IMPACT OF METAL ION HOMEOSTASIS OF GENETICALLY MODIFIED *ESCHERICHIA COLI* NISSLE 1917 AND K12 (W3110) STRAINS ON COLONIZATION PROPERTIES IN THE MURINE INTESTINAL TRACT

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Metal ions are integral parts of pro- as well as eukaryotic cell homeostasis. *Escherichia coli* proved a valuable *in vitro* model organism to elucidate essential mechanisms involved in uptake, storage, and export of metal ions. Given that *E. coli* Nissle 1917 is able to overcome murine colonization resistance, we generated several *E. coli* Nissle 1917 mutants with defects in zinc, iron, copper, nickel, manganese homeostasis and performed a comprehensive survey of the impact of metal ion transport and homeostasis for *E. coli* colonization capacities within the murine intestinal tract. Seven days following peroral infection of conventional mice with *E. coli* Nissle 1917 strains exhibiting defined defects in zinc or iron uptake, the respective mutant and parental strains could be cultured at comparable, but low levels from the colonic lumen. We next reassociated gnotobiotic mice in which the microbiota responsible for colonization resistance was abrogated by broad-spectrum antibiotics with six different *E. coli* K12 (W3110) mutants. Seven days following peroral challenge, each mutant and parental strain stably colonized duodenum, ileum, and colon at comparable levels. Taken together, defects in zinc, iron, copper, nickel, and manganese homeostasis do not compromise colonization capacities of *E. coli* in the murine intestinal tract.

Keywords: metal ion homeostasis and transport, metal ion mutant strains, iron homeostasis, zinc homeostasis, copper homeostasis, periplasmic copper stress, manganese homeostasis, enterobactin, *E. coli* Nissle 1917, *E. coli* K12 (W3110), colonization resistance, gnotobiotic mice, bacterial fitness, bacterial colonization capacity

Introduction

Metal and transition metal ions play pivotal roles in the homeostasis of any living organism [1]. Many enzymes, co-enzymes, and vitamins, for instance, use metal ions as integral parts of their catalytic domain. *Escherichia coli* proved a valuable model organism to elucidate essential mechanisms involved in uptake, storage, and export of metal ions [2]. Even though prokaryotes are capable of surviving under extreme and often rather hostile conditions, there are relatively low thresholds of tolerance levels for environmental metal ion concentrations compatible with bacterial survival [3]. For instance, relatively high metal ion concentrations may exert toxic effects to living cells,

whereas deprivation inhibits basic metabolic cell function and thus cell proliferation. Several studies have shed light onto metal ion homeostasis and related mechanisms [4–9]. These studies, however, have been performed under *in vitro* conditions and so far (if at all) only very limited knowledge exist about metal ion homeostasis of *E. coli* within its natural habitat, the vertebrate intestinal tract [10]. Therefore, we generated a plethora of *E. coli* mutant strains deficient in distinct factors of metal ion homeostasis, such as zinc uptake and efflux transporters, iron efflux transporters, nickel and efflux transporters, manganese uptake transporters, enterobactin synthesis, and periplasmic protection factor from copper stress. These metal ion mutants were generated in two different

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E. coli parental strains, namely, *E. coli* Nissle (EcN) 1917 and *E. coli* K12 (EcK12) W3110. *E. coli* Nissle 1917 was detected almost 100 years ago and ever since was appreciated as a “safe” strain capable of colonizing the vertebrate intestinal tract and exerting probiotic effects in acute enteritis in children [11] and inflammatory bowel diseases, such as ulcerative colitis and Crohn’s disease [12, 13]. *E. coli* K12 (EcK12) W3110 is considered an apathogenic security strain extensively used in molecular biological laboratories for genetic cloning properties, for instance [14, 15].

In the experiments presented here, we performed a comprehensive *in vivo* survey of the impact of distinct factors in metal ion transport and homeostasis of mutant *E. coli* Nissle 1917 and *E. coli* K12 (W3110) strains on colonization capabilities in the murine small as well as large intestinal tract applying conventionally colonized as well as gnotobiotic mice.

Materials and methods

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the “Landesamt für Gesundheit und Soziales” (LaGeSo, Berlin). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice.

Generation of gnotobiotic mice

Female C57BL/6 wildtype mice were bred and housed under specific pathogen-free (SPF) conditions in the Forschungsinstitut für Experimentelle Medizin (Charité, Berlin, Germany). To eradicate the commensal gastrointestinal microbiota, conventionally colonized mice were transferred to sterile cages and treated by adding ampicillin (1 g/l; Ratiopharm), vancomycin (500 mg/l; Cell Pharm), ciprofloxacin (200 mg/l; Bayer Vital), imipenem (250 mg/l; MSD), and metronidazole (1 g/l; Fresenius) to the drinking water ad libitum for 8–10 weeks as described earlier [16] starting at 8 weeks of age.

E. coli mutant strains

In the *in vivo* experiments presented here *E. coli* Nissle 1917 and *E. coli* K12 (W3110) strains carrying a chloramphenicol resistance cassette ($\Delta rcnB$) were used. Thus, we were able to unequivocally detect the applied strain among the other enterobacterial commensal strains in the intestinal tract by culture on solid Luria–Bertani (LB) agar supplemented with chloramphenicol.

The strains lacking specific genes involved in trace metal homeostasis or signaling were as follows:

E. coli Nissle 1917 mutant strains

$\Delta rcnB$ (control strain, RcnB is not involved in trace metal homeostasis [9]), $\Delta znuABC \Delta zupT$ (deletion of all known zinc uptake transporters [9]), $\Delta znuABC$ (deletion of a zinc uptake transporter [9]), $\Delta zupT$ (deletion of a zinc and other divalent metal cation uptake transporter [9]), $\Delta entC$ (deletion of enterobactin biosynthesis, impairment of iron uptake system [17]) (Table 1).

E. coli K12 (W3110) mutant strains

$\Delta rcnB$ (control strain [9]), $\Delta znu \Delta zupT$ (deletion of all known zinc uptake transporters [9]), $\Delta zntA \Delta zitB$ (deletion of all known zinc efflux transporters [9]), $\Delta cus \Delta cueO$ (deletion of periplasmic protection from copper stress [5]), $\Delta fieF$ (deletion of iron efflux transporter [18]), $\Delta rcnA$ (deletion of nickel / cobalt efflux transporter [8, 19]), $\Delta mntH \Delta zupT$ (deletion of all known manganese uptake transporters [20]) (Table 2).

Recolonization of gnotobiotic mice

Recolonization of gnotobiotic mice was performed as described earlier [16, 21]. In brief, 2 days prior to re-colonization experiments, the antibiotic cocktail was withheld and substituted by sterilized tap water. Respective *E. coli* strains were grown in supplemented brain heart infusion broth to a turbidity equivalent of 6 McFarland units (a bacterial load of approximately 10^9 – 10^{10} colony forming units (CFU) per ml), harvested by centrifugation, washed once, resuspended

Table 1. Metal ion mutants of *E. coli* Nissle 1917. All strains carry a chloramphenicol resistance cassette ($\Delta rcnB::cat$)

Mutation	Description	References
$\Delta znuABC \Delta zupT$	Deletion of all known zinc uptake transporters	Grass, 2006
$\Delta znuABC$	Deletion of a high-affinity zinc uptake transporter	Grass, 2006
$\Delta zupT$	Deletion of a low affinity zinc uptake transporter	Grass, 2006
$\Delta entC$	Deletion of enterobactin biosynthesis, impairment of iron uptake	Grass, 2006
$\Delta rcnB$	Control strain; no phenotype	Grass, 2001

Table 2. Metal ion mutants of *E. coli* K12 (W3110) strain. All strains carry a chloramphenicol resistance cassette ($\Delta rcnB::cat$)

Mutation	Description	References
$\Delta zntA \Delta zitB$	Deletion of all known zinc efflux transporters	Grass, 2001
$\Delta cus \Delta cueO$	Deletion of periplasmic protection from copper stress	Grass, 2001
$\Delta fieF$	Deletion of an iron efflux transporter	Munkelt, 2004
$\Delta rcnA$	Deletion of a nickel/cobalt efflux transporter	Rodrigue, 2005, Koch, 2007
$\Delta znuABC \Delta zupT$	Deletion of all known zinc uptake transporters	Grass, 2001
$\Delta mntH \Delta zupT$	Deletion of all known manganese uptake transporters	Makui, 2000
$\Delta rcnB$	Control strain; no phenotype	Koch, 2007

in 5 ml phosphate-buffered saline (PBS), and administered by gavage in a final volume of 0.3 ml on three consecutive days. Bacterial loads of oral suspensions were determined by cultivation of serial dilutions on solid MacConkey and LB agar supplemented with chloramphenicol (50 μ g/ml; Sigma-Aldrich, Steinheim, Germany). Gnotobiotic mice were perorally infected with 10^9 CFU *E. coli* on three consecutive days by gavage as described earlier [16, 21].

Sampling procedures

Mice were sacrificed by isoflurane inhalation (Abbott, Wiesbaden, Germany). Luminal samples taken from duodenum, terminal ileum, and colon of each mouse were removed under sterile conditions.

Cultural analysis of the intestinal microbiota

Cultural analyses of luminal intestinal contents were performed in serial dilutions on solid MacConkey (Oxoid, Wesel, Germany) and LB agar supplemented with

chloramphenicol (50 μ g/ml; Sigma-Aldrich, Steinheim, Germany) under aerobic conditions (2 days incubation at 37 °C). The bacterial species were morphologically and biochemically identified as described earlier [16].

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney *U* Test. Two-sided probability (*p*) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

Results

Impact of bacterial metal ion homeostasis on colonization capacities of *E. coli* Nissle 1917 in conventional mice

In vitro studies have shown that tolerance against varying metal ion concentrations is limited for bacterial fitness [3–9]. So far, detailed knowledge about the role of metal

Fig. 1. Colonization resistance against *E. coli* Nissle 1917 and its mutant strains lacking specific factors of metal ion homeostasis. On three consecutive days conventionally colonized mice were perorally recolonized either with 10^9 *E. coli* Nissle 1917 ($\Delta rcnB$ control strain) or comparable loads of its isogenic mutant strains $\Delta znuABC \Delta zupT$ (deletion of all known zinc uptake transporters), $\Delta znuABC$ (deletion of a high-affinity zinc uptake transporter), $\Delta zupT$ (deletion of a low-affinity zinc uptake transporter), $\Delta entC$ (deletion of enterobactin biosynthesis, impairment of iron uptake system). (A) One, (B) three, and (C) seven days following infection, colonization densities were determined in the colonic lumen by quantification of live *E. coli* Nissle 1917 and its mutant strains by cultural analysis (CFU, colony forming units). Numbers of mice harboring the respective *E. coli* Nissle 1917 strain out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated. Data shown are representative for three independent experiments

ion homeostasis of *E. coli* within its natural habitat, the intestinal tract, however, is scarce due to the physiological colonization resistance displayed by mice against *E. coli* strains. We were therefore interested in whether mutants of *E. coli* Nissle 1917, a probiotic strain known for its intestinal colonization capacities under physiological conditions [11–13] were compromised regarding colonization properties in conventionally colonized mice. We here focused on mutant strains defective in zinc or iron homeostasis, namely, enterobactin biosynthesis and iron uptake (ΔentC) as summarized in Table 1. For this purpose, conventional mice were perorally colonized with 10^9 CFU of the respective mutant *E. coli* Nissle 1917 strain or the parental wildtype strain on three consecutive days. Given that all strains were carrying a chloramphenicol resistance cassette (ΔrcnB), we were thus able to unequivocally detect the applied mutant or wildtype strain among the other enterobacterial commensal strains abundant in the intestinal tract of conventionally colonized mice. One day following the last infection, all mutant and control strains were detected in the colonic lumen at median loads of between 10^6 and 10^8 CFU/g (Fig. 1A). At day 3 p.i., mice harbored overall lower bacterial numbers compared to the previous time point and displayed a trend toward higher colonic loads of the parental versus the respective mutant strains (median difference between 3 and 4 orders of magnitude; not significant; Fig. 1B). Thereafter, the colonic loads of all *E. coli* Nissle 1917 strains decreased toward comparable and rather low median levels of between 10^2 and 10^4 CFU/g at day 7 following infection (Fig. 1C). Interestingly, bacterial numbers of the ΔentC strain (with defective iron uptake function) tended to be lower compared to the parental control strain at days 1 and 3 post-infection (p.i.) (n.s.). In some animals, the ΔentC mutant could not

be reisolated from the colon at day 3 or 7 p.i. at all (Fig. 1). Collectively, these data indicate that in mice harboring a conventional microbiota, defects in zinc or iron uptake do not significantly compromise the principal colonization capacity of *E. coli* Nissle 1917.

Impact of bacterial metal ion homeostasis on colonization capacities of *E. coli* K12 (W3110) in gnotobiotic mice

Under normal conditions, the physiological colonization resistance exerted by the microbiota composition of conventionally colonized vertebrate hosts interferes with stable establishment of *E. coli* K12 strains within the intestinal tract upon peroral infection. Therefore, we generated gnotobiotic mice by antibiotic treatment in order to completely eradicate the conventional intestinal microbiota of the host. For reassociation studies, we chose the security strain *E. coli* K12 (W3110), which is not able to colonize conventional mice, but readily establishes in mice treated with broad-spectrum antibiotics [22]. This “bad colonizer” carried a chloramphenicol resistance cassette (ΔrcnB). *E. coli* K12 (W3110) and its isogenic mutants with distinct defects in zinc efflux, periplasmic copper stress response, iron efflux, zinc uptake, manganese uptake, iron efflux, and nickel/cobalt efflux (Table 2) were detected in fecal samples due to their chloramphenicol resistance.

Mice with abolished colonization resistance were perorally replenished with approximately 10^9 CFU of one of the respective seven different metal ion mutants (Table 2) on three consecutive days (Fig. 2). Remarkably, the parental as well as the metal ion mutant strains were able to stably colonize the terminal ileum and the colon at com-

Fig. 2. Colonization resistance against *E. coli* K12 and its mutant strains lacking specific factors of metal ion homeostasis is abrogated following antibiotic treatment. Conventionally colonized mice were subjected to quintuple antibiotic therapy for 8 to 10 weeks to eradicate the gastrointestinal microbiota as described in methods. Generated gnotobiotic animals were perorally recolonized either with *E. coli* K12 (EcK12) W3110 ΔrcnB control-strain or its isogenic mutant strains: $\Delta\text{znu } \Delta\text{zupT}$ (deletion of all known zinc uptake transporters), $\Delta\text{zntA } \Delta\text{zitB}$ (deletion of all known zinc efflux transporters), $\Delta\text{cus } \Delta\text{cueO}$ (deletion of periplasmic protection from copper stress), ΔfieF (deletion of iron efflux transporter), ΔrcnA (deletion of nickel/cobalt efflux transporter), $\Delta\text{mntH } \Delta\text{zupT}$ (deletion of all known manganese uptake transporters). Seven days following recolonization, luminal bacterial loads were assessed in (A) duodenum, (B) ileum, and (C) colon by culture (CFU, colony forming units). Numbers of animals harboring the respective *E. coli* K12 (W3110) strain out of the total number of analyzed animals are given in parentheses and medians (black bars) are indicated. Data shown are representative for three independent experiments

parably high densities of 10^8 – 10^9 CFU per gram until day 7 post challenge (Fig. 2B, C). Similarly, all strains were detected at similar, but lower median densities of 10^6 – 10^7 CFU per g in the proximal small intestine (duodenum) (Fig. 2A). Taken together, defined defects in bacterial zinc, iron, copper, manganese, and nickel homeostasis do not impact colonization properties of *E. coli* K12 (W3110) in the upper and lower intestinal tract of gnotobiotic mice.

Discussion

So far, many studies have been performed to elucidate prokaryotic metal ion homeostasis [3]. Whereas dozens of genes involved in regulating metal ions homeostasis in *E. coli* in particular have been investigated *in vitro*, only little – if anything – is known about its role within the intestinal ecosystem. We here present a comprehensive survey of the impact of distinct factors involved in metal ion homeostasis on bacterial colonization in the murine intestinal tract. For this purpose, we generated *E. coli* Nissle 1917 mutant strain defective in zinc or iron uptake systems [9, 17]. The probiotic *E. coli* Nissle 1917 strain is known to colonize even the intestinal tract of vertebrates harboring a conventional microbiota that is responsible for the physiological colonization resistance [23–25]. These properties are due to the existence of four genetic islands encoding certain fitness factors which are essential for colonization [26] and also for an intense cross-talk and quorum sensing between *E. coli* Nissle 1917 and intestinal epithelial cells [27, 28]. Thus, using *E. coli* Nissle 1917, we were able to investigate the impact of bacterial zinc and iron transport systems on colonization capacities within the naive, healthy murine host harboring a conventional microbiota. Whereas at day 3 following infection mice exhibited a trend toward lower loads of the respective zinc or iron mutant compared to the parental strain (not significant), by day 7 p.i., bacterial loads were comparable. This (if at all minor) kinetic effect might be due to the existence of further (so far unknown) metal ion transporters which could have been up-regulated “by demand” in order to compensate for the deleted ones in the course of the infection. Interestingly, the *ΔentC* strain with a defect in the iron uptake system (enterobactin synthesis) appeared to exhibit a somewhat compromised colonization capacity as indicated by two orders of magnitude lower median colonic loads compared to the parental strain at days 1 and 3 p.i. (not significant). In support, some animals had completely expelled the mutant strain by days 3 and 7 p.i. Nevertheless, our *in vivo* experiments here revealed that the impact of distinct zinc and iron uptake systems of *E. coli* Nissle 1917 on intestinal colonization capacities seem rather minor.

To further exclude potential interactions with commensal bacteria, we applied a gnotobiotic mouse model in which the conventional microbiota had been eradicated by broad-spectrum antibiotic treatment. Even though regarded as a security strain due to inherent genetic defects preventing it from adhesion to epithelial cells [29–31], we

recently clearly showed that *E. coli* K12 (MG1655) was indeed able to stably colonize the intestinal tract of gnotobiotic mice following abrogation of colonization resistance [32]. Interestingly, also in the study presented here, a very close relative, *E. coli* K12 (W3110), could be cultured from the proximal and distal small intestinal tract as well as from the colon at high loads 1 week following peroral challenge of gnotobiotic mice. It is noteworthy that even when the commensal microbiota that is essentially responsible for the reabsorption of the metal ion burden in the intestinal tract under physiological conditions is completely abolished, no toxic metal ion concentrations seem to affect *E. coli* K12 (W3110) fitness and intestinal colonization properties. It is further remarkable that irrespective of six *E. coli* K12 (W3110) *ΔrcnB* marker-mutant strains investigated, colonization capacities in gnotobiotic mice were not affected by additional defects in zinc, iron, copper, nickel, or manganese homeostasis. Given that any mutant strain with a compromised efflux system, e.g., *ΔzntA* *ΔzitB* [9], *ΔfieF* [18], or *ΔrcnA* [8, 19], was able to colonize as effectively as the parental *ΔrcnB* marker-strain, we conclude that concentrations of zinc, iron, copper as well as nickel and cobalt ions in the intestinal tract conditions are not limiting to support *E. coli* growth. Furthermore, strains deficient in metal ion uptake systems, such as *Δznu* *ΔzupT* [9] or *ΔmntH* *ΔzupT* [9, 20], colonized the intestinal tract of gnotobiotic mice at comparable concentrations to the control strain. This suggests that sufficient amounts of zinc and manganese ions may be taken up by alternative, unknown transport systems. In *Salmonella enterica* serovar Typhimurium, for instance, closely related to *E. coli*, another manganese uptake system was identified (SitABC [10]). It was further shown that copper concentrations do not reach concentration toxic for *E. coli* within the intestinal tract, given that abolishing periplasmic protection from copper stress does not compromise colonization capacities of mutant strains compared to wildtype strains (*Δcus* *ΔcueO* [5]). One needs to take into consideration, however, that virtually all bacteria had been removed from the intestinal ecosystem of conventional mice following 8–10 weeks of a quintuple antibiotic treatment. Obviously, concentrations of metal ions bound to nutrients (e.g., amino acids) by far exceed the amount reabsorbed in the intestines. Given that commensal bacterial numbers largely exceed those of human epithelial cell in the intestines [33], the vast majority of metal ion users had been eradicated through the antibiotic treatment. In turn, an excess amount of free metal ions would have been made available for *E. coli* K12 (W3110) in our gnotobiotic model system. Thus, the gnotobiotic mouse model has to be considered a defined, but to some extent artificial, *in vivo* system. One should therefore be rather cautious not to over-interpret the presented data. Conversely, it is tempting to speculate that the impact of metal ions on colonization capacity might have been over-interpreted in the past. Nevertheless, results derived from our conventionally colonized mice infected with *E. coli* Nissle 1917 mutant strains support the main conclusions derived from the gnotobiotic mouse model.

Taken together, the study presented here demonstrates that neither defects in enterobacterial zinc, iron, copper, nickel, nor manganese homeostasis are associated with lower intestinal loads of the respective *E. coli* mutant strains in our *in vivo* models. However, future studies using gnotobiotic mice co-colonized with distinct commensal (i.e., human) or pathogenic strains, might further unravel distinct features of metal ions under defined *in vivo* conditions.

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Competing interests

The authors have declared that no competing interests exist.

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