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A Functional and Phylogenetic Comparison of Quorum Sensing Related Genes in *Brucella melitensis* 16M[§]

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A quorum-sensing (QS) system is involved in *Brucella melitensis* survival inside the host cell. Two transcriptional regulators identified in *B. melitensis*, BlxR and VjbR, regulate the expression of *virB*, an operon required for bacterial intracellular persistence. In this work, 628 genes affected by VjbR and 124 by BlxR were analyzed to gain insights into their functional and taxonomical distributions among the *Bacteria* and *Archaea* cellular domains. In this regard, the Cluster of Orthologous Groups (COG) genes and orthologous genes in 789 nonredundant bacterial and archaeal genomes were obtained and compared against a group of randomly selected genes. From these analyses, we found 71 coaffected genes between VjbR and BlxR. In the COG comparison, VjbR activated genes associated with intracellular trafficking, secretion and vesicular transport and defense mechanisms, while BlxR affected genes related to energy production and conversion (with an equal effect) and translation, ribosomal structure and biogenesis, posttranslational modifications and carbohydrate and amino acid metabolism (with a negative effect). When the taxonomical distribution of orthologous genes was evaluated, the VjbR- and BlxR-related genes presented more orthologous genes in *Crenarchaeota* (*Archaea*), *Firmicutes*, and *Tenericutes* and fewer genes in *Proteobacteria* than expected by chance. These findings suggest that QS system exert a fine-tuning modulation of gene expression, by which VjbR activates genes related to infection persistence and defense, while BlxR represses general bacterial metabolism for intracellular adaptations. Finally, these affected genes present a degree of presence among *Bacteria* and *Archaea* genomes that is different from that expected by chance.

Keywords: *Brucella melitensis*, quorum sensing, microarray data, taxonomical distribution, orthologous, genomics

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Introduction

Brucella melitensis is a Gram-negative intracellular pathogen that belongs to the alpha-2 proteobacteria group. As with many other intracellular facultative bacteria, such as *Mycobacterium* spp., and *Salmonella* spp., *Brucella* spp. replicate within macrophages preventing phagosome/lysosome fusion (Baldwin and Winter, 1994; Pizarro-Cerdá *et al*., 1994; Porte *et al*., 1999). In order to adapt to the environmental conditions needed for intracellular survival, *B. melitensis* requires a quorum-sensing (QS) communication system. In this bacterium, the system is composed of a small diffusible molecule, *N*-dodecanoyl-d,l-homoserine lactone (C12-HSL) produced by an autoinducer synthase (LuxI), yet unidentified, and a LuxR-type transcriptional regulator, with an N-terminal C12-HSL-binding domain and a C-terminal DNA-binding domain (Uzureau *et al*., 2007). The QS system coordinates bacterial behavior in a density-dependent manner, producing alterations in gene expression of secretion systems, virulence, biofilm formation, and cell division (Davies *et al*., 1998; Fuqua *et al*., 2001; Anand and Griffiths, 2003). In *B. melitensis*, the two LuxR transcriptional regulators involved in the QS system are VjbR (BMEII1116) and BlxR (BME I1758, also known as BabR) (Uzureau *et al*., 2007). VjbR activates the expression of the *virB* operon, which encodes the type IV secretion system (T4SS) that has been implicated in virulence by means of intracellular trafficking and survival (Wang *et al*., 2009). BlxR is also involved in the regulation of the *virB* operon, together with a large diversity of metabolic genes (Rambow-Larsen *et al*., 2008; Uzureau *et al*., 2010).

To date, two genome microarray and proteomic studies have been conducted in order to understand the differential gene expression of Δ*vjbR* and Δ*blxR* mutant strains with respect to the wild type (Uzureau *et al*., 2010; Weeks *et al*., 2010), and one minimicroarray study that included 289 genes was performed with the Δ*blxR* strain (Rambow-Larsen *et al*., 2008).

Therefore, in this work, an integrative analysis that considered protein and microarray data associated with the QS response and the main functions assigned to the QS-related LuxR regulators was conducted. This evaluation was based on the functional annotations according to the Cluster of Orthologous Groups (COG) classifications with respect to the whole-genome COG distribution. In addition, the taxonomical distribution of the differentially expressed genes associated with this response was evaluated with respect to that expected by random sampling.

Materials and Methods

The differentially-expressed genes for the QS system of *B. melitensis* were analyzed based on microarray and protein data. These data came from the following sources: studies performed by Uruzeau *et al.* (2010) with Δ*vjbR* and Δ*blxR* strains, by Weeks *et al.* (2010) with the Δ*vjbR* strain in the exponential and stationary growth phases and by Rambow-Larsen *et al.* (2008) with the Δ*blxR* strain. The direction of the effect produced by the studied mutant, i.e., activation or repression, was registered for each affected gene. Finally, in the study of Weeks *et al.* (2010), when a gene was affected in the exponential and stationary growth phases, we only registered the effect in the exponential growth phase (considering that the other study in Δ*vjbR* strain was also performed in this phase), it is important to mention that in this study 58 of the 524 genes were affected in both phases in an opposite direction.

Functional annotations

The functional annotations were retrieved from the DOE Joint Genome Institute Integrated Microbial Genomics system (http://img.gji.doe.gov). The COG classification, including that reported in the complete-genome studies (Uzureau *et al.*, 2010; Weeks *et al.*, 2010), was compared with the *B. melitensis* complete-genome COG distribution. The study of Rambow-Larsen *et al.* (2008) was not included in this analysis, considering that it could represent a bias (increasing or diminishing some associations), as it only included 289 genes.

Identification of orthologous genes

The orthologous genes encoding each protein in 789 non-redundant bacterial and archaeal genomes were obtained by using the bidirectional best hits (BDBHs) definition in the protein sequence, through depurated genomes at 95% identity, with an E-value of ≤1e-6, as described elsewhere (Janga and Moreno-Hagelsieb, 2004). The taxonomical distribution of the differentially-expressed genes in each strain (Δ*vjbR*, Δ*blxR*) obtained from the studies analyzed was compared with the taxonomical distribution of 628 randomly selected genes of the *B. melitensis* 16M genome, obtained via the RSA Tools database (http://rsat.ulb.ac.be). This number was selected because the largest set of genes investigated was 628 (for Δ*vjbR*).

Statistical analysis

The descriptive statistics generated in the study consisted of frequencies and percentages. For the comparison of the COG classification and the taxonomical distribution, chi-square and Fisher exact tests were performed. For the comparison of the number of orthologous genes between the Δ*vjbR* and Δ*blxR* strains, we used the Mann-Whitney U test (considering the nonparametric distribution of the data). The statistical analysis was carried out using the software SPSS v 10.0 and Epi-info. Statistical significance was set at $p \leq 0.05$.

Results

From all the studies included, a total of 628 genes were registered to be affected by VjbR and 124 by BlxR.

Overlapping genes among the studies

In order to evaluate the overlapping and specific genes associated with the experimental data analyzed, we show in Supplementary data Table S1 the genes affected in the different mutant strains; in Table 1 we show the number of overlapping genes among the different studies considered in our analysis. In this regard, a total of 71 genes were identified as coaffected by VjbR and BlxR (Rambow-Larsen *et al.*, 2008; Uzureau *et al.*, 2010; Weeks *et al.*, 2010). Twenty-two of these 71 genes were clearly regulated in opposite directions, i.e., activated in one strain and repressed in the other one; 26 genes were identified as regulated in the same direction (activated or repressed by both regulators); finally, 23 genes exhibited an uncertain regulatory effect, i.e., different regulatory effects were observed in different studies with the same mutant strain (Supplementary data Table S1). Among the genes clearly regulated in opposite directions, we identified those related to energy production and conversion (C), translation, ribosomal structure, and biogenesis (J) and amino acid transport and metabolism (E); these genes were mostly repressed by VjbR and activated by BlxR. In contrast, the

Table 1. Number of overlapping genes among the different studies included

Genes sets	Δ*blxR*, microarray, Rambow-Larsen *et al.* (2008)	Δ*blxR*, protein data, Uzureau *et al.* (2010)	Δ*blxR*, microarray data, Uzureau *et al.* (2010)	Δ*vjbR*, protein data, Uzureau *et al.* (2010)	Δ*vjbR*, microarray data, Uzureau *et al.* (2010)	Δ*vjbR*, microarray data, Weeks *et al.* (2010)
Δ*blxR*, microarray, Rambow-Larsen *et al.* (2008)	**41**					
Δ*blxR*, protein data, Uzureau *et al.* (2010)	0	**66**				
Δ*blxR*, microarray data, Uzureau *et al.* (2010)	7	22	**46**			
Δ*vjbR*, protein data, Uzureau *et al.* (2010)	2	2	9	**35**		
Δ*vjbR*, microarray data, Uzureau *et al.* (2010)	7	38	38	18	**103**	
Δ*vjbR*, microarray data, Weeks *et al.* (2010)	12	7	11	7	13	**524**

genes affected in the same direction were mainly classified in the COG groups for translation, ribosomal structure and biogenesis (J), carbohydrate transport and metabolism (G), and amino acid transport and metabolism (E); these genes are mostly repressed by VjbR and BlxR (Supplementary data Table S1). Finally, the genes with an uncertain regulatory effect were mainly associated with intracellular trafficking, secretion and vesicular transport (U) and amino acid transport and metabolism (E); these genes were activated and repressed in VjbR, respectively, with an unclear direction for effects in BlxR.

VirB genes, including BMEII0025, BMEII0026, BMEII 0027, BMEII0028, BMEII0029, BMEII0030, BMEII0032, and BMEII0033, were the genes most frequently represented in the studies that evaluated the Δ*vjbR* and Δ*blxR* mutants (Rambow-Larsen *et al*., 2008; Uzureau *et al*., 2010; Weeks *et al*., 2010). These genes were upregulated by VjbR in the studies of Uzureau *et al*. (2010) and Weeks *et al*. (2010) and downregulated by BlxR in the analysis by Uzureau *et al*. (2010); nevertheless, they were upregulated by BlxR in the study performed by Rambow-Larsen *et al*. (2008).

When we evaluated the overlapping genes among the studies for the same mutant, the highest proportion of overlapping genes was found for the genes in the microarray and proteomic results of Uzureau *et al*. (2010) for the Δ*vjbR* strain (18 genes shared) and for the Δ*blxR* strain (22 genes shared). The lowest proportion of coaffected genes was found in the Δ*vjbR* strain in the 2 different studies (Uzureau *et al*., 2010; Weeks *et al*., 2010), with only 16 shared genes, including 7 that belong to the *virB* genes (BMEII0025, BMEII0026, BMEII0027, BMEII0029, BMEII 0030, BMEII0032, and BMEII0033), BMEI1367 (superoxide dismutase), BMEI0747 (50S ribosomal protein L10), BMEI1435 (polysaccharide deacetylase), BMEI1305 (porin), BMEII0923 (spermidine/putrescine-binding periplasmic protein), BMEI 1900 (cytochrome O ubiquinol oxidase subunit I), BMEII0002 (ribosomal protein serine acetyltransferase), BMEI1267 (dimethyladenosine transferase), and BMEII0374 (alanine racemase). The strain Δ*blxR* also showed a low proportion of overlapping genes in the studies of Rambow-Larsen *et al*. (2008) and Uzureau *et al*. (2010) with 7 shared genes when compared with the microarray data and 0 when compared with the protein data results of the study of Uzurezu *et al*. (2010); all 7 genes are *virB* genes (Table 1).

Additionally, we observed that BlxR expression was activated by VjbR (Uzureau *et al*., 2010), and VjbR expression was affected by BlxR in an uncertain direction. A positive automodulation of VjbR was also observed. Finally, BMEI 0390 (VceA homologue) and BMEI0948 (VceC homologue), which are T4SS effector proteins, were activated and rep-

Table 2. Comparison of COG distribution in the genes affected in the different strains, with the *B. melitensis* genome COG distribution

COG	Δ*vjbR* frequency (%), *P* value		Δ*blxR* frequency (%), *P* value		*B. melitensis* frequency (%)
A	0 (0)	1	0 (0)	1	1 (0.03)
B	0.5 (0.08)	0.29	0 (0)	1	1 (0.03)
C	41.5 (6.61)	0.10	10 (11.11)	0.03*	172 (5.1)
D	6 (0.96)	0.69	0 (0)	1	27 (0.80)
E	62.8 (10)	0.76	17 (18.89)	<0.01*	326 (9.65)
F	14.5 (2.31)	0.75	1 (1.11)	1	74 (2.2)
G	39.8 (6.34)	0.36	10.5 (11.67)	<0.01*	187 (5.53)
H	28 (4.46)	0.54	5.5 (6.11)	0.17	133 (3.93)
I	10.67 (1.70)	0.03*	2.5 (2.78)	1	114 (3.37)
J	35.33 (5.63)	0.46	10 (11.11)	0.02*	165 (4.88)
K	33.4 (5.32)	0.71	3.5 (3.89)	0.63	190 (5.62)
L	20.33 (3.24)	0.98	2 (2.22)	1	107 (3.17)
M	32.5 (5.18)	0.78	4 (4.44)	1	169 (5)
N	6 (0.96)	0.92	0 (0)	1	31 (0.92)
NC	110 (17.52)	0.73	2 (2.22)	<0.001*	573 (16.95)
O	29.5 (4.70)	0.14	8.5 (9.44)	<0.01*	121 (3.58)
P	23.5 (3.74)	0.50	2 (2.22)	0.36	157 (4.65)
Q	7.67 (1.22)	0.30	0.5 (0.56)	1	63 (1.86)
R	41 (6.53)	0.02*	0.5 (0.56)	<0.01*	318 (9.41)
S	37.5 (5.97)	0.10	1 (1.11)	0.02*	268 (7.94)
T	15.5 (2.47)	0.80	2 (2.22)	1	92 (2.72)
U	17.5 (2.79)	0.01*	7.5 (8.33)	<0.001*	51 (1.50)
V	14 (2.23)	0.02*	0 (0)	1	38 (1.13)
W	0.5 (0.08)	0.28	0 (0)	1	1 (0.03)
Total	628 (100)		90 (100)		3379 (100)

P value obtained with chi-squared test and Fisher exact test, **P* value ≤ 0.05.Abbreviations; COG, cluster of orthologous group; A, RNA processing and modification; B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control, cell division, chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination, and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; NC, not in COG; O, posttranslational modifications, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking, secretion and vesicular transport; V, defense mechanisms; W, extracellular structures

ressed by VjbR, respectively (Weeks *et al.*, 2010).

COG classifications and comparisons

In order to have a better approximation of the functional annotations of the studied regulators and genes, all affected genes described in the whole genome studies were classified in terms of their COG classifications, after which their frequencies were evaluated (Table 2). In this regard, we found that for Δ*vjbR*, the COGs with the highest frequencies were amino acid transport and metabolism (E), general function prediction only (R), energy production and conversion (C) and genes not categorized in a COG (NC). It is important to emphasize that for those NC genes there is no functional information in the Gene Ontology (GO) annotations. In the comparison with the COG genome distribution, we found that the significantly overrepresented COGs with predominantly positive or activating effects were intracellular trafficking, secretion and vesicular transport (U), composed predominantly of the *virB* and flagellar genes, and defense mechanisms (V), mainly represented by antibiotic resistance and ABC transporter genes. In contrast, the significantly underrepresented COGs were those associated with lipid transport and metabolism (I) and with general function prediction (R). Finally, the COG of unknown function (S) was underrepresented, with a borderline p value of 0.10.

For Δ*blxR*, we found that the COGs with the highest frequencies were amino acid transport and metabolism (E), carbohydrate transport and metabolism (G), translation, ribosomal structure and biogenesis (J), energy production and conversion (C), and posttranslational modifications, protein turnover and chaperones (O). In the COG comparison, the COG associated with energy production and conversion was the only one with an equal effect, and the rest of the overrepresented COGs exhibited a repressing effect, including effects on translation, ribosomal structure and biogenesis (J), posttranslational modification, protein turnover and chaperones (O), and trafficking, secretion and vesicular transport (U) (mainly composed of *virB* genes), and also carbohydrate transport and metabolism (G) and amino

Table 3. Comparison of the phylogenetic distribution of the genes affected in the different mutated strains with random selected genes of *B. melitensis*

Genes sets	Random genes (N=628)		Δ*vjbR* genes (N=628)			Δ*blxR* genes (N=124)		
Microorganism group (N)	N	% (A. P.)	N	% (A. P.)	p value	N	% (A.P.)	p value
Crenarchaeota (25)	1404	1.17 (1.83)	1637	1.32 (2.01)	<0.001*	527	1.60 (2.32)	<0.0001*
Euryarchaeota (61)	4186	3.49 (2.08)	4333	3.50 (2.00)	0.89	1301	3.95 (2.14)	<0.0001*
Korarchaeota (1)	44	0.04 (1.90)	65	0.05 (2.58)	0.06	21	0.06 (2.95)	0.03*
Nanoarchaeota (1)	67	0.06 (1.56)	69	0.06 (2.04)	0.98	19	0.06 (1.69)	0.90
Thaumarchaeota (1)	13	0.01 (2.61)	16	0.01 (2.41)	0.63	4	0.01 (2.39)	0.77
Acidobacteria (3)	533	0.44 (2.66)	537	0.43 (2.47)	0.69	141	0.43 (2.28)	0.67
Actinobacteria (90)	13785	11.50 (3.04)	14489	11.71 (2.99)	0.10	3990	12.10 (2.98)	<0.01*
Aquificae (5)	569	0.47 (4.64)	623	0.50 (4.67)	0.30	182	0.55 (4.84)	0.07
Bacteroidetes (30)	3874	3.23 (3.30)	4035	3.26 (3.23)	0.68	1140	3.46 (3.39)	0.04*
Chlamydiae (8)	571	0.48 (4.28)	632	0.51 (4.34)	0.22	202	0.61 (4.96)	<0.01*
Chlorobi (11)	1510	1.26 (4.17)	1544	1.25 (3.93)	0.79	437	1.33 (3.92)	0.34
Chloroflexi (12)	1388	1.16 (3.57)	1494	1.21 (3.59)	0.25	438	1.33 (3.84)	0.01*
Cyanobacteria (31)	4329	3.61 (3.16)	4276	3.46 (2.85)	0.03*	1266	3.84 (3.04)	0.05*
Deferribacteres (3)	442	0.37 (4.20)	462	0.37 (4.05)	0.84	127	0.39 (3.98)	0.66
Deinococcus-Thermus (3)	449	0.37 (3.73)	486	0.39 (3.71)	0.46	130	0.39 (3.50)	0.60
Dictyoglomi (2)	210	0.18 (3.98)	237	0.19 (4.14)	0.34	69	0.21 (4.27)	0.19
Elusimicrobia (1)	100	0.08 (4.52)	100	0.08 (4.17)	0.82	33	0.10 (4.86)	0.36
Fibrobacteres (1)	120	0.10 (2.68)	112	0.09 (2.31)	0.44	31	0.09 (2.25)	0.75
Firmicutes (120)	15561	12.98 (3.20)	16786	13.57 (3.22)	<0.0001*	4645	14.09 (3.15)	<0.0001*
Fusobacteria (5)	589	0.49 (3.40)	640	0.52 (3.42)	0.36	152	0.46 (2.87)	0.48
Gemmatimonadetes (1)	181	0.15 (3.17)	187	0.15 (3.02)	0.99	47	0.14 (2.68)	0.72
Nitrospirae (1)	112	0.09 (3.90)	127	0.10 (4.08)	0.46	41	0.12 (4.66)	0.11
Planctomycetes (3)	446	0.37 (1.99)	458	0.37 (1.87)	0.93	107	0.32 (1.56)	0.20
Proteobacteria (316)	65265	54.46 (4.51)	65749	53.15 (4.24)	<0.0001*	16694	50.63 (4.01)	<0.0001*
Spirochaetes (15)	1455	1.21 (3.40)	1604	1.30 (3.59)	0.06	424	1.29 (3.39)	0.29
Synergistetes (2)	227	0.19 (4.36)	242	0.20 (4.27)	0.72	66	0.20 (4.12)	0.69
Tenericutes (25)	963	0.80 (3.75)	1166	0.94 (4.19)	<0.001*	306	0.93 (3.97)	0.03*
Thermobaculum (1)	130	0.11 (3.17)	152	0.12 (3.42)	0.29	37	0.11 (2.95)	0.85
Thermotogae (7)	772	0.64 (3.98)	860	0.70 (4.07)	0.12	238	0.72 (3.99)	0.12
Verrucomicrobia (4)	555	0.46 (3.28)	581	0.47 (3.12)	0.81	157	0.48 (3.06)	0.75
Total (789)	119850	100 (100)	123699	100 (100)		32972	100 (100)	

N, Number; A.P., adjusted percentage; this is the corresponding percentage of the number of orthologous per each 100 genes of each organisms and divided by the number of organisms in each group, *P* value obtained with chi-squared test and Fisher exact test, * *P* value ≤ 0.05. In order to understand the differences in the percentages between the groups, the percentage and not the adjusted percentage must be taken into account, the adjusted percentage should only be considered in descriptive results, i.e, to know the distribution of orthologous in the organisms groups in each set of genes.

acid transport and metabolism (E). The underrepresented COGs were those associated with unknown function (S), general function prediction only (R) and genes not classified in COG (NC) (Table 2).

Taxonomical distribution of the affected VjbR and BlxR genes

In order to evaluate the taxonomical distribution of the QS genes identified in *B. melitensis*, the orthologous genes associated with the affected genes in each mutant (Δ*vjbR*, Δ*blxR*) were evaluated in comparison to the randomly selected genes (Table 3). Therefore, with respect to the number of orthologous genes, the strain Δ*vjbR* exhibited a median (range) of 116.5 (0–1051) genes, Δ*blxR* had 198 (0–791) genes and random genes represented 102.5 (0–796) orthologous genes. From these data, a significantly higher number of orthologous genes among those affected by Δ*blxR* (p= 0.002) and no clear differences in the genes affected by Δ*vjbR* (p=0.79) were observed compared with randomly selected genes.

After adjustments for genome size and number of organisms analyzed per group in the three data sets, the highest proportion of orthologous genes in the set of random genes was found in the *Aquificae*, *Elusimicrobia*, *Proteobacteria*, *Synergistetes*, and *Chlamydiae* divisions, whereas the lowest proportion of orthologous genes was found in the archaeal divisions *Nanoarchaeota, Crenarchaeota*, *Euryarchaeota* and *Planctomycetes,* the other 2 sets of genes presented a similar distribution with slight variations in the order of frequencies (Table 3).

In order to evaluate the significance of the orthologous distributions among all taxonomical divisions, we compared the data against the distribution of random genes. In this regard, the genes affected in the mutant strains presented a slight but significant increase of orthologous genes in *Crenarchaeota*, *Firmicutes* and *Tenericutes*, and a significant decrease in *Proteobacteria*. In addition, the Δ*blxR* strain also exhibited a significant increase of orthologous genes in *Euryarchaeota, Korarchaeota, Actinobacteria, Bacteroidetes, Chlamydiae*, *Chloroflexi*, and *Cyanobacteria*. Finally, Δ*vjbR* showed a slight but significant decrease of orthologous genes in *Cyanobacteria*.

Discussion

To our knowledge, this is the first integrative analysis that has included different studies of expression data involving a specific response or regulator in *B. melitensis* 16M. In this respect, among the overlapping genes observed in the different studies, we noticed a relatively high number of coaffected genes between the transcriptional regulators VjbR and BlxR, with a similar number of genes in the same and the opposite directions. The high number of coaffected genes with an uncertain direction can be explained by the used methodology including the growth phase and the undetected presence of C12-HSL produced by the bacterium.

Considering the 681 genes not coaffected between VjbR and BlxR and the diversity in the functional categories, we suggest that both regulators play a complementary as well as concerted function in order to achieve intracellular adaptation of *B. melitensis*. These results are consistent with the similar diminishment in the intracellular growth rate observed in both Δ*vjbR* and Δ*blxR* mutant strains (Rambow-Larsen *et al*., 2008), although Δ*vjbR* exibits a higher attenuation.

When the affected genes in the same mutant strain were evaluated, the highest number of shared genes was found in the protein and microarray data of the same study (Uzureau *et al*., 2010); however, the number of shared genes with other studies that evaluated the same mutant strain was very low, suggesting that many protein and microarray data obtained during different growth phases, as well as experimental data, are required in order to have a better approximation of the genes controlled by specific transcriptional regulators. Another important observation is that VjbR can be considered a global regulator in *B. melitensis* based on the high number of affected genes (628), representing 18% of the bacterial genome. This number is even higher in the presence of C12-HSL (Uzureau *et al*., 2010).

On the other hand, *virB* genes were the most consistently affected in the Δ*vjbR* and Δ*blxR* strains, which suggests that these genes are the main target genes of both transcriptional regulators. Although the direction of the regulation is still controversial in the Δ*blxR* strain, it is probable that *virB* genes are positively regulated by VjbR and negatively by BlxR, considering the lower virulence of the Δ*vjbR* mutant strain in comparison with the Δ*blxR* mutant strain (Rambow-Larsen *et al*., 2008). Alternatively, the *virB* operon can be upregulated or downregulated by BlxR, depending on the time postinfection and the concentration of C12-HSL.

Also notable is the mutual regulation of both LuxR transcriptional regulators. In this regard, positive autoregulation of both transcriptional regulators has been experimentally demonstrated (Rambow-Larsen *et al*., 2008) and confirmed in this case for VjbR; however, it has been shown that VjbR represses its own expression in the presence of C12-HSL (Uzureau *et al*., 2007). The T4SS effector protein VceC homologue was unexpectedly found to be downregulated by VjbR (Weeks *et al*., 2010), contrary to previous findings with the bacterium *Brucella abortus* (de Jong *et al*., 2008). Nevertheless, neither VceA nor VceC has been found in attenuated mutants.

It has been demonstrated that VjbR activates the expression of *omp25*, *omp31*, and *omp36* in the presence of C12-HSL (Uzureau *et al*., 2007). This suggests that other genes, probably related to virulence, could be also expressed by VjbR or even BlxR in the presence of C12-HSL; nevertheless, Weeks *et al.* (2010) demonstrated a general repressive effect of C12-HSL for VjbR, which has been mainly confirmed for *virB* and flagellar genes (Taminiau *et al*., 2002; Delrue *et al*., 2005; Uzureau *et al*., 2010). In summary, it is possible that a fine modulation of gene expression is achieved by C12-HSL and both LuxR transcriptional regulators.

In our comparison of COG classifications for the Δ*vjbR* and Δ*blxR* strains with respect to the *B. melitensis* genome distribution, we found that the main represented COG genes with an activating effect in the Δ*vjbR* strain were related to defense and intracellular transport. This was expected, considering the important function performed by VjbR in these

activities and the high attenuation observed in the Δ*vjbR* strain (Rambow-Larsen *et al*., 2008). VjbR also performs other important functions, as indicated by the high frequencies of COGs E, R, C, G, and NC. There was an underrepresentation of COGs related to lipid transport and metabolism, general function prediction only, and unknown function. These changes suggest a lower importance of lipid metabolism in *B. melitensis* intracellular adaptation, and also that specific and known functions are promoted by VjbR.

In the case of BlxR, it is noteworthy that for most overrepresented COGs, the direction of the regulation is mainly negative, as these COGs are related to translation, posttranslational modifications, carbohydrate transport and metabolism, and amino acid transport and metabolism; therefore, it is possible that BlxR is responsible for general downregulation of bacterial metabolism, in order to focus the energy and the transcriptional machinery for the functions needed for intracellular adaptations and, additionally, because the intracellular environment is characterized by oxidative stress and starvation conditions. The COG U, related to *virB*, is repressed by BlxR, according to the results of the whole-genome study (Uzureau *et al*., 2010), which indicates an opposite regulation of these genes between VjbR and BlxR and may be related to the moment when these transcriptional regulators are expressed during cellular infection, as VjbR is probably expressed first, with subsequent induction of *virB*, followed by BlxR, which counteracts this effect when it is no longer needed. Nevertheless, the effect of BlxR in *virB* transcription remains to be determined, considering the controversial results of BlxR studies (Rambow-Larsen *et al*., 2008; Uzureau *et al*., 2010). As with Δ*vjbR*, the COGs associated with unknown function, general function prediction only and additionally the genes not categorized in COG (NC) were significantly underrepresented, indicating that BlxR regulates genes with specific and known functions. In this regard, it is important to mention that these results were not previously reported by Uzureau *et al.* (2010), Rambow-Larsen *et al.* (2008), and Weeks *et al.* (2010).

Concerning the taxonomical distribution analysis, we observed that genes affected by BlxR showed a significantly higher number of orthologous genes than in the group of randomly selected genes, which suggests they have an increased presence in different bacterial and archaeal groups. This is in part explained by the important number of genes coding for metabolism proteins, which can be observed with a higher frequency in different organisms in comparison with randomly selected genes.

With the adjusted taxonomical distribution for the number of genes per organism and the number of organisms per group, we found in the 3 sets of genes a high frequency in bacterial groups other than *Proteobacteria*, including *Aquificae*, *Chlamydiae*, and *Elusimicrobia*; this could be explained by events of massive horizontal transfer between the *Brucellaceae* family and these groups, as proposed in discussions on deviations from the genome-wide molecular clock (Novichkov *et al.,* 2004); although it is also important to consider that these groups are composed of a small number of organisms, which diminishes their representativeness and could affect the true distribution.

In the comparison of the taxonomical distribution, we observed that LuxR-regulated genes presented low but significant differences with randomly selected genes. These differences suggest an increased presence of LuxR-associated genes in *Crenarchaeota, Firmicutes*, and *Tenericutes* and a diminished presence in *Proteobacteria* than would be expected by chance. Among the groups with the highest significance for differences in Δ*blxR* genes are the *Chlamydiae* and *Actinobacteria* groups, which include intracellular pathogens, indicating that these genes could be needed for intracellular adaptations.

Conclusions

The quorum-sensing system in *B. melitensis* evolved in order to achieve the intracellular adaptations needed for bacterial persistence within host cells. To date, few studies have been conducted in order to determine the genes regulated by this system, and there has been low agreement between the studies performed. This highlights the need for additional experimental evidence that accurately describes the genes regulated by the QS system in this bacterium. Nevertheless, the information already available in the literature indicates that the transcriptional regulators VjbR and BlxR, which are involved in QS in *B. melitensis*, exert a fine-tuning modulation of gene expression in order to survive inside the host cell. Among these genes, the *virB* operon plays a fundamental role, as its expression is regulated by both transcriptional regulators and its correct expression is crucial for bacterial persistence within the cell. Our taxonomical distribution analysis of QS-related genes indicates small but significant differences compared to randomly selected genes of *B. melitensis*; these differences are related to the presence of these genes in different organisms and are probably related to their functions. Along this line of thought, it is remarkable that QS-related genes present significant increases of orthologous genes in different groups, including the *Firmicutes*, *Crenarchaeota*, and *Tenericutes*, and a significant decrease in *Proteobacteria*. These findings indicate a higher relevance of QS-related genes in different bacterial and archaeal groups than would be expected among randomly selected genes, some of them associated with an intracellular life, as well as a diminished presence in *Proteobacteria*. Further experimental and integrative analysis will increase the knowledge of QS-genes functions and phylogenetic origins in *B. melitensis.*

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