delta rfaD$ mutant bacteria were lysed within 30 min. The modification of lipid A by the $msbB$ mutation did not influence the bacterial behavior toward these innate effector molecules (Fig. 4A).

This was further evaluated by depletion of complement from tumor-bearing mice with cobra venom factor (CVF). Quantitation of the bacteria in organs, blood, and tumors revealed that the reduced fitness of the $\Delta rfaD$ mutant might not be due to the complement system. Colonization was very similar to that of untreated mice (Fig. 4B). However, this experiment might underestimate the role of complement in vivo since complete depletion was not achieved (see Fig. S4 in the supplemental material).

Since the complement system might have only a minor impact on the inner-core LPS mutants in vivo, the role of macrophages was investigated. Resistance to uptake by J774 macrophages was analyzed. The majority of the $\Delta rfaL$ mutant bacteria were phagocytosed within 1 h (Fig. 4C). The $\Delta rfaG$ mutant was also readily

![FIG 3](mbio.asm.org)
taken up by these macrophages, while uptake of the ΔrfαD mutant was low, similar to that of WT salmonellae. Importantly, the numbers of ΔrfαD, ΔrfαG, and ΔmsbB mutant bacteria within the macrophages were reduced significantly at 18 hpi. This indicates that such mutants could not resist the effector mechanisms of the phagocytic cells. In contrast, the ΔrfαL mutant, lacking only the O antigen, was still able to replicate in macrophages although at a lower rate (Fig. 4D). The experiment was corroborated with bone marrow-derived macrophages (BMDM) from BALB/c mice with similar results (see Fig. S5 in the supplemental material).

To analyze the activity of macrophages against such LPS variant bacteria in vivo, macrophages were depleted with clodronate before infection with the ΔrfαD mutant (see Fig. S6 in the supplemental material). Quantitation of the numbers of CFU in organs, blood, and tumors demonstrated that bacteria were still present in the blood and liver at 24 hpi while they were absent from samples

FIG 4 Sensitivities of selected LPS variants to complement and phagocytes. (A) In vitro sensitivities of different LPS variants to complement. A total of 2 × 10^7 bacteria were treated with either untreated or heat-inactivated (HI) human serum for 30 min at 37°C. The lysis effect was determined by plating and correlates with the LPS structure. (B) Influence of in vivo depletion of the complement system on the colonization profile of the ΔrfαD mutant. Mice were treated with 5 IU of CVF at 12 h before infection. Bacterial burdens were assessed by plating tissue homogenates. (C) Sensitivities of selected LPS variants to phagocytes. J774 cells were infected at an MOI of 1, and phagocytic uptake was determined relative to that of the WT after 1 h. The uptake of the ΔrfαG and ΔrfαL mutants is significantly increased, whereas that of the ΔrfαD mutant remains at WT levels. The ΔmsbB mutant was almost not taken up by J774 cells. (D) Intracellular replication of LPS mutants within J774 cells. Cells were allowed to phagocytize the bacteria as in panel C. Infected cells were incubated for 18 h, and the remaining bacterial burden was determined by plating. Except for the ΔrfαL mutant, the LPS mutants cannot survive in J774 cells. (E) To determine the importance of macrophages for the in vivo survival of LPS variants, mice were treated with clodronate (CI) or left untreated and then infected with 5 × 10^6 ΔrfαD mutant bacteria i.v. The number of bacteria was determined by plating of serial dilutions of tissue homogenates. The median and range are displayed. Results are representative of two independent experiments with five replicates per group. *, P < 0.05.
from untreated mice (Fig. 4E). Thus, mainly macrophages appeared to be responsible for the decreased fitness of LPS mutant strains, in particular of the \( \Delta rfaD \) and \( \Delta rfaG \) mutants. Attenuation of the LPS variant strains is apparently achieved by increasing bacterial sensitivity to innate immune effector mechanisms.

**Intrinsic antitumor effect of the \( \Delta rfaD \) and \( \Delta rfaG \) mutants.** While attenuation of the \( rfaD \) or \( rfaG \) mutant was satisfactorily achieved, therapeutic efficacy needed to be established. As shown in Fig. 5, the volumes of the CT26 tumors of mice infected with the *Salmonella* \( \Delta rfaD \) or \( \Delta rfaG \) mutant regressed and some tumors were even cleared (Fig. 5A). In contrast, only growth retardation of RenCa tumors was observed (Fig. 5B). Almost no effect on F1A11 sarcomas was observed (Fig. 5C). In general, the antitumor effect was weaker than that of strain SL7207, which was tested in previous studies (18, 19).

Earlier studies showed the importance of a strong initial TNF-\( \alpha \) response, which is required for successful tumor colonization. Thus, the TNF-\( \alpha \) induction was determined in the blood of infected mice shortly after bacterial application. In comparison to those achieved with the WT, the levels of TNF-\( \alpha \) were greatly reduced when CT26-bearing mice were infected with the \( \Delta rfaD \) or \( \Delta rfaG \) mutant (Fig. 5D). This might explain the weaker antitumor response.

**Improvement of the \( \Delta rfaD \) and \( \Delta rfaG \) mutant strains.** The \( \Delta rfaD \) or \( \Delta rfaG \) mutant strain needed to be improved. Since the specificity of tumor colonization of the \( \Delta rfaD \) variant was the highest, we concentrated on this variant. A delayed-attenuation system was designed for these bacteria. The strategy was to conditionally complement the LPS mutation with an arabinose-inducible construct. Arabinose should be provided in culture for the mutants to exhibit a WT phenotype, whereas in the host, the bacteria should become attenuated since they are no longer able to express the complemented gene. Various possibilities were tested, ranging from high- and low-copy-number plasmids to chromosomally integrated systems. Figure 6A shows silver-stained SDS-PAGE of the noninduced conditionally complemented variants. Most such systems appeared to be leaky. Only chromosomal integration replacing the \( araBAD \) genes with the PBAD-controlled \( rfaD \) construct appeared to fulfill the requirements (Fig. 6A). The LPS structure in its noninduced state appeared to be like that of the original \( \Delta rfaD \) mutant.

To test whether chromosomal complementation of the PBAD \( rfaD \) mutant could restore the WT phenotype to the \( \Delta rfaD \) mutant, arabinose was added to the cultures. Indeed, the LPS pattern on SDS-PAGE resembled the WT LPS pattern (Fig. 6B). Thus, the chromosomal PBAD \( rfaD \) construct was able to conditionally complement the LPS mutation. We next removed arabinose from the culture to test how long complemented LPS could be detected. The first reduction of band intensity was seen after 1.5 h. By 3 h, complementation had ceased and the LPS pattern resembled that of the clean \( \Delta rfaD \) mutant (Fig. 6B).

These findings were corroborated by testing the motility of such conditionally complemented mutants. Motility was strongly reduced in noninduced variants. Leakiness of the constructs was obviously not as apparent as in silver-stained SDS-PAGE. Induction of complementation by the addition of arabinose from the culture to test how long complemented LPS could be detected. The first reduction of band intensity was seen after 1.5 h. By 3 h, complementation had ceased and the LPS pattern resembled that of the clean \( \Delta rfaD \) mutant (Fig. 6B).
upon the addition of arabinose to the complemented variants (see Fig. S7 and S8 in the supplemental material).

The full WT LPS structure was detected for all \( rfaG \) mutant constructs, even in the absence of the inducer arabinose. Such noninduced conditionally complemented variants were lethal for mice. This indicated that conditionally complemented variants of the \( /H9004\ rfaG \) mutant were too leaky to be useful (data not shown).

**Therapeutic potential of improved strains.** The performance of the chromosomally and arabinose-regulated \( rfaD \) mutant was then tested in the mouse tumor models. Bacteria were induced, i.e., complemented in culture and then injected into tumor-bearing mice. Compared to that of the noncomplemented \( rfaD \) mutant, the antitumor response of the conditionally complemented mutant was clearly enhanced for CT26 tumors (Fig. 7A). Similar observations were made in the RenCa model (Fig. 7B). No improved effect on F1A11 was observed (data not shown). F1A11 has been shown previously to be very resistant to this type of therapy (data not shown).

The conditional activation of the complementing gene with arabinose correlated with a slight increase in the pathogenicity of the strain (Fig. 7C). General health, as judged by weight loss, was affected more than with the noncomplemented \( \Delta rfaD \) mutant. However, the effect was less pronounced than with the \( \Delta msbB \) mutant, which had been shown to be safe in human patients (10).

As expected, stronger induction of TNF-\( \alpha \) was observed in the blood after application of the conditionally complemented \( \Delta rfaD \) mutant (Fig. 7D). Taken together, these complementation experiments demonstrated that a conditional-attenuation approach might be a suitable way to circumvent the problem of early clearance of attenuated bacteria by the immune system. Importantly, this approach achieved stronger antitumor responses and more efficient tumor colonization while at the same time rendering the bacteria safe.

**DISCUSSION**

LPS originating from Gram-negative bacteria like salmonellae is one of the major inducers of sepsis. Therefore, modification of LPS represents an attractive way to attenuate salmonellae for use as a systemically applied vector. However, LPS, as the outermost structure of the cell envelope, is also an important barrier and

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**FIG 6** Phenotypes of conditionally complemented LPS mutants. (A) Complementation was carried out by placing the arabinose-inducible construct in high-copy (HC) and low-copy (LC) plasmids or by chromosomal integration (\( araBAD \)). Silver-stained SDS-PAGE of bacterial lysates in a noninduced state is shown. The chromosomal integration \( \Delta araBAD::P_{BAD} rfaD \) was chosen for further studies since no O antigen was produced. (B) Recovery of the WT LPS phenotype by the \( \Delta araBAD::P_{BAD} rfaD \) variant treated with arabinose (+) and kinetics of loss of WT LPS structure upon removal of arabinose. An aliquot of an induced culture was subcultured in arabinose-free medium. The LPS phenotype was assayed at the times indicated. The deletion phenotype was first detected 3 h after the removal of arabinose. (C) Assay of motility on swimming agar plates. Aliquots of the arabinose-induced or untreated \( \Delta rfaD \) variant were used to inoculate swimming plates containing 0.3% agarose and incubated for 4 h at 37°C. Points of inoculation are indicated by black dots. \( araD \) stands for \( \Delta araBAD::P_{BAD} rfaD \). LC indicates that the variant was conditionally complemented with a construct on a low-copy-number plasmid. When the conditionally complementing construct was chromosomally integrated (\( araD \)), motility was similar to that seen upon a clean deletion (the \( \Delta rfaD \) mutant) in the absence of the inducer arabinose.
in vitro colonization is not entirely clear. When using tumor cylindroids types of solid tumors, although the process of bacterial tumor phenotype has not been established yet. However, the precise mechanism resulting in this nonmotile highly decreased number of flagella on the surface of these bacteria indicated that the lack of motility is likely due to a chemotaxis machinery or production and assembly of flagella.

Transmission electron microscopy of negatively stained bacteriophage P22. Furthermore, the lack of an appropriate LPS structure strongly decreased the motility of the ΔrfaD, ΔrfaG, and Δrfal mutants, indicating that altered LPS interferes with either the chemotaxis machinery or production and assembly of flagella. Transmission electron microscopy of negatively stained ΔrfaD mutant bacteria indicated that the lack of motility is likely due to a highly decreased number of flagella on the surface of these bacteria. However, the precise mechanism resulting in this nonmotile phenotype has not been established yet.

All of the LPS mutants tested were able to colonize different types of solid tumors, although the process of bacterial tumor colonization is not entirely clear. When using tumor cylindroids in vitro, chemotaxis and motility of the bacteria were required (21). However, recent in vivo experimental data suggest that colonization of tumor tissue is a passive event. Mutants lacking motility or chemotaxis genes colonized cancerous tissue in mice to the same extent as the WT (22, 23). Consistently, the same was observed in this study in the nonmotile ΔrfaD and ΔrfaG mutants.

FIG 7 Tumor development upon infection with the arabinose-induced conditionally complemented Salmonella ΔrfaD variant. CT26 (A) and RenCa (B) tumor-bearing mice were infected i.v. with 5 × 10^8 bacteria. Tumor volumes were measured with a caliper. PBS served as a negative control, and SL7207 served as a positive control for comparison. (C) Body weight as an indicator of the general health status of infected mice. Induced (+) ΔaraBAD::PBAD ΔrfaD bacteria exhibited greater virulence but also a stronger antitumor effect. (D) TNF-α levels in sera measured by ELISA at 1.5 hpi. Upon induction with arabinose (+), TNF-α was restored to WT levels. The mean and range are displayed. Results are representative of two independent experiments with five replicates per group.
volved, with macrophages probably being the most important component. In accordance, phagocytic uptake by macrophages was strongly increased when the O antigen was missing. This was reversed when the core structure was further truncated, indicating the importance of the core sugars for immune recognition. However, only the ability to replicate intracellularly appears to be important. Replication of the deep rough LPS Δrfad ΔΔrfag mutants was greatly reduced, and especially the Δrfad mutant was cleared efficiently by macrophages. This could explain the in vivo findings. Bacteria that are readily phagocytosed but are able to replicate cause a lethal infection in mice. In contrast, bacteria with WT-like or increased uptake that are not able to replicate intracellularly are highly attenuated.

Thus, because of their safety profile, the Δrfad and ΔΔrfag mutants appeared to be the most promising and were therefore followed up for their intrinsic antitumor effect. Unfortunately, the therapeutic effect of the unmodified Δrfad and ΔΔrfag variants was found to be weaker than that of SL7207, which was previously employed in our experiments (18, 19, 22, 27). These findings are consistent with LPS being an important inductive molecule for the antitumor effect. The lower level of TNF-α and other cytokines induced by infection with these strains most likely explains the weaker tumor-clearing efficiency observed. To improve their antitumor efficacy, these strains were modified to increase the induction of cytokines without increasing the safety risk.

A conditional-attenuation approach was employed. A similar strategy has been intensively elaborated by the Curtiss lab to design potent bacterial vaccines (28, 29). In the present work, different constructs were designed to achieve conditional or delayed attenuation. All of these constructs rely on complementation of the truncated LPS under the control of an inducible promoter.

The leakage of the arabinose-inducible promoter used was a major problem. Even when introduced on low-copy-number plasmids, the LPS structure of the deletion mutants was comparable to that of the WT. Probably because of the long half-life of LPS, no quantitative differences were noticed independent of the copy number. The problem was overcome only when the construct was placed chromosomally under the control of P_BBAD.

Induction of complementation by the addition of arabinose to the bacterial cultures completely restored the LPS of the Δrfad and ΔΔrfag variants to the WT pattern. In addition, resistance to human complement and phagocytic uptake could be restored upon induction. After removal of arabinose from the cultures, the WT LPS pattern disappeared within 3 h. In the case of the Δrfad mutant, the shift was complete and no O-antigen structures could be detected anymore. In contrast, the complementing construct of rfg could not be silenced completely. WT structures were visible, although at lower intensities. In addition, the conditionally complemented rfg variant in a noninduced state remained lethal for mice. Thus, for unknown reasons, constructs complementing rfg were less leaky than the ones complementing rfg. Alternatively, higher rfg expression levels might be needed to restore the WT LPS phenotype. We therefore conclude that the chromosomally inducible ΔaraBAD::P_BBAD ΔΔrfad ΔΔrfag complementation mutant represents a variant with appropriate regulation of the LPS structure and an intrinsic antitumor effect in the induced state. We cannot exclude a contribution of restored flagellum expression to the antitumor effect. Although we find it unlikely that the regained motility contributes to the activity, expression of flagella could boost the adjuvant effect.

In summary, the use of conditional complementation mutants of LPS variants like the rfaD complementation strain appears to be a promising strategy to establish a safe and therapeutically effective strain for tumor therapy. To improve compliance, additional mutations to also metabolically attenuate the bacteria might be required. In addition, the strains should be further evaluated in orthotopic and autochthonous tumor systems to validate the present results. Nevertheless, we consider our findings an important step toward a clinical application.

MATERIALS AND METHODS

Ethics statement. All animal experiments were performed according to guidelines of the German Law for Animal Protection and with the permission of the local ethics committee and the local authority LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) under permission no. 33.9-42502-04/12/0713.

Bacterial strains and growth conditions. For the bacterial strains and plasmids used in this study and their genotypes and sources, see Table S1 in the supplemental material. The bacteria were grown in LB medium at 37°C either to mid-log phase for subculturing (3 h) or overnight. For induction of P_BBAD-controlled expression, 0.2% (wt/vol) arabinose was added to the subculture before inoculation.

Complementing constructs. The vector backbones pH3L30 (30) and pCMVM4A (31) were digested with SdIII/Xhol and PstI/Xbal, respectively (Fermentas). The rfaD and rfg genes from ATCC 14028 were amplified via PCR with primers with the corresponding restriction sites as overhangs. In the case of low-copy-number plasmid pCMVM4A, the P_BBAD promoter had been transferred from pH3L30 before. For chromosomal integration into the araBAD locus, the araBAD locus was exchanged with the rfaD and rfg genes by lambda Red-mediated recombination (32, 33). For the corresponding primers, see Table S2 in the supplemental material.

LPS phenotype of mutants. All strains were cultured overnight in LB medium. Preparation of LPS was done as described in reference 28. LPS was separated by 16.5% Mini-Protein Tris-Tricine Gel (BioRad) SDS-PAGE and visualized by silver staining. As a WT control, the LPS of ATCC 14028 was used.

Motility assay. Semisolid swimming plates containing 0.3% (wt/vol) agar were prepared. Single colonies were picked with a toothpick and spotted into the agar. After 4 h at 37°C, mutant strain motility was assayed by measuring the swarm diameter and compared to WT motility.

Electron microscopy. The mutants were cultured in 5 ml of LB or minimal medium overnight. On the next day, 650 μl of glutaraldehyde (final concentration, 2%) was added to fix the bacteria. The mixture was stored in the refrigerator at 4°C. For transmission electron microscopic observation, bacteria were negatively stained with 2% aqueous uranyl acetate with a carbon film deposited on mica. Samples were examined in a Zeiss TEM 910 at an acceleration voltage of 80 kV with calibrated magnifications. Images were recorded digitally with a Slow-Scan charge-coupled device camera (ProScan, 1,024 by 1,024) with ITEM-Software (Olympus Soft Imaging Solutions).

Complement sensitivity. Human blood was taken from volunteers, and the serum was isolated with Microvette serum tubes (Sarstedt). Bacteria were adjusted to 2 × 10^7 CFU and challenged with the serum by mixing it 1:1. Serum prepared by heat inactivation at 56°C for 2 h served as a control. The reaction mixture was incubated for 30 min at 37°C. The remaining CFU were determined by plating.

Invasion assay. J774 cells and primary BMDM were used to determine the phagocytic uptake and intracellular replication of the bacteria. The assay was performed as described previously (34). A total of 8 × 10^5 cells were seeded into the wells of a 24-well plate and infected with bacteria (multiplicity of infection [MOI] of 1). Uptake was assayed at 2 hpi by removing the supernatant and determining the CFU count inside the macrophages by plating. The intracellular replication was analyzed at 18 hpi by the same procedure. All values were compared to WT levels.
Murine tumor model. Six- to 8-week-old BALB/c mice (Janvier) were intradermally inoculated with $5 \times 10^5$ syngeneic tumor cell lines. CT26 (colorectal cancer, ATCC CRL-2638), RenCa (renal adenocarcinoma), and F1A11 (fibrosarcoma) cells were used in this study. Tumor growth was monitored with a caliper. When the tumors reached a volume of approximately 150 mm$^3$, the mice were infected intravenously with $5 \times 10^6$ salmonellae suspended in phosphate-buffered saline (PBS).

**Therapeutic benefit and bacterial burden.** Tumor development after infection was monitored until the tumors either were cleared or outgrew (reached $\geq 1,000$ mm$^3$). In addition, body weight was measured. Mice were euthanized once their weight dropped below 80% of their initial weight. In order to determine the bacterial burden, blood, spleen, livers, and tumors were harvested at 12 and 36 hpi and treated as described previously (13). CFU were counted, and the bacterial burden was calculated as the total number of CFU per gram of tissue.

TNF-α measurement in serum. Blood samples were collected at 1.5, 3, and 12 hpi. The TNF-α ELISA Max Standard kit (BioLegend) was used to determine the TNF-α level in serum. All steps were done according to the manufacturer’s manual. Three different biological replicates were analyzed, and a PBS-treated group served as a negative control.

**In vivo depletion of macrophages.** Clodronate liposomes (0.3 to 3 μm; Clodrosome) were used to deplete macrophages. Two days before infection, 750 μg of clodronate liposomes was administered intravenously (i.v.) to the mice. The procedure was repeated 1 day before infection (500 μg given intraperitoneally). Empty liposomes served as a control. The efficiency of macrophage (CD11b$^+$ F4/80$^+$ CD11c$^-$ cells) depletion from the blood was monitored by flow cytometry.

**In vivo depletion of complement system.** Twelve hours before infection with salmonellae, the mice were treated i.v. with 5 IU of CVF (Quidel Corporation) to deplete the complement system in their blood. Blood samples were taken 12 h before and 3 and 7 h after CVF administration in order to monitor complement depletion. C3a levels were determined by enzyme-linked immunosorbent assay (ELISA; TECO development GmbH).

**Statistics.** Statistical analyses were performed with the two-tailed Student $t$ test, and $P < 0.05$ was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00254-15/-/DCSupplemental.

- Figure S1, TIF file, 0.8 MB.
- Figure S2, TIF file, 1 MB.
- Figure S3, TIF file, 0.8 MB.
- Figure S4, TIF file, 0.8 MB.
- Figure S5, TIF file, 0.6 MB.
- Figure S6, TIF file, 0.8 MB.
- Figure S7, TIF file, 0.9 MB.
- Figure S8, TIF file, 0.7 MB.
- Table S1, DOCX file, 0.01 MB.
- Table S2, DOCX file, 0.01 MB.

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