

*DMRT1* gene. As a probe, 10 µl of DOP-PCR amplification of paint E4 were used. For *DMRT1* a 287-base-pair (bp) *Pst*I digest of chicken *DMRT1* complementary DNA, containing the DM (Doublesex and MAB-3) domain was hybridized. For both experiments the probe DNA was labelled radioactively with [<sup>32</sup>P]-dATP and the BAC library screened according to the manufacturer's instructions ([http://www.genome.clemson.edu/protocols/hyb\\_filter.html](http://www.genome.clemson.edu/protocols/hyb_filter.html)). Positive clones were ordered from the Clemson University Genomics Institute (CUGI). For *DMRT1*, a final positive clone was verified by PCR using primers from conserved *DMRT1* specific regions<sup>29</sup>. This clone was then sequenced after subcloning into a TOPO Shotgun subcloning kit (Invitrogen) according to the manufacturer's instructions. Sequences were deposited at EMBL. The *DMRT1* BAC as well as the E4 specific BAC clones were labelled by standard nick translation with biotin or digoxigenin and 400–600 ng of labelled DNA were used to map the clones by FISH, as described above.

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## Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron

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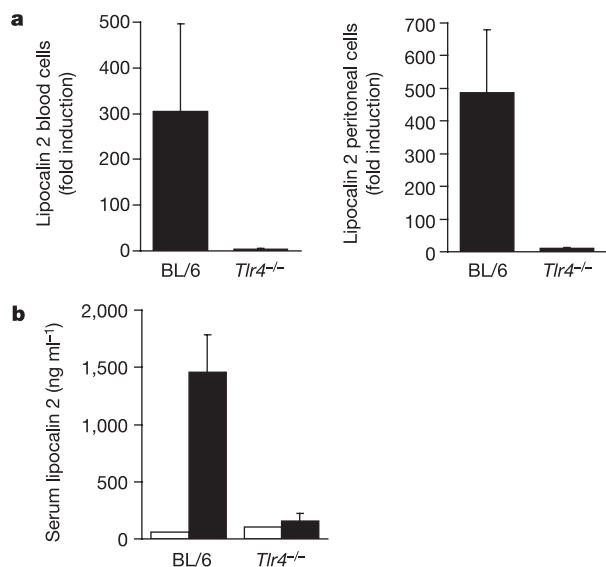
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Although iron is required to sustain life, its free concentration and metabolism have to be tightly regulated<sup>1</sup>. This is achieved through a variety of iron-binding proteins including transferrin and ferritin<sup>2</sup>. During infection, bacteria acquire much of their iron from the host by synthesizing siderophores that scavenge iron and transport it into the pathogen<sup>3,4</sup>. We recently demonstrated that enterochelin, a bacterial catechol siderophore,



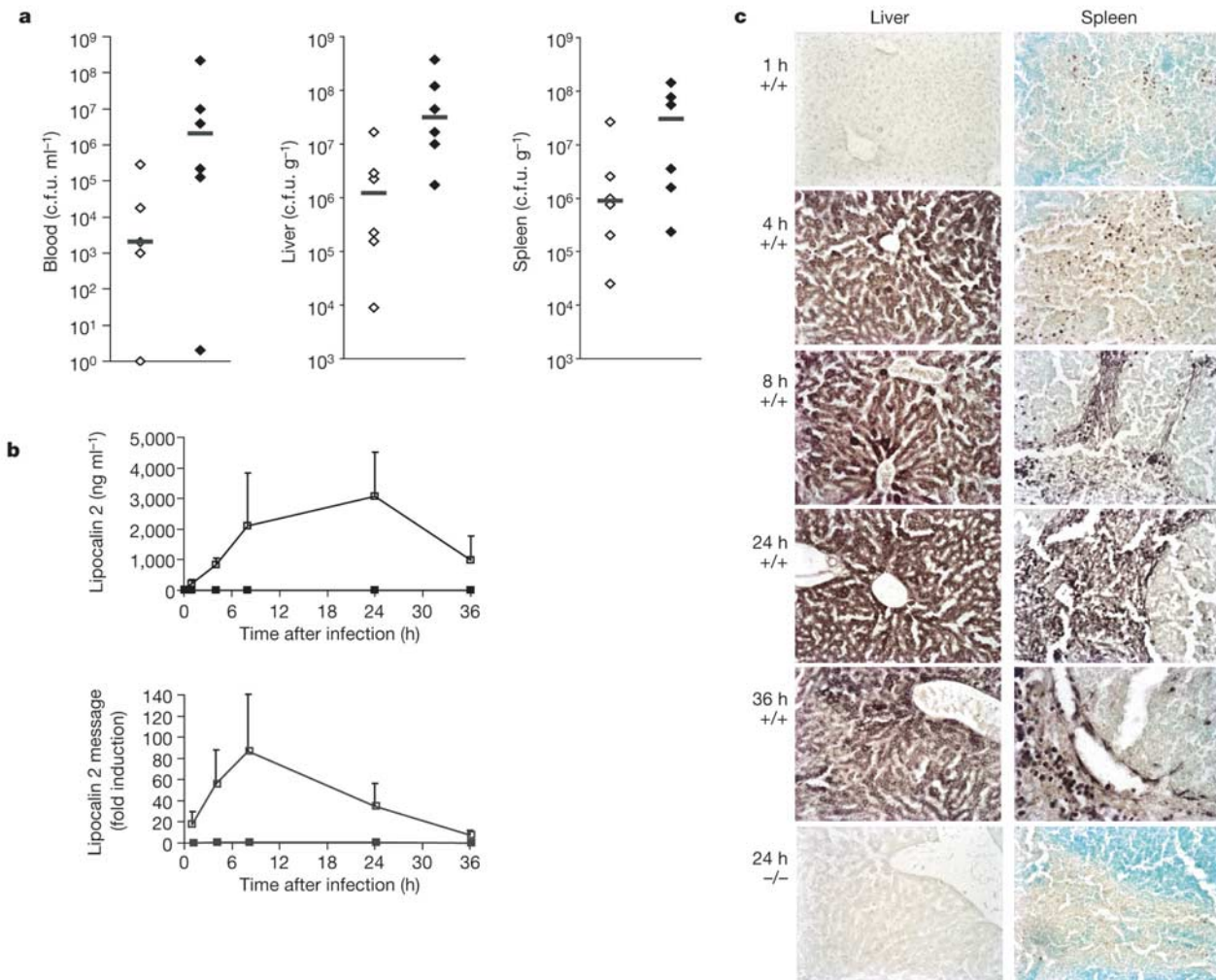
**Figure 1** Lipocalin 2 production is induced through TLRs. Lipocalin 2 mRNA and protein measured 4 h after i.p. injection of C57BL/6 wild-type and TLR4-deficient mice with 10 µg LPS (*n* = 3 mice). **a**, Lipocalin 2 mRNA from blood cells and peritoneal cells is shown as fold induction values relative to the mRNA from PBS-injected mice. **b**, Serum levels of lipocalin 2 protein after LPS injection (filled bars) are compared to the levels in PBS-injected mice (open bars). Errors bars show the s.d. for each experiment.

binds to the host protein lipocalin 2 (ref. 5). Here, we show that this event is pivotal in the innate immune response to bacterial infection. Upon encountering invading bacteria the Toll-like receptors on immune cells stimulate the transcription, translation and secretion of lipocalin 2; secreted lipocalin 2 then limits bacterial growth by sequestering the iron-laden siderophore. Our finding represents a new component of the innate immune system and the acute phase response to infection.

In mammals, distinct combinations of at least ten Toll-like receptors (TLRs) discriminate between the large number of microbial components found in nature<sup>6</sup>. Activation of the mammalian TLRs leads to the induction of inflammatory responses and to the development of adaptive immunity<sup>7</sup>. Complementary DNA microarray analysis demonstrates that lipocalin 2 (also known as neutrophil gelatinase-associated lipocalin, siderocalin, 24p3, or uterocalin<sup>5,8-12</sup>) transcription is markedly increased in macrophages stimulated *in vitro* with lipopolysaccharide (LPS; detected by TLR4 (ref. 13)), Pam<sub>3</sub>CSK<sub>4</sub> lipopeptide (TLR2/1 (refs 14–16)) and flagellin (TLR5 (ref. 17)) (data not shown). *In vivo*, LPS induces a

200-fold increase in lipocalin 2 messenger RNA transcription and a 20-fold increase in protein concentration in a TLR4-dependent manner (Fig. 1a, b).

To assess the role of lipocalin 2 *in vivo*, we generated lipocalin-2-deficient mice (Supplementary Fig. 1). These mice have normal litters and no apparent phenotype when housed in specific pathogen-free conditions. However, intraperitoneal challenge with a sub-lethal dose of a clinical strain of *Escherichia coli*, H9049, results in substantial increases in bacteraemia and bacterial burden in the liver and spleen (Fig. 2a). The peak bacteraemia was on average 1,000-fold ( $P = 0.02$ ) greater in lipocalin-2-deficient mice than in controls, and significant differences in bacterial counts in the liver ( $P = 0.04$ ) and spleen ( $P = 0.03$ ) were also seen (Fig. 2a and not shown). The physical signs of sepsis (lethargy, hunched posture, ruffled fur) correlated well with the bacterial burden, especially in the blood. Despite the marked difference in bacteraemia between wild-type and lipocalin-2-deficient mice there were no apparent differences in leukocyte numbers (Supplementary Table 1) or in neutrophil infiltration and tissue iron distribution (Supplementary



**Figure 2** Lipocalin 2 protects against infection with *E. coli*. **a**, Bacterial burden in blood, liver and spleen 36 h after i.p. infection of wild type (open diamonds) and lipocalin-2-deficient (filled diamonds) mice with  $0.6 \times 10^8$  c.f.u. *E. coli* H9049. Individual c.f.u. values and the median (horizontal line) from one representative experiment out of three are shown ( $n = 6$  mice for each group,  $P < 0.05$  in the three experiments combined, as evaluated by two-sided Student's *t*-test and Fisher's method for combining *P*-values). **b, c**, Induction of lipocalin 2 in serum, blood cells and tissues 1, 4, 8, 24 and 36 h after i.p. infection of lipocalin-2-deficient and wild-type mice with  $0.6 \times 10^8$  c.f.u. *E. coli* H9049

( $n = 5$  mice in each group for each time point). **b**, Serum levels of lipocalin 2 protein (top panel) and lipocalin 2 mRNA in blood cells (bottom panel) measured in wild-type mice (open squares) and in lipocalin-2-deficient mice (closed squares). Data are presented as the mean and s.d. for five mice at each time point. **c**, Tissue induction of lipocalin 2 in wild-type mice detected on frozen sections of liver and spleen with a polyclonal antibody specific for lipocalin 2. From the lipocalin 2 knockouts, only the 24-h time point is shown (bottom panel).



type siderophores such as aerobactin and ferrichrome, or to polycarboxylate-type siderophores such as rhizoferrin (Fig. 3a). This indicates that lipocalin 2 is effective against bacteria that depend exclusively on catecholate-type siderophores for growth in iron-restricted environments. The H9049 strain of *E. coli* used in these studies can use enterochelin and ferrichrome, but not aerobactin, to import iron (Fig. 3b). Consistent with this finding H9049 does not possess the genes for aerobactin biosynthesis (*iucD*) or uptake (*iutA*) (data not shown). As predicted, lipocalin 2 inhibited the growth of H9049 in a dose-dependent manner (Fig. 3c). It also inhibited the growth of H9049 in the presence of exogenously added enterochelin, and this inhibition was only overcome when the enterochelin concentration exceeded that of lipocalin 2 (Fig. 3c), reflecting their equimolar stoichiometry of binding<sup>5</sup>. Taken together, the data demonstrate that the bacteriostatic effect of lipocalin 2 is dependent on its sequestration of enterochelin.

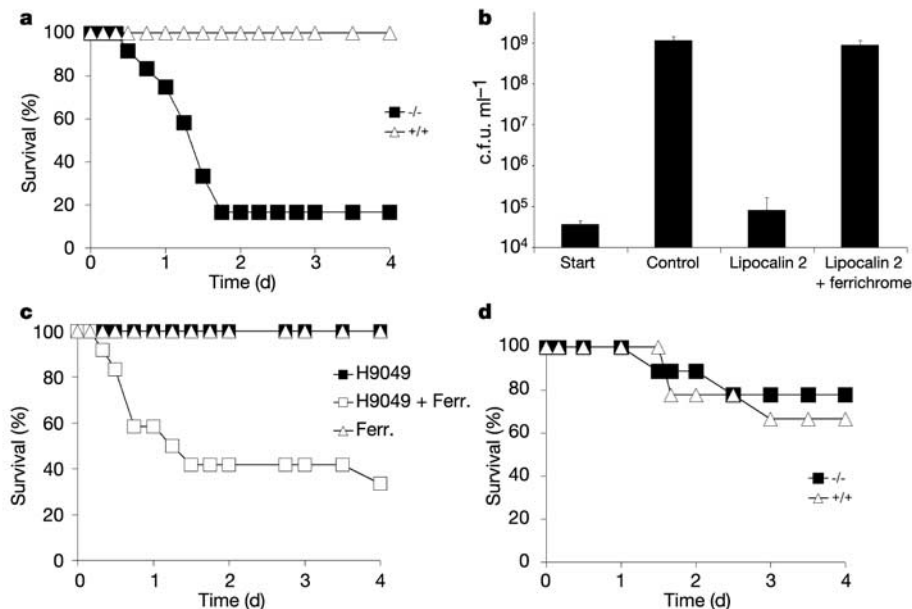
As the most pronounced difference in bacterial burden between infected wild-type and lipocalin-2-deficient mice was seen in the blood, we tested whether bacterial growth was limited by the increased serum lipocalin levels found in wild-type mice. H9049 cultures grew rapidly in acute phase serum from lipocalin-2-deficient mice, reaching levels 1,000-fold higher than cultures grown in acute phase serum from wild-type mice (Fig. 3d). The addition of recombinant lipocalin 2 to the acute phase serum of the knockout mice restored the bacteriostatic activity (Fig. 3d). Acute phase serum from wild-type mice contained 40 nM ( $1 \mu\text{g ml}^{-1}$ ) of lipocalin 2, and an equivalent concentration of recombinant lipocalin 2 conferred 99% growth inhibition of the H9049 strain (Fig. 3c).

The bacteriostatic effect of lipocalin 2 was absolutely specific for bacteria that acquire iron through lipocalin-2-binding siderophores; for example, enterochelin (Fig. 3d) and mycobactin (R. Strong, unpublished data). By contrast, lipocalin 2 deficiency had no effect on bacteria that acquire iron through siderophores that do not bind lipocalin 2; for example, *Staphylococcus aureus*,

which does not require enterochelin-type siderophores (Fig. 3d).

To test the role of lipocalin 2 in an acute lethal infection, we increased the bacterial challenge ( $2 \times 10^8$  colony-forming units (c.f.u.) *E. coli* H9049) and compared the survival of lipocalin-2-deficient mice and wild-type controls (Fig. 4a). Knockout mice showed substantially accelerated lethality: within the first 42 h greater than 80% of lipocalin-2-deficient mice had died compared with 0% of wild-type mice (Fig. 4a). Only 17% of the lipocalin-2-deficient mice recovered completely from the infection, whereas all of the wild-type mice survived.

To explore further the specificity of the protective effect of lipocalin 2 against enterochelin-dependent bacterial infection, we supplied the bacteria with a lipocalin-2-independent route of iron acquisition by adding ferrichrome, a hydroxamate-type siderophore. Ferrichrome is not synthesized by *E. coli*, but can be used by the bacterium for iron acquisition (Fig. 3b). We first showed *in vitro* that ferrichrome is able to circumvent lipocalin-2-dependent bacteriostatic activity (Fig. 4b). We next challenged wild-type mice with intraperitoneal infection of *E. coli* H9049, with or without co-injection of ferrichrome. Mice that received bacteria and ferrichrome demonstrated substantially increased lethality, similar to the lethality of lipocalin-2-deficient mice; ferrichrome alone had no effect (Fig. 4c). Further insight into the specificity of lipocalin 2 function was provided by the observation that lipocalin 2 deficiency has no effect on the survival of mice intraperitoneally infected with *S. aureus* (Fig. 4d), a bacterium whose acquisition of iron is lipocalin-2-independent (Fig. 3d). These results demonstrate that lipocalin 2 confers resistance to bacterial infection by abrogating enterochelin-like, siderophore-dependent iron uptake by bacteria. This host defence mechanism is important because a great many human pathogens including *E. coli*, *Salmonella* spp., *Brucella abortus*, *Bacillus anthracis*, *Burkholderia cepacia*, *Corynebacterium diphtheriae*, *Paracoccus* spp. and *Vibrio* spp. make enterochelin-like siderophores. In addition, the near absolute conservation of residues contributing to the siderophore-binding pocket of lipocalin 2



**Figure 4** Lipocalin 2 protection against bacterial infection is siderophore specific. **a**, Survival curve comparing lipocalin-2-deficient mice and wild-type controls after i.p. challenge with  $2 \times 10^8$  c.f.u. *E. coli* H9049 per mouse ( $n = 13$  for each group). **b**, Growth of bacteria in an iron-restricted environment (RPMI/10% FBS) in the presence of added lipocalin 2 ( $1.5 \mu\text{M}$ ) and ferrichrome (5 nM), as indicated. Errors bars show the s.d. for each experiment. **c**, Survival curve comparing wild-type mice after i.p. challenge

with  $2 \times 10^8$  c.f.u. *E. coli* H9049 per mouse with or without co-injection of ferrichrome (25 nmol) ( $n = 12$  for each group), or for wild-type mice ( $n = 6$ ) injected with ferrichrome (25 nmol) alone. **d**, Survival curve comparing lipocalin-2-deficient and wild-type mice after i.p. challenge with  $3 \times 10^8$  *S. aureus* ( $n = 9$  for each group). No mice died after i.p. infection with  $1 \times 10^9$  *S. aureus*, whereas all mice died after i.p. infection with  $10^9$  *S. aureus* (data not shown).

(ref. 5) suggests that this defence mechanism is conserved across species.

The expression of lipocalin 2 in multiple tissues outside the setting of infection suggests that it may have other functions, such as have been postulated in kidney development<sup>21</sup> and the implantation and parturition stages of pregnancy<sup>12,22</sup>. Although we cannot exclude a role for lipocalin 2 in these functions, the phenotype of lipocalin-2-deficient mice argues against an obligatory role in these biological processes. Alternatively, there may be subtle effects that will only be revealed by more detailed investigation of lipocalin-2-deficient mice.

Lipocalin 2 has also been implicated as a factor that induces apoptosis through interaction with an as yet undefined receptor<sup>23</sup>. It is formally possible that lipocalin-2-deficient mice are more susceptible to infection because of a defect in apoptosis. However, two findings argue against this possibility. First, our experiments demonstrate that lipocalin 2 deficiency renders mice susceptible to bacteria that depend on enterochelin-based iron uptake (Fig. 4). Second, we have not detected an apoptotic defect in lipocalin-2-deficient mice (Supplementary Fig. 3).

We have described a new mechanism by which the innate immune system curbs bacteraemia during the early stage of infection. Upon encountering bacteria, innate immune cells produce and secrete lipocalin 2, which, in turn, limits bacterial growth by iron sequestration. Our data in the mouse model indicate that the absence of this defence mechanism can lead to sepsis and death; these findings, therefore, have substantial implications for the clinical treatment of bacteraemia and bacterial sepsis. □

## Methods

### Generation of lipocalin-2-deficient mice

The genomic DNA fragment containing the *Lcn2* gene was screened from a 129/Sv mouse genomic library and characterized by restriction enzyme mapping and sequence analysis. The targeting vector was constructed by replacing a 1.9-kilobase fragment of the *Lcn2* gene, including exons 2–5, with a neomycin-resistance gene cassette. A herpes simplex virus thymidine kinase gene, driven by an MC1 promoter, allowed for negative selection. The targeting vector was transfected into embryonic day (E)14.1 embryonic stem (ES) cells. Targeted ES cells, identified by polymerase chain reaction (PCR) and Southern blotting, were subsequently injected into C57BL/6 blastocysts. Male chimaeric mice were then mated to C57BL/6 female mice and the heterozygote F<sub>1</sub> progeny were intercrossed to generate lipocalin-2-deficient mice. Knockout mice and their wild-type littermates were then used in separate breeding programmes to generate populations of lipocalin 2 knockouts and wild-type controls for subsequent experiments.

### Lipocalin 2 mRNA and protein measurements

Lipocalin 2 messenger RNA was measured in freshly drawn blood cells and extracted peritoneal cells using a PAXgene blood RNA kit (PreAnalytix) and an RNeasy mini kit (Qiagen), respectively, both in accordance with the manufacturer's protocols. cDNA was synthesized and quantified by real-time PCR using a lipocalin-2-specific probe and primer set. All data were normalized to a housekeeping gene (*Efla*) measured in the same sample.

For detection of lipocalin 2 protein we generated polyclonal antibodies by immunizing rabbits with recombinant mouse lipocalin 2 protein<sup>5</sup> (R&R Research and Development). Affinity-purified antibody was used both as capture (4 µg ml<sup>-1</sup>) and detection (biotinylated, 0.1 µg ml<sup>-1</sup>) antibody in a sandwich ELISA. The detection limit was 100–150 pg ml<sup>-1</sup> lipocalin 2. The same anti-lipocalin 2 antibody was used in western blots at 1 µg ml<sup>-1</sup> and in immunohistochemistry on frozen sections at 3 µg ml<sup>-1</sup>.

### Mouse intraperitoneal injections and infection model

TLR4 (backcrossed eight generations onto the C57BL/6 background) or lipocalin-2-deficient mice were age- and sex-matched with C57BL/6 or lipocalin 2 wild-type controls, respectively. The mice were kept in specific pathogen-free housings. For measurements of lipocalin 2 protein and mRNA, TLR4-deficient mice and C57BL/6 controls were injected intraperitoneally (i.p.) with 10 µg LPS (List Biological Laboratories) in 0.5 ml PBS. The mice were killed 4 h later and the blood was used for RNA extraction and for generation of serum. Acute phase serum was produced by i.p. injection of 10<sup>8</sup> c.f.u. heat-killed *E. coli* (clinical isolate H9049; provided by S. Swanzey) into lipocalin 2 wild-type and knockout mice, and after 5 h mice were killed and bled. For infections with live bacteria, log phase *E. coli* H9049 or *S. aureus* (American Type Culture Collection 25923) were washed, re-suspended in PBS to the desired concentration and injected i.p. in 0.5 ml per mouse using a 30-gauge needle. All injected doses were verified by counting c.f.u. Mouse behaviour was carefully monitored every 12 h. During survival experiments the mice were not left alone for more than 3–4 h. All dead mice were analysed by necropsy. For measuring the bacterial burden, heparinized blood samples and homogenized liver and spleen were diluted in PBS and plated on LB agar to determine c.f.u. Blood samples were also used for RNA extraction and measurements of serum lipocalin 2 protein. Tissue samples were fixed for paraffin sectioning and immunohistochemistry (frozen sections).

### Siderophore binding to lipocalin 2

Binding of siderophores to either human or mouse lipocalin 2 was determined as previously reported<sup>5</sup> or by a surface plasmon resonance assay, in HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% P-20) using a Biacore 3000 system (Biacore AB). Siderophores were obtained from Sigma-Aldrich, Biophore Research Products or as a gift of K. N. Raymond. We classified a binding interaction as an interaction with a dissociation constant (*K<sub>d</sub>*) estimated at less than 100 nM; non-binding siderophores interact with protein with a *K<sub>d</sub>* estimated to be greater than 1 mM. The kinetics of binding are complex and multiphasic, currently obviating quantification of the affinities. Owing to the marked solution instability of enterochelin, a non-hydrolysable synthetic analogue<sup>24</sup>, differing only in backbone chemistry, was used as a surrogate in the binding assay.

### In vitro growth assay

For *in vitro* growth measurements of *E. coli* H9049 or *S. aureus* we added 10<sup>3</sup>–10<sup>4</sup> c.f.u. of log phase bacteria in 100 µl per well, 96-well plates. The bacteria were grown in RPMI with 10–20% heat-inactivated fetal bovine serum (FBS) or acute phase serum from lipocalin 2 wild-type or knockout mice. Purified siderophores (EMC microcollections GmbH) and/or recombinant mouse lipocalin 2 (ref. 4) were added at the indicated concentrations, and growth was measured as c.f.u. from serial dilutions plated onto LB agar.

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