

# Real-Time Quantitative Reverse Transcription-PCR Analysis of Expression Stability of *Actinobacillus pleuropneumoniae* Housekeeping Genes during In Vitro Growth under Iron-Depleted Conditions

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The aims of the present investigation were to develop and test a sensitive and reproducible method for the study of gene expression in the porcine lung pathogen *Actinobacillus pleuropneumoniae* by real-time quantitative reverse transcription (RT)-PCR and to evaluate a number of suitable internal controls, as such controls have not been defined yet for this bacterium. Bacterial gene expression was studied during in vitro exponential and early stationary growth in medium with and without sufficient iron, respectively. First, the stability of expression of five genes, the *glyA*, *tpiA*, *pykA*, *recF*, and *rhoAP* genes involved in basic housekeeping, was evaluated on the basis of the mean pairwise variation. All the housekeeping genes included were stably expressed under the conditions investigated and consequently were included in the normalization procedure. Next, the geometric mean of the internal control genes was used to correct five genes of interest. These genes were three genes involved in iron acquisition (*thpA*, *exbB*, and *fhuD*), the heat shock protein gene *groEL*, and a putative quorum-sensing gene (*luxS*). The level of *thpA*, *exbB*, and *fhuD* expression in *A. pleuropneumoniae* showed significant up-regulation under iron-restricted conditions compared to bacteria grown in medium with sufficient iron. The observed expression patterns of the genes of interest were consistent with previous observations. This study therefore lends further support to the use of real-time quantitative RT-PCR, with the *glyA*, *tpiA*, *pykA*, *recF*, and *rhoAP* genes as internal controls, for future similar gene expression studies in *A. pleuropneumoniae*.

An infected host represents a complex and dynamic environment to which the invading pathogen constantly needs to respond. Understanding how bacteria survive and multiply in this hostile environment and which genes are involved in this process is a prerequisite for the rational design of novel approaches for the treatment and prevention of disease.

Porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is one of the important bacterial diseases of the respiratory tract of the pig; it is distributed worldwide and results in severe losses in the pig-rearing industry (8, 23). The pathogenesis of this disease, which is complex and still not completely understood, was chosen as a model for the study of the interrelated host and pathogen gene expression patterns during an infection. In this context, real-time quantitative reverse transcription-PCR (qRT-PCR) is the method of choice, both as an independent method for expression analysis and as a method to confirm results of differential gene regulation obtained by microarray analysis.

The accuracy of a relative comparison by qRT-PCR depends largely on the normalization of raw values by means of stably expressed internal reference genes, which are used to eliminate sample-to-sample variation of RNA isolation and reverse transcription. However, the “housekeeping” metabolism of prokaryotes has been reported to be highly variable depending on the experimental procedure (26). Identification of candidate

genes that are at least minimally regulated under the conditions investigated and preferably the inclusion of more than one reference gene in the analysis are therefore important for the accuracy of the qRT-PCR test (20, 22, 27).

In order to validate internal reference genes for a qRT-PCR assay and to test the reproducibility of the method, the expression of five housekeeping genes and five genes of interest in response to low-iron conditions was examined during in vitro exponential and early stationary growth in the porcine lung pathogen *A. pleuropneumoniae*.

Iron is essential for bacterial growth and replication, and the ability to acquire this micronutrient from the very limited free iron sources in the mammalian host is an important component of bacterial virulence (11). The five genes of interest that were analyzed by qRT-PCR included transferrin binding protein gene A (*thpA*) (5, 7) and inner membrane protein complex gene B (*exbB*), both encoding proteins involved in iron binding and transport (25) and considered to be important for *A. pleuropneumoniae* virulence (1, 2). Furthermore, previous studies of *A. pleuropneumoniae* expression by polyacrylamide gel electrophoresis and immunoblotting have demonstrated that proteins encoded by *thpA* and *exbB* are expressed only under iron-limiting growth conditions (5, 25). Another *A. pleuropneumoniae* iron transport system, via ferrichrome, was represented in the analysis by a gene coding for ferric hydroxamate uptake protein (*fhuD*), which is part of the *fhuCDBA* operon (14). Previous results indicated that the regulation of *fhuA* appears to be independent of the iron supply (15).

Also included in the analysis were the heat shock protein-

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TABLE 1. Primers used in real-time qRT-PCR study

Target	Forward primer (5'-3')	Reverse primer (5'-3')
<i>recF</i>	TATGCCGAGATTCTTGCTCA	AATTTAAGCTGCCACGAGA
<i>glyA</i>	CAAGCGAATGCAGCTGTTTA	CTGTGATGCCGTAGAGGACA
<i>rhoAP</i>	AATACCGTGCAGCTGTTC	ACTAATGCCGTGCGATAAT
<i>tpi</i>	CTACGAACCGATTTGGGCTA	CCGCCGTATTGGATAATCAC
<i>pykA</i>	GTACGGATGCGGTAATGCTT	ACCTTCCATACGGGTACGAG
<i>groEL</i>	CGCAAATCAAAGCACGAGTA	ACCGGCTAATTTGCAACAC
<i>luxS</i>	CGTGTGCAAAAACGATGAC	GCATAAAGCCGCAAAATAAG
<i>tbpA</i>	ATTACGCTGCTCGCTATGT	GGCAAAACCGACTTGTTTCAT
<i>exbB</i>	CCGTTTCATTGGGTTATTTGG	AAATAGTCCCATTGCCGTTG
<i>fluD</i>	TTTTATACGGAAGGCGATGC	ACAAGCGGTGCAATTTTCAT

encoding gene *groEL* (28) and a putative quorum-sensing protein-encoding gene (*luxS*) responsible for the production of autoinducer 2, which has been reported to be important for aerobic growth of *Actinobacillus actinomycetemcomitans* under iron limitation conditions (6).

The putative reference genes included in the analysis were genes encoding recombinase F (*recF*) (12), glycine/serine hydroxymethyl transferase A (*glyA*), rho factor (*rhoAP*) (17), triosephosphate isomerase A (*tpiA*), and pyruvate kinase A (*pykA*) (13). The expression stabilities of these genes were measured as proposed by Vandesompele et al. (27), and the resulting normalization factor was used to correct the raw values for the five genes of interest.

#### MATERIALS AND METHODS

**Bacterial strain and growth conditions.** Type strain L20 (serotype 5b) of *A. pleuropneumoniae* was used in this investigation. For the iron depletion study, an overnight culture was diluted 1:10 in brain heart infusion supplemented with 0.03% NAD (Sigma-Aldrich, Copenhagen, Denmark) and incubated until the optical density at 660 nm was 0.7. The culture was split into two 50-ml portions. Iron-depleted conditions were established in one of the flasks by addition of the iron chelator 2,2'-dipyridyl (Sigma-Aldrich) (300  $\mu$ M). The cultures were grown in a shaking incubator at 37°C. Samples (0.5 ml) were removed from both cultures (control and sample) 0, 10, 20, 30, 40, and 60 min after addition of the iron chelator, and these samples represented the exponential and early stationary growth phases (the optical density changed from 0.7 at time zero to 1.3 after 1 h of growth). Samples were harvested by centrifugation, immediately resuspended in RNAlater RNA stabilization reagent as described by the manufacturer (Ambion, Cambridgeshire, United Kingdom), and stored at 4°C until they were used; however, they were not stored for more than 1 month.

**RNA isolation and reverse transcription.** Total RNA was isolated from 0.5-ml portions of bacterial samples by using an RNeasy mini kit (QIAGEN, Hilden, Germany) as described by the manufacturer. Genomic DNA was eliminated by RNase-free DNase I treatment during the isolation procedure. Reverse transcription was performed at 42°C for 120 min using random hexamer primers and 100 U of Moloney murine leukemia virus reverse transcriptase (Ambion). For each time extraction was performed in duplicate. Furthermore, serial dilutions containing from approximately 1.8  $\mu$ g to 0.6 ng of total RNA extracted from a control sample at an optical density at 660 nm of 0.58 were reverse transcribed.

**Quantitative real-time PCR.** Gene quantification was performed with a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Primers were designed from GenBank (<http://www.ncbi.nlm.nih.gov/>) sequences with the aid of DNASIS MAX V1.0 (Hitachi Software Engineering Company, Ltd., San Francisco, CA). The sequences of the primers are shown in Table 1. Each PCR was performed in a 20- $\mu$ l reaction mixture containing SYBR Green I (stock diluted 1:20,000; BMA, Rockland, ME) and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The final concentrations of primers and Mg<sup>2+</sup> were 0.5  $\mu$ M and 4 mM, respectively. The thermal cycling conditions were as follows: 6 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Data collection was performed during each extension phase. Positive controls (DNA), a negative control (distilled water), and RT-negative controls (total RNA sample) were included in each run. For each of the RNA extractions, measurements of gene expression were obtained in triplicate, and the mean of these values was used for further analysis.

TABLE 2. geNorm stability results<sup>a</sup>

Gene	geNorm stability measure ( <i>M</i> )	
	Run 1	Run 2
<i>rhoAP</i>	0.554 (3)	0.868 (4)
<i>pykA</i>	0.615 (4)	0.346 (1)
<i>tpiA</i>	0.401 (2)	0.821 (3)
<i>recF</i>	0.170 (1)	0.508 (2)
<i>glyA</i>	0.170 (1)	0.346 (1)

<sup>a</sup> geNorm stability values were determined for the five housekeeping genes included in this study. Genes with the lowest *M* values have the most stable expression values. *M* is the standard deviation of the logarithmically transformed expression ratios with all other control genes. The ranks of the genes are indicated in parentheses.

**Primer specificity.** A Rotor-Gene 3000 melting curve analysis was performed, which for all primer sets resulted in single product-specific melting curves. Gel electrophoresis and a melting curve analysis of PCR products showed that very little or no primer-dimers were generated during the runs.

**Relative quantification.** The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real-time PCR efficiency of the individual transcripts (18), as follows:

$$\text{Ratio} = (E_{\text{target}})^{\Delta C_{\text{target}}(\text{control} - \text{sample})} / (E_{\text{ref}})^{\Delta C_{\text{ref}}(\text{control} - \text{sample})}$$

The relative expression ratio of a target gene was computed based on its real-time PCR efficiencies (*E*) and the crossing point difference ( $\Delta C_{\text{t}}$ ) for an unknown sample versus a control. For each gene, cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies ( $E = 10^{(-1/\text{slope})}$ ). The geometric mean of the five internal reference genes was used to correct the raw values for the genes of interest.

**Statistical analysis.** The stability of the mRNA expression of the reference genes was evaluated by using the freely distributed MS Excel application geNorm (27). This approach is based on the assumption that the expression ratio of two ideal control genes is the same for all samples, regardless of the experimental conditions, and that increasing variation in this ratio corresponds to decreasing expression stability. For each reference gene, the program generates the expression stability measure *M*, which represents the average pairwise variation for a particular reference gene with all other reference genes tested (27). This measure is used to select the most stable genes in a particular experimental situation. In general, the lower the *M* value, the more stable the housekeeping genes expressed and the more precise the quantification of the experiment. Stepwise exclusion of genes with the highest *M* values allows ranking of the genes tested according to their expression stabilities. A reliable normalization factor, averaging individual housekeeping gene-specific differences, is obtained by using the geometric mean of multiple relatively stable control genes (27). The geometric mean of the best-scoring reference genes was used as a normalization factor in the analysis.

The total expression ratio of the five genes of interest over 1 h was tested for significance by a randomization test implemented in the relative expression software tool (REST) ([www.gene-quantification.info](http://www.gene-quantification.info/)), which is an Excel-based application for groupwise comparison and statistical analysis of relative expression results in qRT-PCR (19).

## RESULTS

**Validation of reference genes.** To compare the expression levels of each reference gene, bacterial samples were taken at six different times, 0, 10, 20, 30, 40, and 60 min after addition of the iron chelator to the media. qRT-PCR analysis was performed twice for different RNA extracts from the same growth experiment. The geNorm program was used to evaluate the expression stability of each reference gene at the different times during growth. The results of the two qRT-PCR runs are ranked and shown in Table 2. Genes with the lowest *M* values have the most stable expression values. The stability of the genes as assessed by geNorm analysis was between 0.17 and 0.55 in the first run and between 0.35 and 0.87 in the second

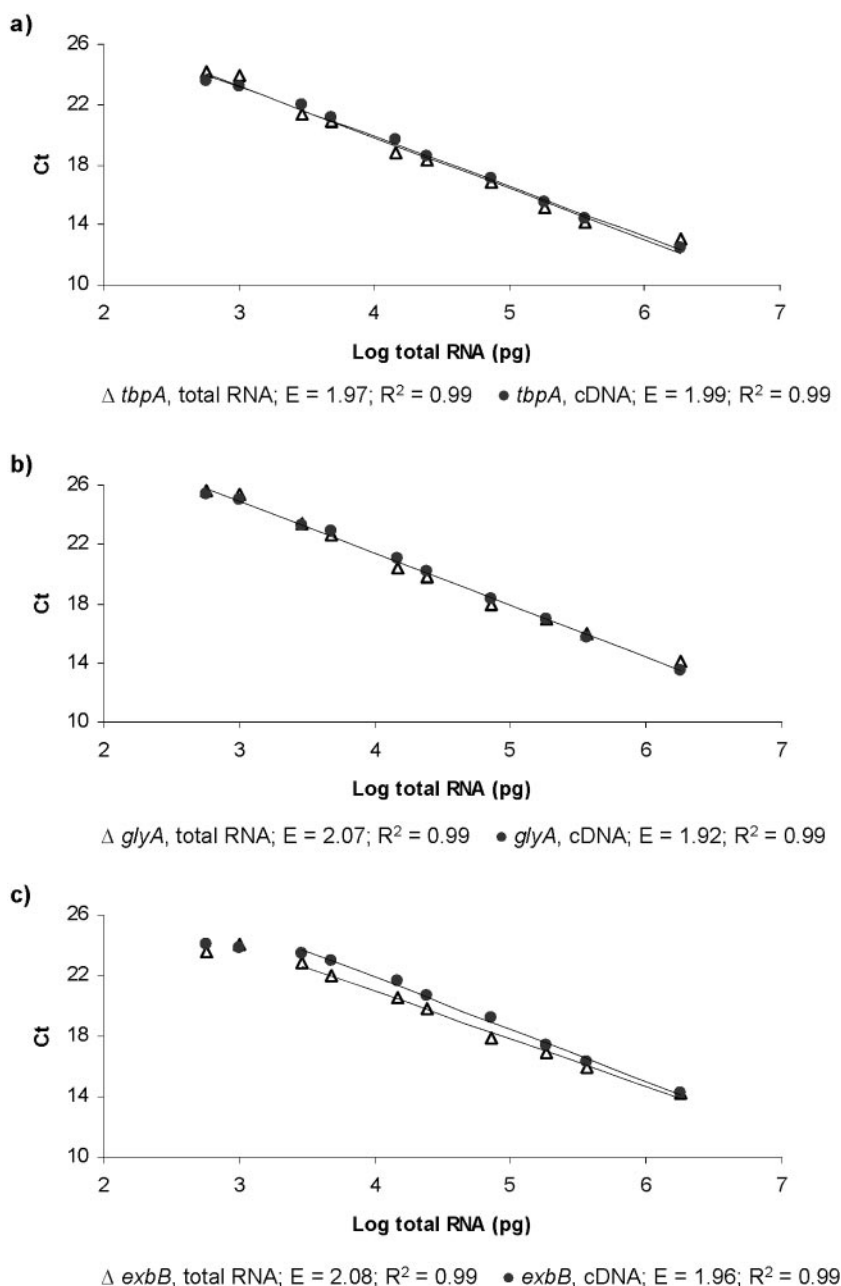


FIG. 1. Standard curves for total RNA and cDNA of *tbpA* (a), *glyA* (b), and *exbB* (c) generated by plotting the log of the reverse-transcribed total RNA concentration versus the threshold cycle ( $C_t$ ). Linear regression analysis was used to determine the slope which corresponded to the amplification efficiency,  $E = 10^{(-1/\text{slope})}$  (18).

run; *recF* and *glyA* were the most stably expressed genes in the first run, and *pykA* and *glyA* were the most stably expressed genes in the second run.

**Test reproducibility for RNA isolation and qRT-PCR.** Test precision (intra-assay variation) and test variability (interassay variation) were evaluated by the  $C_t$  variation from the mean  $C_t$  value. RNA isolation was performed with samples containing approximately  $10^7$  CFU. Repeated RNA extractions of the same bacterial sample resulted in an interassay variability between 0.04 and 13.9% and an average variability of 4.10%. Normalization by using the geometric mean of the five refer-

ence genes reduced the standard deviation of the total inter-assay variations for all genes of interest between one-third and one-fifth. The intra-assay precision was determined for three repeats within one Rotor-Gene run and was found to be between 0 and 6.3%, with an average variability of 1.14%.

**Validation of reverse transcriptase inhibition and dynamic range.** For three of the genes, *tbpA*, *exbB*, and *glyA*, standard curve analyses were performed for both cDNA and total RNA dilution series. The standard curves were all highly linear ( $R^2 > 0.98$ ) over a wide dilution range, with slopes greater than  $-3.1$  and less than  $-3.6$ , indicating a PCR efficiency of 1.92 to 2.08

TABLE 3. Data from a pairwise fixed reallocation randomization test (2,000 randomizations) of the relative expression of the five genes of interest, before and after normalization

Run	Parameter	<i>groEL</i>	<i>luxS</i>	<i>tbpA</i>	<i>exbB</i>	<i>fhuD</i>
1	Expression ratio	0.84	0.87	3.67	8.30	1.82
	<i>P</i> value	0.501	0.223	0.016	0.002	0.016
	Expression ratios (not normalized)	0.82	0.85	3.57	8.10	1.78
	<i>P</i> value (not normalized)	0.560	0.314	0.009	0.002	0.004
2	Expression ratio	1.04	0.94	4.00	8.11	1.94
	<i>P</i> value	0.911	0.624	0.014	0.006	0.047
	Expression ratio (not normalized)	0.89	0.80	3.40	6.94	1.66
	<i>P</i> value (not normalized)	0.740	0.280	0.008	0.006	0.041

Figure 1 shows the  $C_t$  values for the two standard curves for the *tbpA*, *exbB*, and *glyA* genes. The input amount for the real-time PCR ranged between 1.8  $\mu$ g and 0.6 ng of reverse-transcribed total RNA per reaction mixture. No significant difference in the efficiency levels between the cDNA and total RNA dilution series were observed for any of the genes. All the dilutions used are within the dynamic range of the *glyA* and *tbpA* genes. For the *exbB* gene, the dynamic range is 1.8  $\mu$ g to 3.0 ng of reverse-transcribed total RNA.

**Expression of genes of interest after iron deprivation.** The total expression of the five genes of interest during 1 h after the addition of the iron chelator 2,2'-dipyridyl was analyzed by using the relative expression software tool (REST) (19), and the geometric means of the  $C_t$  values for each time for the five housekeeping genes were used to normalize the data. The results for the two runs of the experiment based on separate RNA extractions are shown in Table 3. In both runs, a significant increase ( $P < 0.05$ ) in the expression of the *tbpA*, *exbB*, and *fhuD* genes was observed under iron-deprived conditions compared to growth in iron-replete media, and the mean levels of *fhuD*, *tbpA*, and *exbB* were up-regulated approximately two-, four-, and eightfold, respectively. The remaining two genes, *luxS* and *groEL*, showed no significant regulation (Table 3). For none of the genes did normalization alter the relative expression ratios significantly.

Figure 2 shows the mean normalized expression ratios of the three significantly up-regulated genes 0, 10, 20, 30, 40, and 60 min after addition of the iron chelator. The expression of the *tbpA* and *exbB* genes increased rapidly during the first 20 min, after which *exbB* expression remained relatively stable at the same level, while the expression of *tbpA* seemed to peak after 30 min of growth under iron-deprived conditions, after which it slowly started to level off. Besides a small drop after 10 min of growth under iron deprivation conditions, the level of expression of *fhuD* remained slightly up-regulated during the whole observation period.

## DISCUSSION

The aims of the present investigation were to develop and test a sensitive and reproducible method for expression analysis of *A. pleuropneumoniae* and, as it has been shown that housekeeping gene expression can vary considerably (3, 24, 26), to validate that the reference genes used were minimally regulated in response to experimental treatment. In this context, qRT-PCR was used to monitor the expression of 10 selected genes from *A. pleuropneumoniae* cultivated under iron-restricted conditions. Previous studies of *A. pleuropneumoniae* expression by other methods (5, 25) have shown that the *exbB* and *tbpA* genes are expressed only under iron-limiting conditions. Consequently, these two genes were chosen with the aim of verifying previous results. Two other genes, *fhuD* and *luxS*, which might be expected to be up-regulated in response to diminished levels of iron, were included as genes of interest along with a chaperone gene, *groEL*, involved in protein folding and assembly.

In order to avoid coregulation, the five housekeeping genes in this study were selected from different functional groups. The *tpiA* gene codes for a protein involved in gluconeogenesis, while the *pykA* gene product is a key enzyme in glycolysis. The *recF* gene is involved in the minor recombination pathway, the *rhoAP* gene encodes a protein factor involved in the termination of transcription, and finally, the *glyA* gene product is a catabolic protein involved in converting serine to glycine.

A reliable normalization factor, which is the average of the individual specific expression differences of the genes, is ob-

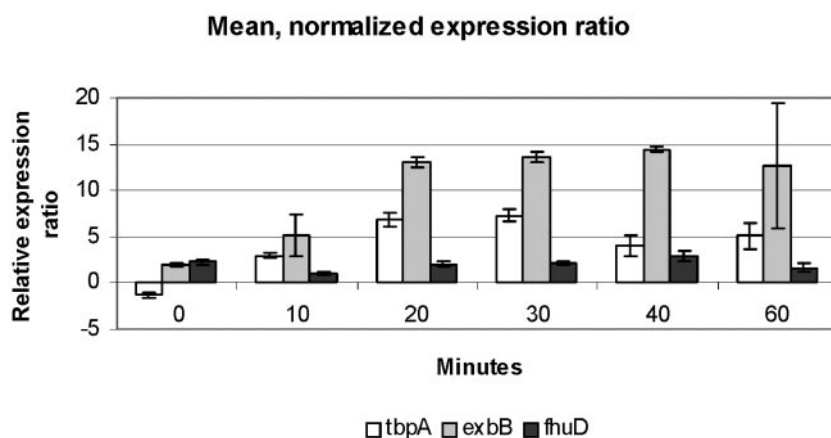


FIG. 2. Mean relative expression levels for the three significantly regulated genes at each time after addition of the iron chelator 2,2'-dipyridyl, normalized by the geometric mean for the five housekeeping genes. The data are the means  $\pm$  standard deviations ( $n = 2$ ).

tained by using the geometric mean of multiple relatively stable housekeeping genes (27). As the levels of expression of the five candidate reference genes in the present study varied less than onefold from each other, they were all included in the calculation of a gene expression normalization factor. However, except for a reduction in the interassay variation, normalizing the genes of interest does not alter the results of the expression analysis significantly in this experimental setup.

As expected from previous investigations (5, 25), the *exbB* and *tbpA* genes are significantly up-regulated in response to diminished levels of iron, both before and after normalization. Also, the observed up-regulation of the two genes within 15 to 20 min after establishment of iron starvation (Fig. 2) is in accord with previous observations made by Deneer and Potter (5), who detected a similar rapid induction of iron-repressible outer membrane proteins. Although the *exbB* and *tbpA* genes are most likely transcribed on a single polycistronic mRNA (described for serotype 7 by Tonpitak et al. [25]), the observed mean expression of *tbpA* increased approximately fourfold, while that of the *exbB* gene increased eightfold in this study. Such a discrepancy has been observed in a number of prokaryotes (9, 10, 16) and might be explained by differential expression of cotranscribed genes due to variation in posttranscriptional mRNA degradation, which seems to be an important step in regulation of prokaryotic gene expression (21).

A previous study found that regulation of the ferrichrome receptor, encoded by *fhuA*, is independent of the iron supply (15). In this investigation, the mean expression of the *fhuD* gene, which belongs to the same operon as *fhuA*, was found to be up-regulated twofold in response to low iron levels. Again, this might be explained by differences in the rate of mRNA degradation, or the observation could be due to the higher sensitivity of the real-time qRT-PCR assay, which might have been able to detect changes in gene expression smaller than changes detected by the traditional RT-PCR method used by Mikael et al. (15). However, to clarify this, further investigation by real-time qRT-PCR, including all genes of the *fhu* operon, is probably needed. The levels of expression of the *groEL* and *luxS* genes did not increase significantly in response to low-iron conditions. Although *luxS* expression is apparently not stimulated by a reduction in the iron concentration, further investigations, involving construction of a *luxS* null mutant, are needed to evaluate the role of this gene in *A. pleuropneumoniae* growth under iron-restricted conditions.

Reverse transcriptase is known to inhibit PCR at low concentrations of template (4). In order to assess the effects of reverse transcription over the dynamic range of potential mRNA expression levels, comparative dilution series of total RNA and cDNA, ranging from approximately 1.8 µg to 0.6 ng of reverse-transcribed total RNA per reaction mixture, were amplified by one control gene, *glyA*, and two genes of interest, *tbpA* and *exbB*.

In this study no inhibitory effect of reverse transcriptase on the PCR were observed at any template concentration. Assuming that mRNAs rarely make up more than 5% of the total RNA, it was possible to detect and amplify at least down to 30 pg of mRNA and likely much less mRNA, considering that with no iron deprivation of the bacteria, two of the genes tested, *tbpA* and *exbB*, are expressed at very low levels in vitro (5, 25).

In conclusion, as previously stated (20, 26) ideal and universal housekeeping genes do not exist. Therefore, one should search for stably expressed genes specific to each experimental system. Although the geometric mean of the five housekeeping genes did not alter the results significantly in the present investigation, the genes were all found to be minimally regulated in this study. With this in mind, the present investigation demonstrated that qRT-PCR is an easy, applicable, and reproducible tool with which to study *A. pleuropneumoniae* gene expression under various biological conditions which this bacterium may encounter when it invades a host organism and that it is an independent method for verification of microarray expression data.

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