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Borrelia burgdorferi genes*, bb0639-0642,* encode a putative putrescine/spermidine transport system PotABCD that is spermidine specific and essential for cell survival

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Summary

Polyamines are an essential class of metabolites found throughout all kingdoms in life. *Borrelia burgdorferi* harbors no enzymes to synthesize or degrade polyamines yet does contain a polyamine uptake system, *potABCD*. In this report, we describe the initial characterization of this putative transport system. After several unsuccessful attempts to inactivate *potABCD*, we placed the operon under the control of an inducible LacI promoter expression system. Analyses of this construct confirmed that *potABCD* was required for *in vitro* survival. Additionally, we demonstrated that the *potABCD* operon were upregulated *in vitro* by low osmolarity. Previously, we had shown that low osmolarity triggers the activation of the Rrp2/RpoN/RpoS regulatory cascade which regulates genes essential for the transmission of spirochetes from ticks to mammalian hosts. Interestingly, induction of the *pot* operon was only affected in an *rpoS* mutant but not in a *rpoN* mutant, suggesting that the genes were RpoS-dependent, RpoN-independent. Furthermore, *potABCD* was upregulated during tick feeding concomitant with the initiation of spirochete replication. Finally, uptake experiments determined the specificity of *B. burgdorferi's* PotABCD for spermidine.

Graphical Abstract

Borrelia burgdorferi, Lyme disease agent, harbors no enzymes to synthesize or degrade polyamines yet does contain the genes encoding a putative polyamine uptake system (*potABCD*).

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Author Contributions

S.B-G. designed and performed experiments, interpreted data and wrote manuscript.

K. L. and C. L. R. performed experiments and edited manuscript.

F. C. G. contributed to experimental design, data analysis, and wrote manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest with the contents of this article.

Ethics statement

Mouse infection studies were carried out in accordance with the Animal Welfare Act (AWA1990) and followed the guidelines of the National Institutes of Health, Public Health Service Policy on Humane Care (PHS 2002) and Use of Laboratory Animals and the United States Institute of Laboratory Animal Resources, National Research Council, Guide for the Care and Use of Laboratory Animals. All animal work was done according to protocols approved by the Rocky Mountain Laboratories, NIAID, NIH Animal Care and Use Committee. The Rocky Mountain Laboratories are accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All efforts were made to minimize animal suffering.

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Here, we demonstrated that the PotABCD is a spermidine-specific transporter system that is essential for survival. The genes are upregulated during tick feeding concomitantly with the decrease of osmoliarity which has been shown to trigger virulence factor expression. The *potABCD* genes are regulated in a RpoN-dependent, RpoS-independent, BosR-independent fashion.

Keywords

Lyme disease; polyamine; *potABCD*; tick; regulation

Introduction

Borrelia burgdorferi, one of the causative agents of Lyme disease (Pritt *et al.*, 2016), is maintained in a life cycle that involves a vertebrate host (*e.g.* rodent) and a hard tick: *Ixodes scapularis* (Stewart & Rosa, 2017). During its infectious cycle, *B. burgdorferi* adapts its physiology and metabolism in response to changing environmental conditions in the mammal or the arthropod vector (Caimano *et al.*, 2016, Gherardini *et al.*, 2010). The mammalian host offers this spirochete a nutrient-rich, stable environment (temperature, pH, osmolarity, etc.) (Waymouth, 1970, The staff of the Jackson Laboratory, 1966). In addition, *B. burgdorferi* uses several mechanisms for evading the aggressive host immune system, allowing it to successfully colonize various mammalian species, including humans (Norris, 2014, Petzke & Schwartz, 2015, Radolf *et al.*, 2012). In contrast, environmental parameters inside the arthropod vector are quite dynamic over the course of *B. burgdorferi*'s life cycle. Studies have shown that the *I. scapularis* midgut has shifting physio-chemical parameters (temperature: 28°C–> 37°C; osmolarity: 600–> 300–> 600 mOsM) (Bontemps-Gallo *et al.*, 2016, Schwan *et al.*, 1995, Stevenson *et al.*, 1995) and, nitrosative and oxidative bursts that peak during the feeding process (Bourret *et al.*, 2016, Sonenshine & Anderson, 2014). In response to fluctuating environmental conditions, *B. burgdorferi* modulates its gene expression program to allow it to successfully colonize both host and vector.

A small set of regulators allow *B. burgdorferi* to adapt to changing conditions throughout the infectious process. Upon colonization of *I. scapularis*, the spirochetes upregulate the expression of genes involved in carbohydrate utilization, cell envelope biosynthesis and long-term survival through the two-component system, Hk1-Rrp1 (Caimano *et al.*, 2011, He *et al.*, 2011, Kostick *et al.*, 2011). This two-component system modulates the levels of c-di-GMP due to the diguanylate cyclase activity on Rrp1 (Caimano *et al.*, 2011, He *et al.*, 2011). *In vitro*, Hk1-Rrp1 is stimulated by high osmolarity (600 mOsM) (Bontemps-Gallo *et al.*, 2016). Long-term survival of the spirochetes, after digestion of the blood meal and subsequent molt, is coordinated via Rel_{Bbu} (RelA/SpoT homolog) (Drecktrah *et al.*, 2015), BosR and RpoS (Samuels, 2011, Skare *et al.*, 2010). After the molt, *I. scapularis* begins its next feeding which provides an influx of nutrients and affects multiple environmental parameters inside the midgut, including temperature and osmolarity (Blevins *et al.*, 2009, Bontemps-Gallo *et al.*, 2016, Caimano *et al.*, 2004, Caimano *et al.*, 2007, Elias *et al.*, 2000, Fisher *et al.*, 2005, Hubner *et al.*, 2001). Growth and changes in environmental conditions activate the Rrp2/RpoN/RpoS regulatory cascade which is required for successful

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transmission from a tick to a mammal (Caimano *et al.*, 2016). Over the last two decades, several signals responsible for activating this pathway have been identified including a temperature shift (23–> 34°C) (Schwan *et al.*, 1995, Stevenson *et al.*, 1995), cell density (3 x 10^8 cells ml^{-1}) (Indest *et al.*, 1997, Ramamoorthy & Philipp, 1998, Yang *et al.*, 2000), low osmolarity (250 mOsM) (Bontemps-Gallo *et al.*, 2016) and acid stress (Dulebohn *et al.*, 2017).

In other bacteria, polyamines (*e.g.* putrescine, spermidine, spermine) are involved in both the oxidative and osmotic stress responses (Altendorf *et al.*, 2009, Chattopadhyay *et al.*, 2003, Cohen, 1998, Munro *et al.*, 1972, Shah & Swiatlo, 2008), both of which are encountered by *B. burgdorferi* during tick feeding. In general, the polyamine content in bacterial cells is regulated by both biosynthesis and transport (Igarashi & Kashiwagi, 1999). In *E. coli*, the most commonly used bacterial model for polyamine research, the intracellular concentration of putrescine (22.1 nmol/10^9 cells) is higher than spermidine (3.8 nmol/10^9 cells) (Morris & Jorstad, 1970). Uptake experiments in *E. coli* demonstrate that spermidine/putrescine transport (via PotABCD) is a spermidine-preferential uptake system (Kashiwagi *et al.*, 1993) that also transports putrescine but with lower affinity. Both molecules can bind nucleic acids and proteins to stabilize their interactions (Childs *et al.*, 2003), modulate the rate of transcription (Thomas *et al.*, 1995) and stimulate growth (Igarashi & Kashiwagi, 2010). Despite extensive research efforts, the role of putrescine and spermidine in bacterial cells remains unclear (Miller-Fleming *et al.*, 2015).

Since *B. burgdorferi's* genome does not appear to contain the genes encoding enzymes with the capacity to synthesize, modify or degrade either putrescine, spermidine or spermine, the bacteria is thought to be a spermidine auxotroph (Fraser *et al.*, 1997, Wyss & Ermert, 1996). Only one putative spermidine/putrescine ABC-transporter, called PotABCD (encoded by *bb0639-642*) is annotated in *B. burgdorferi's* genome (Fraser *et al.*, 1997, Lin *et al.*, 2017). *bb0642* (*potA*) encodes a potential ATP-binding protein while *bb0641* (*potB*) and *bb0640* (*potC*) encode proteins with 6 transmembrane domains suggesting a role in membrane translocation. Finally, *bb0639* (*potD*) encodes a putative spermidine/putrescine periplasmic binding protein which could bind polyamines facilitating transport from the periplasm space into the cytosol. It has been shown that these four genes are expressed as an operon in a polycistronic mRNA (Lin *et al.*, 2017).

In this study, we further characterize PotABCD, the spermidine/putrescine transport system from *B. burgdorferi,* using an inducible expression system (*lacUV5* p/o) to regulate *potABCD* expression. Using this genetic construct, we were able to demonstrate that this system was essential for survival *in vitro*. Interestingly, analyses of specific RpoS, BosR and RpoN mutants indicated that *potABCD* was regulated in an RpoS-dependent, RpoN-independent manner, and that regulation was concurrent with the drop in osmolarity measured in the midgut of feeding ticks (600–250 mOsM). Finally, uptake experiments strongly suggested that PotABCD was specific for spermidine in *B. burgdorferi.*

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Results

The putative PotABCD transport system is essential for B. burgdorferi survival

Spermidine and spermine have been shown to trigger the expression and synthesis of proteins required for mammalian invasion through RpoS and BosR (Lin *et al.*, 2017). To better understand the significance of this transport system, we attempted to inactivate the *potABCD* operon. Unfortunately, multiple attempts were unsuccessful. In addition, the comprehensive STM mutant library did not detect any transposon insertions in any of the genes in the operon (Lin *et al.*, 2012) supporting the assumption that the *potABCD* system could be essential to *B. burgdorferi*. Therefore, we generated an inducible *pot* operon using the inducible *lac* promoter expression system developed by Gilbert *et al.* (Gilbert *et al.*, 2007). In this system, *lacI* under control of the *flgB* promoter leads to constitutive synthesis of the LacI repressor which binds the *flacp* promoter and represses the expression of the *potABCD* operon. When added, IPTG will bind LacI in a dose-dependent manner, allowing controlled expression of the *potABCD* operon (Fig. S1). Using *B. burgdorferi* strain B31-68-LS (Chu *et al.* 2016), we obtained three clones harboring the *flacp* promoter fused to *potABCD* and confirmed these putative mutants by sequencing. One clone, designated BBi-*potABCD*, was selected for further characterization.

To confirm that polyamine utilization and the *pot* operon were essential, the wild-type (B31-68-LS) parent and BBi-*potABCD* strains were grown in BSK-II, pH 7.5 with 0.5 mM IPTG to 5 x 10^7 cells/ml. Cells from the cultures were harvested, washed, sub-cultured (to ~ 5 x 10^5) and grown in BSK-II with or without IPTG. Growth of each culture was monitored every 24 h by dark-field microscopy and aliquots were removed and the cells were grown and quantified on BSK-II plates with IPTG (Fig. 1). In the presence of IPTG, the cells exhibited a normal growth rate and reached 10^8 cells/ml, while without IPTG, growth completely stopped. More importantly, the number of cells that could be recovered from cultures without IPTG decreased dramatically with time suggesting that the cells lost viability without the expression of *potABCD.* These data indicated that *potABCD* is essential in *B. burgdorferi*.

Spermidine is specifically transported by *B. burgdorferi*

Previous studies of *potABCD* systems in other bacteria have demonstrated that these transporters are capable of a broad range of uptake selectivity for polyamines (Kashiwagi *et al.*, 1993, Yao & Lu, 2014). Some transport spermine, spermidine and/or putrescine with equal efficiency while others demonstrate a selective preference. To determine if *potABCD* in *B. burgdorferi* demonstrated preferential uptake of polyamines, we performed kinetic uptake assays with tritium (^{3}H) labelled polyamines. *B. burgdorferi* cells were grown to a density of ~5 x 10^7 cells/ml, harvested, washed thoroughly and resuspended to a cell density of 6 x 10^8 cells/ml. Cells were incubated with either ^{3}H-spermidine or ^{3}H-putrescine and cell-associated radioactivity was analyzed at 1, 2, 6 and 10 min (Fig. 2). Uptake of ^{3}H-spermidine increased linearly 2.4-fold between 1 and 10 min, while no ^{3}H-putrescine uptake was observed during the same time interval. Because the inducible strain lysed when IPTG was removed, we were unable to assay the uptake selected polyamines in BBi-*potABCD*. These data indicated that, unlike the characterized polyamine transport systems in other

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bacteria (Kashiwagi *et al.*, 1993, Yao & Lu, 2014), *potABCD* in *B. burgdorferi* seems to be specific for the uptake of spermidine.

potABCD is up-regulated during nymph tick feeding

To shed light on the role of *potABCD* during tick feeding, we analyzed the expression of the four genes in the operon by RT-qPCR during nymph feeding. Additionally, *rpoS* and *ospC* were assayed as markers for the activation of the Rrp2/RpoN/RpoS pathway (Fig. 3). Ticks were removed daily from mice infected with wild-type *B. burgdorferi* (B31-A3) and the scutal index was measured to standardize feeding progress (Bontemps-Gallo *et al.*, 2016, Falco *et al.*, 1996) before pooling for RNA extraction. For each time-point examined, RNA was extracted from 3 groups of 5 *I. scapularis* ticks. The expression level of *enoS* was used as a housekeeping gene. As expected, *rpoS* transcript increased (6.1-fold) at the first day post attachment (scutal index 2.09) and stayed at a similar level during the feeding process (Fig. 3). Activation of the Rrp2-RpoN-RpoS pathway increases expression of RpoS-dependant genes (e.g. *ospC*) (Caimano *et al.*, 2004). The expression of *ospC* increased 5.6-fold on day one post-attachment and 5.9-fold more on the second day post-attachment after which the expression slowly decreased. The four genes of the *potABCD* operon followed the same pattern of transcription (Fig. 3). On the second day of tick feeding (scutal index of 2.9), the expression of *potABCD* increased 1.9-fold, 2.3-fold, 3.1-fold and 1.8-fold, respectively. In contrast, the expression of the *potABCD* operon decreased about 7-fold in the replete ticks (scutal index of 5.1) compared to the value measured on the second day of feeding (scutal index of 2.9). These data showed that during tick feeding *B. burgdorferi* upregulated the expression of genes encoding the polyamine transport system (*potABCD*) and this expression corresponded with the increase of RpoS-dependent genes.

potABCD is regulated by low osmolarity

During the feeding process of the tick vector, *B. burgdorferi* encounters and must survive two significant stresses in the midgut: oxidative and osmotic (Bontemps-Gallo *et al.*, 2016, Sonenshine & Anderson, 2014). Since putrescine and spermidine have been shown to be involved in protecting bacteria from both stresses (Chattopadhyay *et al.*, 2003, Cohen, 1998, Munro *et al.*, 1972, Shah & Swiatlo, 2008), we hypothesized that one or both stresses could be responsible for the activation of the *potABCD* operon observed during tick feeding. To test this hypothesis, we followed the expression of each gene by RT-qPCR at 250, 450, and 650 mOsM and after exposure to *tert*-butyl hydroperoxide (Fig. 4). Expression of the *potA*, *potB* and *potC* genes encoding the putative ATP-binding protein and the two membrane transport proteins increased 10-fold at low osmolarity compared to medium or high osmolality (Fig. 4A). Surprisingly, expression of the *potD* gene, encoding the periplasmic binding protein, increased 19-fold (Fig. 4A). The higher expression of *potD* compared to the three other genes suggested an internal promoter for *potD*. These data may also indicate that increased expression of the putative periplasmic binding protein (PotD) is required to increase the uptake of polyamines during a time of rapid growth in the tick midgut.

No induction of the *potABCD* operon was observed under oxidative stress while *coADR* and *napA*, both known to be involved in the oxidative stress response (Boylan *et al.*, 2003, Hyde *et al.*, 2009), were induced (Fig. 4B). Additionally, we tested the potential of spermidine and

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putrescine to function as a protective molecule against osmotic or oxidative stress (Fig. S1). No effects on the growth or on the susceptibility to both osmotic and oxidative stresses were observed. We were unable to use our *BBi-potABCD* mutant to assay if the PotABCD system was required for protection against oxidative or osmotic stresses, because the strain did not grow without the IPTG (Fig. 1). It seems very likely that the *in vitro* conditions tested do not accurately reflect the combination of stresses and changing conditions that challenge *B. burgdorferi in vivo.*

potABCD is regulated by RpoS independent of RpoN

Because *potABCD* expression followed a similar pattern to genes regulated by the Rrp2/RpoN/RpoS regulon in a feeding tick under low osmolarity, we hypothesized that this regulatory pathway controlled *potABCD* expression. To test this hypothesis, we assayed the expression levels of *potABCD* in mutant strains deficient in the key regulatory factors (*rpoN*, *rpoS*, and *bosR*) known to directly or indirectly modulate the Rrp2/RpoN/RpoS cascade (Caimano *et al.*, 2016, Samuels, 2011). Wild-type *B. burgdorferi*, *rpoN*, *rpoS*, and *bosR* mutant strains were grown in BSK-II at 250 or 450 mOsM to approximately ~5 x 10^7 cells/ml and RNA was harvested for RT-qPCR analyses. As observed previously, the *potABCD* operon was induced in wild-type cells at low osmolarity (Fig. 4 and 5). *potABCD* induction in the *rpoN* mutant was similar to levels observed in WT cells, while the *rpoS* mutant exhibited significantly reduced transcript levels under low osmolarity (Fig 5). These data were surprising since the current understanding is that the inactivation of either *rpoN* or *rpoS* results in a very similar phenotype (Caimano *et al.*, 2016, Samuels, 2011). Our understanding of the regulation of this small set of genes by RpoS, independent of RpoN, is incomplete. More investigation will be required to evaluate the importance of this regulation beyond Rrp2/RpoN/RpoS regulation (Samuels, 2011).

It has been shown that *rpoS* expression can be modulated by BosR, a transcription factor that is activated by oxidative stress (Boylan *et al.*, 2003, Ouyang *et al.*, 2011). We found that inactivation of *bosR* had no effect on the induction of *potABCD* at low osmolarity. This result was expected since oxidative stress had no effect on *potABCD* gene expression (Fig. 4). Taken together, these data suggest that *potABCD* is RpoS-dependent and BosR- and RpoN-independent. These results also demonstrate that various *in vivo* stresses can be regulated independently [e.g. oxidative stress via BosR (Boylan *et al.*, 2003, Hyde *et al.*, 2009) and acid stress via RpoS (Dulebohn *et al.*, 2017)] from the Rrp2/RpoN/RpoS cascade.

Discussion

Polyamines such as putrescine, spermidine, spermine and cadaverine are found in both prokaryotes and eukaryotes and are essential for normal cell growth, modulating the rate of transcription (Childs *et al.*, 2003), promoting efficient translation, altering and stabilizing the structure of RNA (Thomas *et al.*, 1995) and protecting the cell from deleterious effects of acid, oxidative and nitrosative stresses (Miller-Fleming *et al.*, 2015). Typically, bacterial cells transport, synthesize and/or modify polyamines to maintain an intracellular concentration that is optimal to promote maximum function and these processes are coordinately regulated (Miller-Fleming *et al.*, 2015). Most bacteria maintain higher

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intercellular levels of spermidine than putrescine. *E. coli* is a notable exception in that higher levels of putrescine (22.1 nmol/10^9 cells) are detected compared to spermidine (3.8 nmol/10^9 cells) (Morris & Jorstad, 1970). The synthesis of putrescine and spermidine is accomplished by the decarboxylation of precursor amino acids such as ornithine (via ornithine decarboxylase, encoded by *speC*) or arginine (arginine decarboxylase, encoded by *speA*), while the synthesis of spermine or cadaverine are rare (Miller-Fleming *et al.*, 2015).

The genome of *B. burgdorferi* lacks the genes encoding proteins for the *de novo* synthesis of putrescine or spermidine and it has been proposed that *B. burgdorferi* is a spermidine auxotroph (Wyss & Ermert, 1996). However, it does harbor genes encoding a single putative putrescine/spermidine transport system, *potABCD*, suggesting that this putrescine/spermidine system may be responsible for polyamine utilization *in B. burgdorferi*. Recently, Lin *et al.* showed that high concentration of spermidine (4 mM) *in vitro* triggers the activation of the Rrp2/RpoN/RpoS cascade (Lin *et al.*, 2017), suggesting that this polyamine may affect the regulation of key virulence factors during the infectious cycle. During the infectious process, *B. burgdorferi* switches between an arthropod vector and a mammalian host. To be successful during this transition, the bacteria needs to adapt to take advantage of the resources available, optimize growth, correctly alter gene expression and protect itself from deleterious environmental conditions. The purpose of this study is to determine the role of *potABCD* and polyamines in these progressions.

To determine the role of *potABCD* in *B. burgdorferi,* we initially attempted to generate a mutant by deleting the entire operon. However, multiple attempts to inactivate the putative polyamine transport system were unsuccessful. Therefore, an inducible *potABCD* (strain BBi-*potABCD*) was generated. Growth studies indicated that without inducer, cell division ceased, and the cells lost viability after 24 h, suggesting that *potABCD* was required for bacterial replication and survival (Fig. 1) *in vitro*. Compared to other bacteria which can synthesize and/or transport putrescine, spermidine or spermine, *B. burgdorferi* is quite metabolically limited. Uptake experiments indicate that the bacteria use the PotABCD transport system to specifically transport spermidine Previous studies have shown that spermidine is more abundant in human blood (9 uM) than putrescine (0.13 uM) (Wishart *et al.*, 2007, Wishart *et al.*, 2009, Wishart *et al.*, 2013) and is the only polyamine available in the midgut of nymphal ticks. The availability of spermidine in the various hosts and vectors harboring *B. burgdorferi* and its selective transport strongly suggests that spermidine is the essential polyamine required by *B. burgdorferi* for survival (Narasimhan *et al.*, 2017).

Since we could not introduce strain BBi-*potABCD* directly into ticks, we evaluated the expression patterns of *potABCD* in ticks colonized with strain B31-A3. In the tick, spirochetes up-regulated the *potABCD* operon during the early stage of feeding concomitant with the activation of RpoS-dependent genes (Fig. 3). Recently, Arnold *et al.* demonstrated by RNA-seq that the *potABCD* operon used only one promoter regardless of the phase of growth (Arnold *et al.*, 2016). However, Adams *et al.* showed by using an *in vivo* expression technology-based approach that the *potABCD* operon had one primary promoter and multiple secondary promoters (Adams *et al.*, 2017). These data together with our data from feeding ticks (Fig. 3), strongly suggested that the *potABCD* operon could be expressed as one polycistronic mRNA from a single promoter or as multiple transcripts from secondary

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promoters to control the ratio of the mRNA of each gene and the stoichiometry of the different proteins. Additionally, we demonstrated that the *potABCD* operon is part of a small subset of genes that are RpoS-dependent, RpoN-independent (Fig. 5). Fisher *et al.* previously demonstrated using microarray analyses that a small set of genes in the Rrp2/RpoN/RpoS regulon were RpoS-dependent, RpoN-independent (Fisher *et al.*, 2005). In their study, they found that *potD* expression decreased in an *rpoS* mutant but not in an *rpoN* mutant. Those previous observations and the data presented here demonstrate that *potABCD* is regulated in an RpoS-dependent, RpoN-independent manner.

Finally, we investigated the potential biological roles of polyamines in *B. burgdorferi* as well as the cues that trigger the activation of the *potABCD* in *B. burgdorferi.* Polyamines, such as putrescine or spermidine, are required for cell growth and, in some bacteria, for protecting cells from oxidative or osmotic stress (Altendorf *et al.*, 2009, Igarashi & Kashiwagi, 2010, Miller-Fleming *et al.*, 2015). First, we tested the ability of polyamines to protect *B. burgdorferi* in protecting the bacterium from the adverse effects of osmolarity or oxidative stress (Fig. S2). Under the conditions tested, spermidine was not effective at expanding the survivability of cells from osmotic conditions outside the range of those which have been shown to exist in the tick midgut before, during or after feeding (250–600 mOsM) (Bontemps-Gallo *et al.*, 2016). Additionally, it did not increase the resistance of cells to ROS.

However, *potABCD* was up-regulated when the cells were exposed to low osmolarity but not when cells were exposed to ROS (Fig. 4). Also, *potABCD* was upregulated in the tick midgut during feeding, paralleling the drop in osmolarity that has been measured in ticks at the initial stages of feeding (Bontemps-Gallo *et al.*, 2016). While these data suggest a role for *potABCD* in the tick, we cannot assign a specific function for spermidine in *B. burgdorferi.* It is interesting to note that the increase in expression of *potABCD* occurs at a time during tick feeding which corresponds with maximum cell growth. Because of the complexity of the environment in the tick midgut, it seems likely that the conditions tested *in vitro* do not accurately reflect the multiple parameters (osmolarity, ROS, RNS) to which *B. burgdorferi* is exposed in the tick midgut. While spermidine is essential for *B. burgdorferi* survival and growth *in vitro,* its exact biochemical role remains to be determined.

Experimental Procedures

Bacterial strains, media and growth conditions

The bacterial strains used in this study are described in the Table 1. *B. burgdorferi* strains were grown in BSK-II medium (Barbour, 1984) at 34°C under microaerobic conditions (3–5% O_2, 5% CO_2, 90% N_2). Cell densities were determined by dark-field microscopy. The osmolality of BSK-II medium is 450 mOsM. High and low-osmolality media, were generated as previously described (Bontemps-Gallo *et al.*, 2016). Every 24 h, an aliquot from each culture was examined by dark-field microscopy and plated on BSK-II. Plates were incubated at 34°C under a microaerobic atmosphere for 7–14 d to determine CFU. Spermidine and putrescine (Sigma-Aldrich, St. Louis, MO, USA) were added to BSK-II as indicated. Osmolarity of the growth media (mOsM) was measured with a vapor pressure osmometer (Model 3320, Advanced Instruments, Inc., Norwood, MA, USA).

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Construction of the BBi-potABCD (*B. burgdorferi* inducible–pot operon) strain

Plasmids and primers designed for PCR are listed in Tables 1 and 2, respectively. A fragment containing *bb0643-flaBp-Kan-flacp-potA* was synthesized by Genscript USA and cloned into pUC57 and was named pBBi-*potABCD*. The plasmid was transformed into *B. burgdorferi* strain B31-68-LS (Chu *et al*., 2016) as described previously (Samuels, 1995) and kanamycin-resistant colonies were analyzed by PCR and sequenced to confirm that *potABCD* had been replaced by the inducible *potABCD* (*lacUV5-potABCD*) (Table 2) (Fig. S1). The mutant strain was designated as BBi-*potABCD*. The plasmid profiles of the wild-type and the BBi-*potABCD* were determined using the multiplex PCR system from Bergström laboratory (Bunikis *et al*., 2011). Wild-type strain B31-68-LS was missing cp9 and lp5 while strain BBi-*potABCD* was missing cp9, lp28-1 and lp5.

Uptake of ^{3}H-Spermidine and ^{3}H-Putrescine

Polyamine transport was determined using a previously described assay by Tilly *et al.* (Tilly *et al*., 2004). Briefly, cells were grown in BSK-II to mid log phase (~5 x 10^7 cells/ml), harvested by centrifugation, washed three times with an equal volume of HN buffer (50 mM Hepes, 20 mM NaCl, pH 7.6) supplemented with 6 mM glucose and resuspended to a cell density of 6×10^8 cells/ml in HN buffer supplemented with 6 mM glucose. A 50 μl aliquot was reserved for protein quantification. Tritium labeled polyamine (5 μCi) was added to 1.0 ml cell suspensions and incubated at 34°C for the duration of the experiment. 100 μl aliquots were removed at each time point and applied to a 0.22 μm AcetatePlus filter (Osmonics, Inc., Minnetonka, MN) using a vacuum manifold and washed three times with 5 ml HN buffer before measuring scintillation counts. Spermidine [terminal methylene-^{3}H] trihydrochloride (ART1749) and Putrescine [2,3-^{3}H] dihydrochloride (ART0279) were purchased from American Radiolabeled Chemicals, Inc, (St. Louis, MO). The polyamine concentrations (pmol) in samples were determined against a calibration performed with labeled authentic substrates. The protein concentrations were determined using the Pierce™ BCA Protein Assay with bovine serum albumin as a standard (ThermoFisher Scientific, Rockford, IL, USA).

Tick rearing and feeding

I. scapularis egg masses (Oklahoma State University) were allowed to hatch and mature in a controlled temperature, humidity and photoperiod environment. RML mice, an outbred strain of Swiss-Webster mice reared at the Rocky Mountain Laboratories breeding facility, were needle inoculated by intradermal injection with 100 μl of BSK-II containing 1 x 10^5 *B. burgdorferi* B31-A3 and after three weeks, infection was confirmed by re-isolation of spirochetes from ear punch biopsies. Larval ticks were fed to repletion on infected mice, collected and allowed to molt into nymphs in a controlled environment. Nymphs were then fed on naïve RML mice, mechanically removed during the feeding and the scutal index was determined to standardize the feeding progression (Bontemps-Gallo *et al*., 2016, Falco *et al*., 1996).

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RNA purification and RT-qPCR

RNA samples were extracted from *B. burgdorferi* cultures using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Three independent culture samples were used for each condition. RNA samples were also extracted from *B. burgdorferi* infected ticks during feeding. Ticks were frozen at −80°C, crushed and resuspended in TRIzol (Life technologies, Carlsbad, CA) with chloroform. After centrifugation, the upper phase was mixed with 70% ethanol (1:1) and loaded onto RNeasy column (Qiagen) according to the manufacturer's protocol. The removal of contaminating DNA was accomplished using TURBO DNA-free DNase I (Life technologies, Carlsbad, CA). The cDNA was synthesized from the purified RNA using Superscript IV reverse transcriptase with random primers (Invitrogen, Carlsbad, CA). To compare gene expression, a relative quantification method was employed using *enoS* as a reference gene (Bontemps-Gallo *et al.*, 2016, Pfaffl, 2001). All samples were analyzed in triplicate on a Roche LightCycler 480 System (Indianapolis, IN) using *Power* SYBR® Green PCR Master Mix according to the manufacturer's instructions (Life technologies, Carlsbad, CA). The primers used for the qPCR are listed in Table 2.

ROS susceptibility and protection assays

Strains were grown in pyruvate-free BSK-II medium under microaerobic conditions at 34°C to a cell density of 2 x10^7 cells/ml and cells were exposed to the different concentrations of *tert*-Butyl hydroperoxide (Luperox® TBH70X, Sigma-Aldrich, St. Louis, MO, USA) for 3 h in pyruvate-free BSK-II. Samples were plated in BSK-II and incubated at 34°C under microaerobic conditions for 7–14 d to allow enumeration of CFU. When indicated, Spermidine (Sigma-Aldrich, St. Louis, MO, USA) was added to the BSK-II.

Statistical Analysis

Prism 7 software (GraphPad Software, Inc.,La Jolla, CA, USA) was used. Data were analyzed by using a One-Way ANOVA with a Geisser-Greenhouse correction; a value of $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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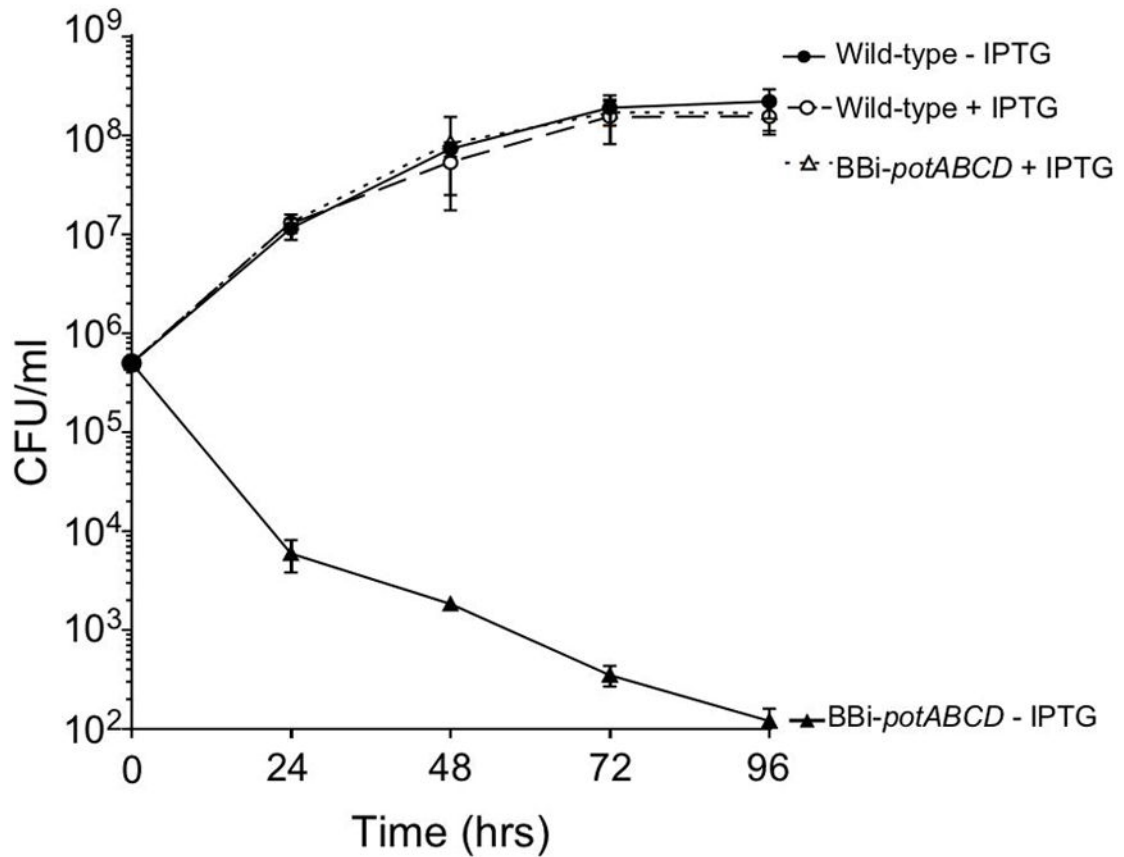

Fig. 1. The PotABCD uptake system is essential for *B. burgdorferi*

Growth and survival curve of wild-type B31-68-LS and BBi-*potABCD* strains with or without 500 μM IPTG. Cells were enumerated by plating on BSK-II plating. Data represent means ± standard deviation of three independent experiments.

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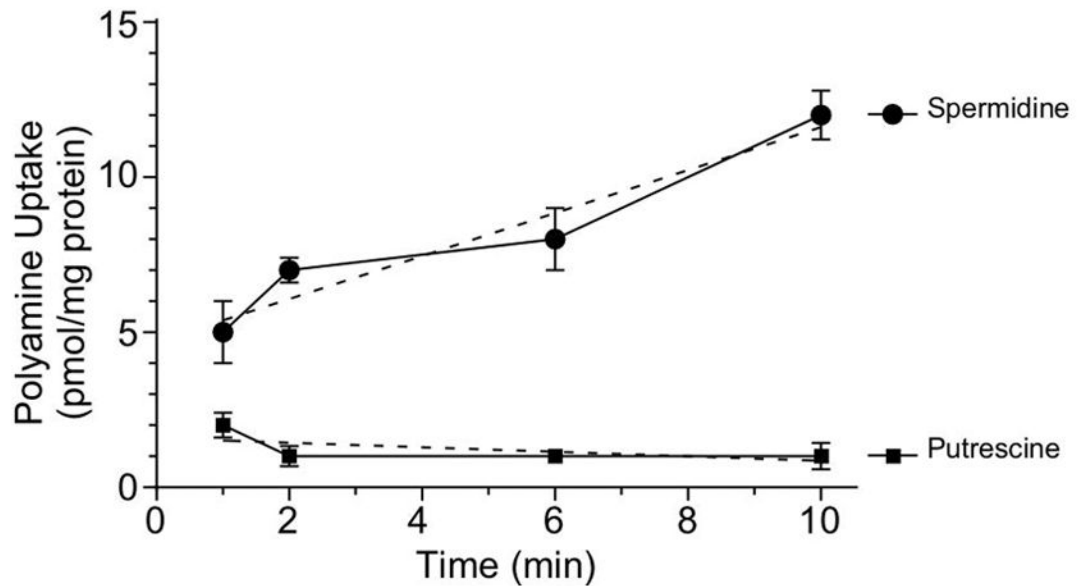

Fig. 2. Spermidine is the preferred polyamine transported by *B. burgdorferi*

Curve of uptake of radioactive spermidine and putrescine by *B. burgdorferi* B31-A3 strain was measured over a 10-min time course. Cells were grown to mid-log phase, washed and resuspended to a cell density of 6 x 10^8 cells/ml in HN buffer supplemented with 6 mM glucose. Tritium labelled polyamine (5μCi) was added to the cell suspensions and incubated at 34°C for the duration of the experiment. Polyamine amounts (pmol) in samples were determined against a calibration performed with authentic labeled substrate. The protein concentration was determined using a BCA Protein Assay with bovine serum albumin as a standard. Dotted lines represent the linear regression. Data represent means ± standard deviation of three independent experiments.

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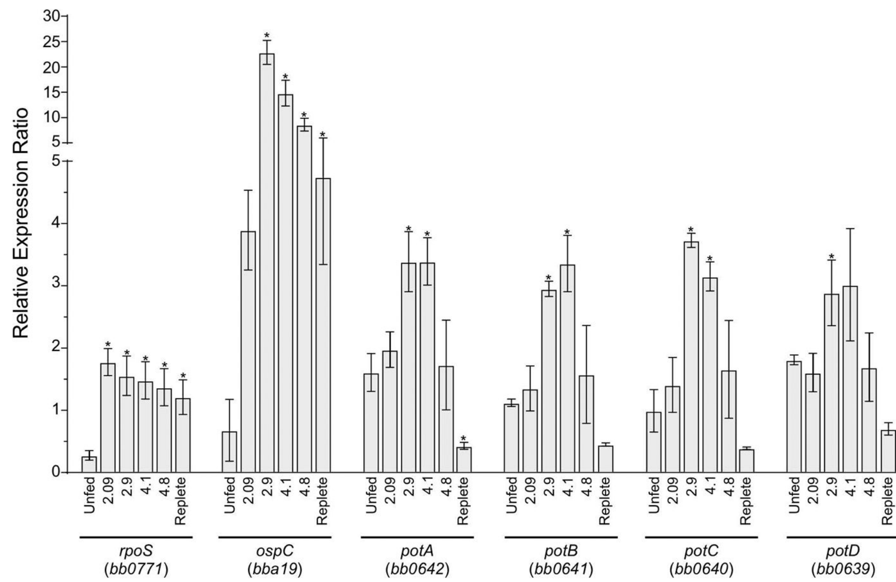

Fig. 3. Relative expression of *potABCD* operon genes during nymph feeding

Graph of the expression of *rpoS* and *ospC* (as markers for the induction of the Rrp2-RpoN-RpoS pathway), and the four genes of the *potABCD* operon were analyzed by RT-qPCR. RNA was extracted from 3 pools of 5 ticks and cDNA was generated as described in the methods section. Scutal index was used to standardize feeding progress (Bontemps-Gallo *et al.*, 2016, Falco *et al.*, 1996). The scutal index is indicated under each bar. Relative gene expression was calculated using levels expression of *enoS* as reference. Data represent means ± standard deviation of three independent pools for each condition.

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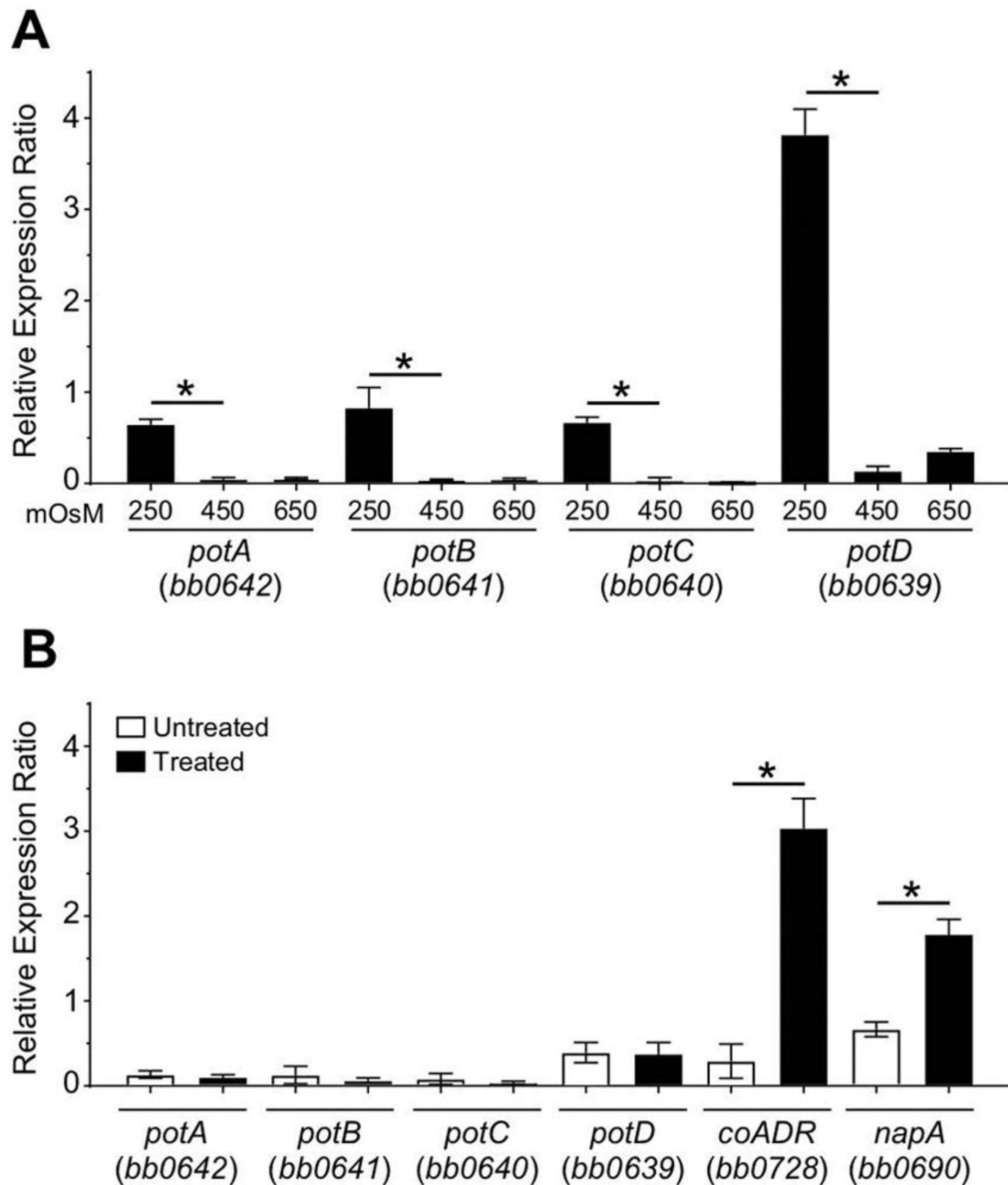

Fig. 4. Expression of *potABCD* operon under osmotic or oxidative stress

(A) Graph of the expression analyses of the *potABCD* operon of *B. burgdorferi* grown in BSK-II at 250, 450 and 650 mOsM. (B) Graph of the expression analyses of the *potABCD* operon of *B. burgdorferi* grown in BSK-II after a treatment with 5 mM *tert*-butyl hydroperoxide compared to an untreated culture. The expression of *coADR*, *napA* (as marker of the induction of the oxidative stress response), and the four genes of *potABCD* operon were analyzed by RT-qPCR. Relative gene expression was calculated using levels expression of *enoS* as reference. Data represent means ± standard deviation of three independent experiments.

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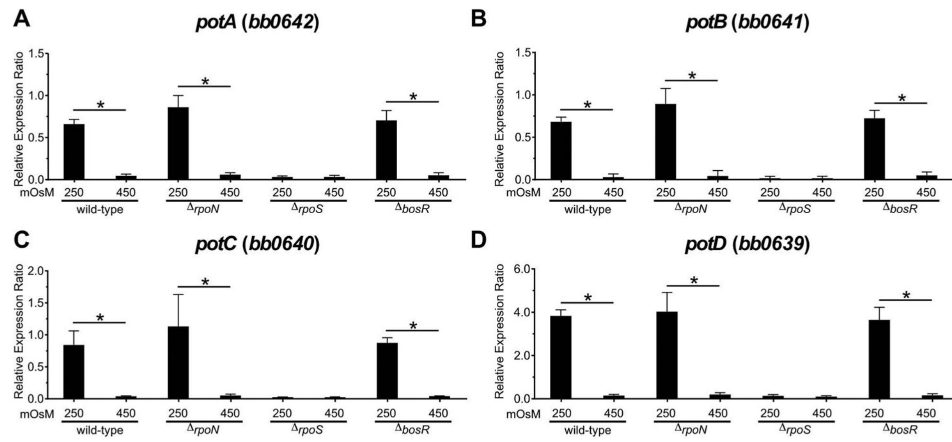

Fig. 5. Expression of *potABCD* operon in wild-type, *rpoN*, *rpoS* and *bosR* mutants

Graph of the expression analyses of the *potABCD* operon of *B. burgdorferi* grown in BSK-II at 250 and 450 mOsM. Relative gene expression was calculated using levels expression of *enoS* as reference. Data represent means ± standard deviation of three independent experiments.

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Table 1

Bacterial Strains and Plasmids used in this study

Strains	**Source**
B. burgdorferi	
B31-A3	(Elias *et al.*, 2002)
B31-A3 *rpoN*	(Fisher *et al.*, 2005)
B31-A3 *rpoS*	(Burtnick *et al.*, 2007)
B31-A3 *bosR* (clone K18)	(Katona, 2015)
B31-68-LS	(Chu *et al.*, 2016)
B31-68-LS–BBi-*potABCD*	This study
Escherichia coli	
Top10F′: *mcrA*, *(mrr-hsdRMS-mcrBC)*, Φ80*lacZ* M15, *lacX74*, *recA1*, *araD139*, *(ara- leu)7697*, *galU*, *galK*, *rpsL*, *endA1*, *nupG*	Invitrogen
Plasmids	
pUC57	Genscript
pBBi-*potABCD*	This Study

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Table 2

PCR primers used in this study.

PCR Primers[a]	Sequence	Efficiency
potAF	GCCATATGGATAATTGTATTATCCTAGAGATTAA	
potAR	CGGATCCTTATTCCTTATGCATAACATGAAT	
GTPF-*Eco*RI	ACTGCTGAATTCTCCACCTATTTTGGCTGCCA	
GTPR-*aat*II	ACTGCTGACGTCACATCCGGATGGGCCTAGTA	
QrpoSF	CTGGACAAAGAAATAGAGGGATCTG	1.838
QrpoSR	CAAGGGTAATTTCAGGGTTAAAAGAA	
QospCF	TGGTACTAAAACTAAAGGTGCTGAAGAA	1.951
QospCR	GCATCTCTTTAGCTGCTTTTGACA	
QpotAF	GCTGGTTGTAAGTTTGCTTGG	1.958
QpotAR	ATCTTCTGGGCGTATTACAAGG	
QpotBF	AGATCTTGGAGCAAGAATGTG	1.704
QpotBR	GTTTAGAGCCTCCTAGCAAATC	
QpotCF	CGGAAATCGCAGGAAGCATAG	1.891
QpotCR	ATCCCTGTCCAGTGGTGAAA	
QpotDF	GCACAAAGCGCTATGCTAAA	1.796
QpotDR	TGGAGCATCAATAGGAATTACA	
QnapAF	CCCTCAATGGAAAGCATTGTTTG	1.831
QnapAR	GCATCCATAAATGTTTCTCAAGATCAC	
QcoADRF	AGCTGGGAATCATACAGCATTTA	1.748
QcoADRR	GTCCTGTTCTTGCAGCTTCT	
QenoSF	GTGCACACTCTGACAACTCT	1.927
QenoSR	ACCTCTGCTGCCATTCTTATT	

[a]All primers were generated for this study.