Chapter 5
Nickel Homeostasis in *Brucella* spp.

James A. Budnick and Clayton C. Caswell

Abstract Nickel is an important cofactor for microbial proteins, such as urease, that are involved in the adaptation of bacteria to stressful conditions, as well as other proteins related to general metabolism. Therefore, successful acquisition of nickel from the environment is essential for microbes to survive. Like most metals though, acquisition of nickel is a double edge sword, as high intracellular concentrations of nickel can be toxic to microbes. Thus, bacteria have developed ways to tightly control intracellular nickel concentrations. Much of what is known about the mechanisms of nickel uptake, export, and regulation have been determined in *Escherichia coli* and *Helicobacter pylori*, but parallels between these systems and *Brucella* spp. can be drawn. This chapter will outline what is currently known about nickel acquisition by the NikABCDE and NikKMLQO systems, as well as propose the role of a putative nickel exporter and transcriptional regulators of genes encoding Ni import and export systems in *Brucella* biology and virulence.

Keywords *Brucella* · Nickel · Urease

5.1 Nickel Import by NikABCDE and NikKMLQO

With regards to nickel homeostasis in *Brucella*, nickel import has been the most studied system to date. *Brucella* spp. contain two loci encoding nickel import systems. NikABCDE is an ABC-type transporter that was first described in 2001 by Jubier-Maurin et al., and NikKLMQO is an energy coupling factor (ECF)-type transporter transcribed with the *ure*2 operon (Sangari et al. 2010; Jubier-Maurin et al. 2001). *nikABCDE* encodes an archetypal ABC-type transporter with *nikD* and *nikE* encoding ATPases, *nikB* and *nikC* inner membrane proteins, and *nikA* as a periplasmic nickel-binding protein (Fig. 5.1). The functions of these proteins have

J.A. Budnick · C.C. Caswell (✉)
Department of Biomedical Sciences and Pathobiology, Virginia–Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061, USA
e-mail: caswellc@vt.edu

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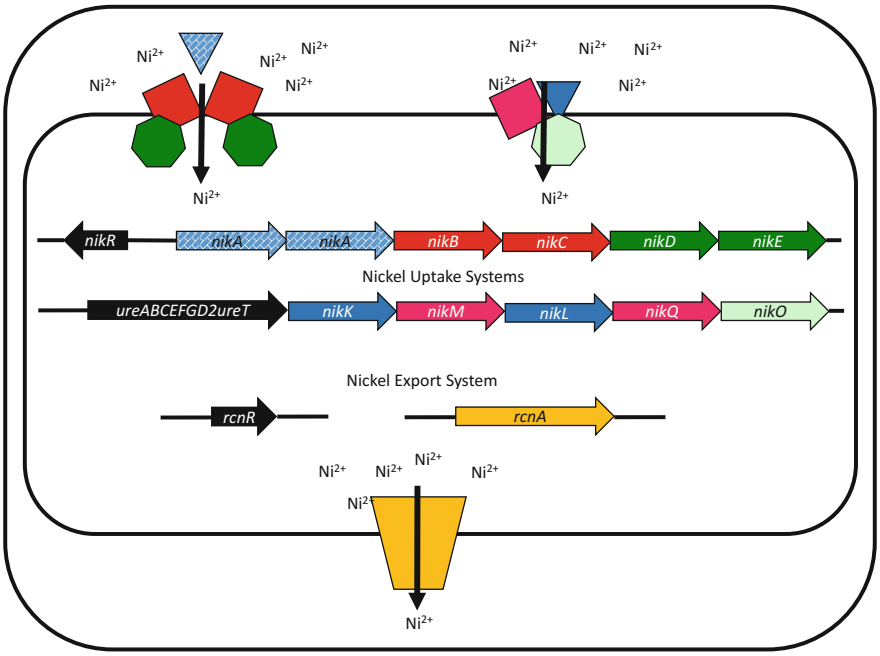

Fig. 5.1 Nickel uptake and export system

not been experimentally characterized in *Brucella* but can be inferred based on homology to other characterized nickel transporters (Rodionov et al. 2006). However, *B. suis* NikA has been crystallized and specific nickel binding sites were determined, adding to the evidence that NikA is the periplasmic binding protein of the *nikABCDE* system (Lebrette et al. 2014).

Utilizing a GFP-transcriptional fusion, it was shown that the *nikA* promoter in *B. suis* 1330 was induced during infection of J774.A1 macrophage-like cells, and similarly, the *nikA* promoter was activated when bacteria were grown in culture medium supplemented with EDTA or under microaerobic conditions (Jubier-Maurin et al. 2001). Despite being induced upon infection of J774.A1 cells, strains containing a partial deletion of *nikA* showed similar replication rates in both human monocytes and THP-1 cells, indicating that this system is not necessary for the survival and replication of *B. suis* during macrophage infection (Table 5.1).

Interestingly, the *nikABCDE* gene locus is dissimilar amongst *Brucella* spp. *nikD* is a pseudogene in *B. ovis* ATCC 25840 (Tsolis et al. 2009); and in *B. abortus* strains 2308, 9-941, and S19, there is a nonsense mutation about halfway through *nikA*, rendering it a pseudogene (Sangari et al. 2010). However, the presence of a second nickel import system likely compensates for these mutations in *B. ovis* and *B. abortus*.

While studying the *ure2* gene locus, Sangari et al. described the presence of an ECF-type nickel transporter gene cluster downstream of the *ure2* operon that encodes

Table 5.1 Nickel-related genes and connections to virulence in *Brucella*

Gene	Locus tag*	Description of phenotypes observed by mutant	Reference
nikA	BAB2_0433 and BAB2_0434	Not attenuated in THP-1 macrophages**	Jubier-Maurin et al. (2001)
nikB	BAB2_0435 (BAB_RS28430)	No data currently available	N/A
nikC	BAB2_0436 (BAB_RS28435)	No data currently available	N/A
nikD	BAB2_0437 (BAB_RS28440)	No data currently available	N/A
nikE	BAB2_0438 (BAB_RS28445)	No data currently available	N/A
nikK	BAB1_1384 (BAB_RS22535)	No data currently available	N/A
nikM	BAB1_1385 (BAB_RS22540)	No data currently available	N/A
nikL	BAB1_1386 (BAB_RS22545)	No data currently available	N/A
nikQ	BAB1_1387 (BAB_RS22550)	No data currently available	N/A
nikO	BAB1_1388 (BAB_RS22555)	Decreased urease activity and higher sensitivity to acidic pH stress	Sangari et al. (2010)
nikR	BAB2_0432 (BAB_RS28420)	Induced 12 hours post-infection of HeLa cells***	Rossetti et al. (2011)
rcnA	BAB2_0094 (BAB_RS26805)	No data currently available	N/A
rcnR	BAB1_0129 (BAB_RS16535)	No data currently available	N/A
*ureC*1	BAB1_0300 (BAB_RS17360)	Attenuated upon oral infection of BALB/c mice	Sangari et al. (2007)
*ureC*2	BAB1_1378 (BAB_RS22505)	Not attenuated upon oral infection of BALB/c mice	Sangari et al. (2007)

*Locus tags coorespond to *Brucella melitensis* biovar Abortus 2308 genome sequence and annotation. The old (i.e., BAB1/2_####) and new (BAB_RS#####) locus tag designation are provided

**Experiment was conducted using a deletion of *nikA* in *B. suis* 1330

***Experiment was conducted using *B. melitensis* 16M

the putative nickel transporter *nikKMLQO* (Sangari et al. 2010). ECF transporters lack a extracytoplasmic periplasmic binding protein and instead contain a membrane-embedded substrate binding protein (Wang et al. 2013; Erkens et al. 2011). Bioinformatic homology analyses predict *nikM* and *nikQ* to encode inner membrane proteins, *nikO* to encode an ATPase, and *nikK* and *nikL* to encode substrate-binding proteins (Fig. 5.1) (Sangari et al. 2010). Again, none of these functions have been experimentally demonstrated. Sangari et al. showed that the *ure*2 operon encodes 3 separate systems, *ureABCEFGD2*, *ureT*, and *nikKMLQO*. In their study, they

constructed a *nikO* mutant to determine the role of this transporter on urease activity. It was shown that the *nikO* mutant had lower urease activity and was more sensitive to acidic pH in culture (Table 5.1). The *nikO* mutant was not used to infect cells in vitro.

Altogether, these data show that *Brucella* spp. contain two separately encoded nickel import systems, which likely compensate for one another. None of the above mutants were used to define the role of the *nik* genes in vivo. Further studies are necessary to characterize both import systems and to understand the potential for them to serve redundant functions in vivo. One method to do this would be to construct mutants containing mutations in each system individually and then construct a mutant containing mutations in both systems. These mutants could be used to elucidate the proposed redundancy of the systems and understand the role of nickel in *Brucella* virulence. As discussed below, the studies provide evidence that they are necessary for the import of nickel and for the efficient activity of urease during infection.

5.2 The Nickel and Cobalt Exporter RcnA

The expression of a nickel and cobalt export system, RcnA, is a mechanism utilized by bacteria to counter metal toxicity and was first described by Rodrigue et al. in 2005. That study demonstrated that *E. coli rcnA* was induced upon addition of nickel or cobalt to the media; strains containing a deletion of *rcnA* were more sensitive to nickel and cobalt toxicity and contained more intracellular nickel and cobalt; and strains harboring a multicopy plasmid expressing RcnA contained less intracellular nickel and cobalt than strains containing an empty multicopy plasmid (Rodrigue et al. 2005). *Brucella* spp. Encode a putative nickel and cobalt export permease RcnA (Fig. 5.1). However, *Brucella* RcnA shows less than 40% amino acid similarity to that of *E. coli* RcnA and is missing the distinctive histidine cluster found within *E. coli* RcnA sequence. This begs the question whether the ortholog of RcnA in *Brucella* is truly a nickel and cobalt exporter, or are there other mechanisms to detoxify the cells of these metal cations?

5.3 Nickel-Responsive Regulators NikR and RcnR

While nickel is an essential cofactor for several proteins in bacteria, an excessive amount of intracellular nickel can be toxic by causing oxidative damage, by replacing essential metal ions in metalloenzymes, or by binding to non-metalloenzyme active sites or secondary sites leading to decreased enzyme activity (Macomber and Hausinger 2011). To combat this problem, bacteria possess mechanisms to regulate intracellular nickel concentrations. *Escherichia coli* and *Helicobacter pylori* encode the well characterized nickel responsive regulator, NikR, which is a nickel dependent ribbon-helix-helix transcriptional regulator.

NikR regulates *nik* genes in response to intracellular metal concentrations and other stimuli, and *E. coli* also encodes a repressor of the nickel exporter RcnA, called RcnR (Iwig et al. 2006; Schreiter and Drennan 2007).

In *E. coli*, NikR solely regulates the *nik* operon (Li and Zamble 2009). In *H. pylori*, NikR is a repressor of the nickel uptake gene *nixA* and an activator of the *ure* operon (Ernst et al. 2005). A putative nickel responsive regulator is encoded adjacent to the *nikABCDE* locus in *B. abortus* 2308. The ability of NikR to regulate the nickel import systems and potentially the *ure2* operon has not been experimentally characterized, and there is little data describing the role of this regulator in *Brucella*. While the aim of the study was not to characterize NikR, Jubier-Maurin et al. identified a sequence upstream of *nikA* that closely resembles the NikR binding site in *E. coli* (Jubier-Maurin et al. 2001). Rossetti et al. were the first to identify a potential role of NikR in virulence during *B. melitensis* infection of HeLa cells. Their data demonstrated that *nikR* expression increased 12 h post-infection of HeLa cells infected with *B. melitensis* (Table 5.1) (Rossetti et al. 2011). This study also revealed increased expression of the *ure2* operon, but no differential expression of any of the *nik* genes during HeLa cell infection. Thus, the authors suggested that NikR could be both a repressor of the *nik* genes and an activator of the *ure*2 genes (Rossetti et al. 2011). However, since this was not a targeted experiment, rather a discovery tool for genes differentially regulated during infection of HeLa cells, the link between NikR and the regulation of the *ure*2 operon remains unknown. It is possible that another regulator is responsible for the induction of *ure2*, and it is coincidental that both *ure2* and *nikR* are induced upon infection of HeLa cells. It is clear that further studies are necessary to deduce the function of NikR in *Brucella* spp.

RcnR is a repressor of *rcnA* and *rcnR* in a nickel and cobalt responsive manner in *E. coli* (Blaha et al. 2011; Iwig et al. 2006). The two genes are transcribed divergently from one another in *E. coli*, and Blaha et al. identified a specific palindromic RcnR binding box, TACT-N7-AGTA, in the intergenic region of the two genes. Upon binding either of the metals, RcnR dissociated from the RcnR binding box, allowing for the expression of *rcnA* and *rcnR* (Blaha et al. 2011). Deletion of *rcnR* showed constitutively expressed *rcnA* (Iwig et al. 2006). *Brucella* spp. also encode a putative RcnR protein that is 40% identical and over 60% similar to the RcnR protein of *E. coli*. Contrary to the situation in *E. coli*, *rcnR* in *Brucella* strains is not located divergently to *rcnA,* but rather is located on a different chromosome from *rcnA*. Interestingly, an identical sequence to that of the *E. coli* RcnR binding box is located upstream of *rcnR*, but this putative RcnR-binding sequence is not observed upstream of *rcnA* in *Brucella*. It should be noted that *Brucella abortus* RcnR is 54% identical to the formaldehyde stress response regulator FrmR in *E. coli*, and the genomic organization of RcnR and surrounding genes in *B. abortus* is similar to that of the *E. coli frmR* and *N. gonorrhoeae nmlR* (an *fmrR* ortholog) loci (Chen et al. 2016). To date, no studies have characterized RcnR in *Brucella* strains, and empirical evidence will be needed to support the hypothesis that RcnR is a transcriptional regulator of *rcnR* and/or *rcnA* in *Brucella* spp. and not, in fact, an ortholog of FrmR related to formaldehyde resistance.

5.4 Nickel-Dependent Proteins in *Brucella*

Bacterial proteins that require nickel include urease, NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl-coenzyme A decarbonylase, methyl-coenzyme M reductase, nickel dependent superoxide dismutases and glyoxylases (Mulrooney and Hausinger 2003; Li and Zamble 2009). Of the above proteins, urease is the only protein directly linked to virulence in *Brucella* spp.

5.4.1 Urease

Urease enzymes hydrolyze urea into carbon dioxide and ammonia and thus, play a key role in nitrogen metabolism, as well as acid resistance as microbes pass through the acidic environment of the stomach (Li and Zamble 2009). Urease was one of the first proteins demonstrated to require nickel for catalysis (Alagna et al. 1984). Since then, extensive biochemical analyses of this protein have exposed much about specific binding sites for nickel within the protein (Mulrooney and Hausinger 2003). The *Brucella ure* operons can be split into two groups: structural proteins (encoded by *ureA*, *ureB*, and *ureC*) and accessory proteins (*ureD*, *ureE*, *ureF*, and *ureG*) (Sangari et al. 2007). This genetic organization is very similar to that of the genes encoding the trimeric urease of *Klebsiella aerogenes* (Mulrooney and Hausinger 1990; Sangari et al. 2007). It was shown that *K. aerogenes* UreE directly binds to nickel and is thought to function as a nickel carrier for the urease enzyme (Mulrooney et al. 2005). The *Brucella* genome contains two urease operons, *ure*1 and *ure*2. While both have been shown to contribute to urease activity and are activated in acidic conditions, only *ure*1 has been shown to be necessary for the virulence of *B. suis* or *B. abortus* in mice infected via the oral route (Bandara et al. 2007; Sangari et al. 2007). Upon deletion of either *ureC*1 (*ureC* from operon 1) or *ureC*2 (*ureC* from *ure* operon 2), *ureC*1 was deemed less fit for oral infection of a mouse model compared to *B. abortus* 2308 (Sangari et al. 2007). This has been predicted to be due to the dissimilar genetic identities of the *ure* operons. In *B. suis* 1330, the amino acid similarity between *ure1* and *ure2* is about 70%, and most of the urease activity in this strain is predicted to be due to *ure1* (Bandara et al. 2007). Mutations in the *ure* genes does not affect the ability of *Brucella* spp. to survive and replicate in cell lines in vitro (Sangari et al. 2007; Bandara et al. 2007). This evidence supports the claim that *Brucella* is most likely utilizing urease to combat a low pH environment during the biologically relevant oral route of infection. Altogether, it has been shown that while *ure2* contributes to urease activity, it is not necessary for infection of a host via the oral route. Therefore, it is possible that *Brucella* spp. have maintained the *ure2* operon due to necessity of other genes (i.e., *ureT*, *nikK*, *nikM*, *nikL*, *nikQ*, *nikO*) located downstream of the *ure2* genes in the operon for infecting the host.

5.4.2 *Other Potential Nickel-Containing Proteins in* Brucella

As stated above, several proteins other than urease have been identified as nickel-binding metalloproteins in microbes. However, *Brucella* urease is the only nickel binding protein that has been extensively studied in *Brucella* biology.

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