Selection of reference genes for measuring the expression of aiiO in Ochrobactrum quorumnocens A44 using RT-qPCR

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Reverse transcription quantitative PCR (RT-qPCR), a method of choice for quantification of gene expression changes, requires stably expressed reference genes for normalization of data. So far, no reference genes were established for the Alphaproteobacteria of the genus Ochrobactrum. Here, we determined reference genes for gene expression studies in O. quorumnocens A44. Strain A44 was cultured under 10 different conditions and the stability of expression of 11 candidate genes was evaluated using geNorm, NormFinder and BestKeeper. Most stably expressed genes were found to be rho, gyrB and rpoD. Our results can facilitate the choice of reference genes in the related Ochrobactrum strains. O. quorumnocens A44 is able to inactivate a broad spectrum of N-acyl homoserine lactones (AHLs) – the quorum sensing molecules of many Gram-negative bacteria. This activity is attributed to AiiO hydrolase, yet it remains unclear whether AHLs are the primary substrate of this enzyme. Using the established RT-qPCR setup, we found that the expression of the aiiO gene upon exposure to two AHLs, C6-HLS and 3OC12-HSL, does not change above the 1-fold significance threshold. The implications of this finding are discussed in the light of the role of quorum sensing-interfering enzymes in the host strains.

Reverse transcription quantitative PCR (RT-qPCR) is the most widely used approach for gene expression studies. However, despite being powerful, the method involves multiple steps and is sensitive to both flaws in experimental design and to technical variation.

One of the crucial decisions to make while performing a RT-qPCR experiment is the choice of stably expressed reference genes (RGs) for the normalization of data. In case of model or well-studied organisms, the selection of suitable RGs can be facilitated based on previous studies. For bacteria, literature provides a list of candidate RGs of potential general use due to their occurrence in multiple species. The most popular include rho, 23S rRNA, rpoD, gyrB⁴, recA¹–⁴, 16S rRNA¹–⁴, dnaK, rpoB⁵–⁷, groEL⁷, gyrA⁸. However, these candidates are not equally suitable for all species under all growth conditions and one should always experimentally verify the stability of a set of candidates when first-time applying RT-qPCR in the given (micro)organisms⁹,¹⁰. In 2009, a set of guideline was published called ‘Minimum Information for publication of Quantitative real-time PCR Experiments’ (MIQE)¹¹. Its goal was to unify the quality of RT-qPCR results published in scientific journals, mainly concerning medical studies. In prokaryotic studies, with the exception of a few earlier works (i.a.), the pursuit to meet the MIQE standards intensified only recently¹⁰,¹²,¹³. Still, in many publications concerning RT-qPCR in bacteria, the expression of the studied gene(s) is arbitrary normalized to certain RGs without validation of their expression stability or even without a clear explanation for the choice.

One of the bacterial genera for which no data has been available on the suitable RGs is Ochrobactrum spp. These ubiquitous Alphaproteobacteria, related to Brucella, Agrobacterium and Rhizobium¹⁴–¹⁷, attract scientific attention as opportunistic human pathogens¹⁴,¹⁷ but also as beneficial members of plant and nematode microflora and bioremediation agents¹⁸–²⁰. In this study, we focused on the Ochrobactrum quorumnocens strain A44⁴⁰,
an isolate from the rhizosphere of potato formerly known as *Ochrobactrum* sp. A44\(^\text{[31]}\). As a result of the activity of AiiO hydrolase, A44 inactivates a broad spectrum of bacterial signaling molecules from the group of N-acyl homoserine lactones (AHLs)\(^\text{[32]}\). Many Gram-negative bacteria secrete and autodetect AHLs, responding at transcriptional level when a threshold concentration is reached. This regulatory mechanism, termed quorum sensing (QS), enables populations of single-celled organisms to perform coordinated actions such as bioluminescence, biofilm formation or production of virulence factors\(^\text{[33,34]}\). The functioning of QS can be disturbed by AHL-cleaving microorganisms like *O. quorumnocens* A44, resulting in the loss of the QS-dependent phenotype\(^\text{[35–37]}\). The latter phenomenon is known as the quorum quenching (QQ). For example, A44, when co-inoculated on plant tissue with the plant pathogenic bacterium *Pectobacterium parmentieri* SCC3193, attenuates the QS-governed virulence of this pathogen\(^\text{[31,38]}\).

To date, numerous AHL-inactivating enzymes have been described\(^\text{[39,40]}\). Discovery of many of them, including AiiO, resulted from screening studies designed for the selection of new QS-disrupting agents. It is unclear whether the cleavage of AHLs in some bacterial species is the primary function of the involved proteins\(^\text{[41]}\). For some of these enzymes, additional, unrelated functions were identified in the respective hosts, indicating that AHL cleavage may be the effect of catalytic promiscuity (reviewed in\(^\text{[10,41]}\)). For example, BIIc (AtM) from *Agrobacterium tumefaciens* was found to be involved in the metabolism of gamma-aminobutyrate\(^\text{[42]}\), and PvdQ from *Pseudomonas aeruginosa* is necessary for the maturation of the pyoverdine siderophore\(^\text{[43]}\). Even if the function of certain enzymes is assumed to be inactivation of AHLs, the predicted biological purpose of this activity is not uniform among the QS-interfering strains. Depending on whether the strain produces its own AHL or not, the predicted functions include, but are not limited to, self-regulation of own QS, using AHLs as a source of nutrients and providing an advantage in the environment over the AHL-producing competitors (reviewed in\(^\text{[44]}\)). The role of the AHL-cleaving enzyme AiiO in the metabolism and environmental fitness of *O. quorumnocens* A44 remains unclear.

In this study, we selected stably expressed RGs for RT-qPCR analyses in *O. quorumnocens* A44. Next, we applied RT-qPCR to measure changes in the expression levels of *aiiO* in A44 in response to AHLs and factors such as growth phase, growth temperature, the medium pH the addition of potato root extract. The study is a part of our investigation on the role of AiiO in the metabolism and fitness of *O. quorumnocens* A44.

### Results

#### Selection of reference genes (RGs).

A *gfp*-tagged variant of *O. quorumnocens* A44 was used throughout the study (hereafter referred to as A44)\(^\text{[45]}\). Eleven candidate genes were explored as potential RGs. Nine candidate genes (16S rRNA, 23S rRNA, *dnaK*, *groEL*, *gyrB*, *recA*, *rho*, *rpoB* and *rpoD*) were chosen based on previous reports on RGs used in bacteria\(^\text{[1–7]}\) (Supplementary Table S1). The remaining two candidates were: *gfp*, constitutively expressed by the tagged strain from an artificially introduced vector pPROBE-GTkan\(^\text{[46]}\), and CES85_4722, an A44 gene encoding a hypothetical protein with no data concerning its stability (a blind control). The *rpoD* gene was explored as an RG candidate due to its rare occurrence in the environment, a feature that would be valuable for potential studies on complex samples. Primers targeting the aforementioned genes in A44 were designed and specificity in amplification of fragments of expected sizes (139–148 bp) was confirmed by gel electrophoresis and a melt curve analysis (Supplementary Fig. S1AB). In parallel, the performance of the primers targeting the *aiiO* gene was verified.

#### Expression stability of candidate RGs.

A pilot assay was conducted to establish the expression stability of 11 candidate RGs under 10 different conditions (Table 1).

According to geNorm\(^\text{[46]}\), the most stably expressed candidate RGs, characterized by the lowest geNorm M values (≤0.6), were found to be *rho*, *gyrB* and *rpoD*, followed by *recA* (Fig. 1A). The commonly applied RG encoding 16S rRNA was considerably less stable (M = 0.913). The heterologously expressed *gfp* gene, with M = 1.058, was also not among the top-ranking candidates. The mean threshold quantification cycle (C\(_T\)) obtained for the 16S rRNA was 10.26 and 8.79 for the 23S rRNA. Therefore, the C\(_T\) for these RG candidates was 6–13 and 7–15 cycles lower, respectively, than that of the other candidates, reflecting the high abundance of the rRNA species in the cells (Fig. 1B).

The results obtained with geNorm were compared with RG ranks obtained with two other popular algorithms: NormFinder\(^\text{[46]}\) and BestKeeper\(^\text{[47]}\). The 4 genes selected by geNorm were also among the top 5 ranked by NormFinder, accompanied by the 16S rRNA gene (Fig. 1C). For three best-ranking genes, *gyrB*, *rpoD* and *recA*, NormFinder assigned stability values of 0.19, 0.28 and 0.28, respectively. BestKeeper ranked all analyzed genes except for *gfp* and *recA* as well-performing, that is with a statistically significant (α = 0.05) correlation between the correlation coefficients of individual genes with the BestKeeper index calculated for all genes in the analysis (Fig. 1D). A juxtaposition of the results from the three algorithms ranked *rho*, *rpoD* and *gyrB* as the most stable genes (Supplementary Table S2).

Apart from ranking genes by their stability, the geNorm tool can also suggest the optimal number of RGs to be included in the normalization factor (NF)\(^\text{[48]}\). In this study, geNorm tool suggested using three RGs (V value <0.15 threshold) (Fig. 2). Therefore, the complete set of samples (11 conditions, 2–3 biological replicates) for the analysis of the expression of *aiiO* (gene of interest) was analyzed by targeting *aiiO* alongside the three most stable RG candidates: *rho*, *rpoD* and *gyrB*. Next, the stability of expression of the applied RGs was validated on the complete dataset. geNorm analysis indicated the highest stability for *rho* (M = 0.441, CV = 0.187), followed by *gyrB* (M = 0.453, CV = 0.185) and *rpoD* (M = 0.5, CV = 0.221).

#### PCR efficiency.

PCR efficiency is a parameter included in the final calculation of the relative abundance of the investigated targets. With the primers designed in this study, PCR efficiency for *gyrB* and *rho* were 95.9% and 93.5%, respectively, and similar to the efficiency for the gene of interest *aiiO* (94.9%). The efficiency of PCR for *rpoD* was slightly lower (89%) (Table 2; Supplementary Fig. S2).
Table 1. Conditions for the growth of A44 strain applied for the RT-qPCR experiment. 1(L) and (S) stand for liquid and solid medium, respectively. aThe medium was buffered only in case of pH 5.5. In other cases, 7 is the initial pH of the medium. bIf provided, growth phase was determined based on the growth curve of A44 GFP in LB at 28 °C. O/N – stands for an overnight culture (16–20 h). early stat. – stands for early stationary growth phase. cCulture condition not included in the geNorm pilot experiment (initial ranking of RG candidates). dC6-HSL – N-hexanoyl homoserine lactone. e3OC12-HSL – N-3-oxododecanoyl homoserine lactone. fThe medium contained water extract from the roots of soil-grown, 3-week-old potato plants (final concentration 25%).

Comparison of the performance of the selected normalization factors (NFs). GeNorm analysis recommended applying 3 RGs for data normalization in the developed RT-qPCR assay. To determine whether the change of RGs included in the NF may influence the interpretation of the expression data for our gene of interest (aaiO), we compared the consistency of results obtained using 3 different NFs: NF1 (rho, the most stably expressed...
RG based on geNorm), NF2 \((\text{rho}, \text{gyrB})\) and NF3 \((\text{rho}, \text{gyrB} \text{ and } \text{rpoD})\). For the comparison of the single-gene NF1 and the 2-gene NF2, the results were significantly different \((p < 0.05)\) under 3 out of 11 culture conditions tested \((\text{LB agar 28 °C, LB early stationary, LB middle exponential})\) (Fig. 3). For the comparison of the single-gene NF1 and the 3-gene NF3, the results were significantly different in 4 conditions \((\text{LB agar 28 °C, LB agar 20 °C, LB early stationary, LB middle exponential})\). On the contrary, the difference between applying the 2-RG NF2 and the 3-gene NF3 was significant only under a single culture condition \((\text{M63 + 3OC12-HSL})\) (Fig. 3).

**Changes in the expression level of aiiO in response to AHLs and other culture conditions.**

Expression of aiiO was investigated in three types of media: lysogeny broth \((\text{LB})\) with peptone as the main energy source, LB solidified with agar and a defined mineral medium M63 supplemented with 0.4% glucose. Based on these media, a set of 11 conditions was established, 10 of which were included in the study on the stability of RGs (Table 1), in order to test whether the expression of aiiO in A44 is affected by factors such as: (i) the growth
**Figure 4.** Fold change in relative expression of aiiO under different culture conditions. Relative expression (CNRQ) of aiiO in all samples was calculated with respect to normalization NF3 comprising rho, gyrB and rpoD. The fold change (log2) was calculated independently for each type of basal medium (LB agar, LB and M63 0.4% glucose) where the median CNRQ of one of the samples was treated as a reference (‘control’). If not indicated otherwise, the cells were grown at 28 °C. SCC3193 – growth in the presence of AHLs secreted by P. parmentieri SCC3193; no suppl. – without supplementation; early stat. – early stationary growth phase; middle exp. – middle exponential growth phase; C6-HSL, 3OC12-HSL – aiiO expression 90′ after the addition of 50 µM·mL⁻¹ of the respective AHL; root extract – growth in the presence of 25% water-based extract obtained from potato roots. *denotes \( p < 0.05 \) *** denotes \( p < 0.0005 \) calculated using BootstRatio.

Two of the AHL types, N-3-oxo-octanoyl homoserine lactone (3OC8-HSL) and N-3-oxohexanoyl homoserine lactone (3OC6-HSL), were delivered as compounds secreted by the co-cultured cells of *P. parmentieri* SCC3193. N-hexanoyl homoserine lactone (C6-HSL) and N-3-oxododecanoyl homoserine lactone (3OC12-HSL) were delivered as commercially synthesized pure compounds in 50 µM concentration. All AHLs included in the assay are known to be degraded by A44. To prevent complete depletion of AHLs prior sampling of cells for RNA extraction, the sampling was performed 90 min post addition of AHLs. The time point was chosen based on a previous experiment in which C6-HSL, supplemented to the culture in a 5-time lower concentration (10 µM), could still be detected in the supernatant of culture of A44 at 40 min (strong signal) and 100 min (weak signal) post addition.

The expression data for aiiO was normalized with respect to NF3 and the resulting CNRQs (Supplementary Table S3) were grouped by the type of basal medium (LB, LB agar, M63 0.4% glucose). For each of the 3 types of medium, one sample was established as a reference (‘control’). The control samples were derived from cells grown in a respective medium without any additives and at 28 °C. When compared to the control samples, expression of aiiO was not altered above 1.5-fold in any of the tested conditions (Fig. 4). The >1-fold threshold was breached for LB culture in acidic pH 5.5, with respect to non-buffered LB medium of initial pH 7 (downregulation, \(-0.116, p < 0.0005\)). The 1-fold threshold was also exceeded for LB agar cultures grown at 37 °C (upregulation, \(1.320 \pm 0.228\)) and at 28 °C in the presence of SCC3193 (1.286 \(\pm 0.159\)), with respect to cultures grown separately at 28 °C, and in the middle exponential phase cells in LB with respect to early stationary phase cells in the same medium (1.023 \(\pm 0.147\)). However, the latter three changes were not statistically significant (\( p > 0.05 \)). Difference between M63% glucose and M63% glucose supplemented with 3OC12-HSL, although significant from a statistical standpoint, was below the 1-fold change cut-off (\(-0.115 \pm 0.026, p < 0.05\)) (Fig. 4, Supplementary Table S4). In general, the expression of aiiO was not considerably induced under any of the conditions tested. In contrast, the expression of groEL, one of the least stable RG candidates in this study, was nearly 6-fold higher in samples from the mid-exponential culture in LB than in samples from LB agar at 37 °C, again with no significant induction observed for aiiO (Supplementary Fig. S3).

**Discussion**

The QQ activity of *O. quorumnocens* A44 is attributed to the \( \alpha/\beta \) hydrolase protein AiiO. However, little is known concerning the role of this enzyme in the metabolism and environmental fitness of this strain.

Here, we developed a RT-qPCR assay to study the expression of aiiO gene in *O. quorumnocens* A44. A key consideration in RT-qPCR is the choice of stably expressed reference genes (RGs), also known as internal controls. To identify RGs suitable for RT-qPCR analyses in *O. quorumnocens* A44, we tested the expression stability of 11 candidate genes, 9 of which were previously reported as RGs in other bacterial species (i.e. [-7]). Despite it is known that RGs suitable for one microorganism often do not show sufficient stability in others (RGs are not universal)\(^\text{9}^\), and that the incorporation of inadequate genes to the normalization factor (NF) can significantly alter
the results\textsuperscript{12}, the experimental validation of the stability of RGs tends to be neglected in gene expression studies in prokaryotes.

Ideally, the expression stability of a potential RG should be validated in all physiological or experimental conditions intended for the study of the gene of interest\textsuperscript{13}. Here, we ranked the stability of expression of 11 candidate RGs in a pilot assay including 10 different conditions, on samples originating from a single experiment, and then verified the performance of best RGs in the final dataset (11 culture conditions, all biological replicates). The strategy for applying a pilot assay to choose the most promising RG candidates is in line with practical recommendations for the implementations of MIQE\textsuperscript{49}. When human clinical samples are analyzed, a minimum of 10 samples from different patients can be used to rank candidate RGs and then the best scoring RGs, usually 2–5, are targeted alongside the gene(s) of interest for all samples/patients under investigation\textsuperscript{50}. Although a sample set derived from a bacterium cultured in 10 different conditions is not equivalent to a sample set from 10 human individuals, the tools developed for the evaluation of gene expression stability usually require an input of this approximate size. Therefore, this approach is also used for prokaryotic studies\textsuperscript{10}.

The expression stability of the candidate RGs was evaluated using geNorm\textsuperscript{46} and two other popular algorithms, NormFinder\textsuperscript{47} and BestKeeper\textsuperscript{48}, each relying on a different statistical approach\textsuperscript{53,54}. According to geNorm, the most stable RG candidates were rho, gyrB and rpoD. These genes were also among the five top ranking genes according to NormFinder. The ranking of gene stability suggested by BestKeeper was different from that suggested by the other two algorithms. This method also yielded the smallest resolution, assigning good performance to the majority of tested genes. Interestingly, recA, listed among the top 4 genes by geNorm and 3 top genes by NormFinder, was among two with the worst ranks according to BestKeeper. A discrepancy between the results from BestKeeper and the results from both geNorm and NormFinder were reported in other studies (i.e.\textsuperscript{51,52}). The reason for that is different input data (raw C\textsubscript{q} values for BestKeeper and relative expression data for geNorm and NormFinder) and different stability criteria considered by the three programs – a matter thoroughly summarized in the works of De Spiegelaere et al.\textsuperscript{53} and Gomes et al.\textsuperscript{13}.

Based on the juxtaposition of the results from geNorm, NormFinder and BestKeeper, we chose rho, rpoD and gyrB as the most promising RG candidates to be used for the normalization of RT-qPCR data in *O. quorumnocens* A44. Importantly, these genes do not belong to a common functional group (rho – encoding the transcription termination factor Rho, rpoD – encoding the primary sigma factor $\sigma^1$, gyrB – encoding the B subunit of DNA gyrase\textsuperscript{4}) what reduces the chances that they are co-regulated\textsuperscript{34,55}.

Apart from ranking the potential RGs based on their stability, the geNorm tool also suggests the optimal number of genes to be included to the normalization factor\textsuperscript{46}. In this study, based on the pilot assay, geNorm suggested including three RGs in gene expression studies in A44. This value, however, is a guideline and does not have to be treated as a strict threshold\textsuperscript{13,46}. We investigated whether and to what extent the number of genes in the NF influences our expression data. The results showed moderate impact of switching form 2-gene (rho, gyrB) to 3-gene (rho, gyrB, rpoD) NF, with a difference observed only for a single out of 11 investigated culture conditions. However, application of a single-gene NF (rho) significantly influenced the outcome under 3/11 and 4/11 culture conditions when compared to 2-gen and 3-gen NFs, respectively. These results are in line with the MIQE guidelines, according to which expression of the target gene(s) should be normalized to more than one RG\textsuperscript{13}.

The expression stability of rho, rpoD and gyrB, the most promising RG candidates selected in the pilot assay, was validated on the final dataset (11 culture conditions, all biological replicates). The geNorm M and CV values obtained for these genes were, respectively, $M = 0.441$ and $CV = 0.187$ for rho, $M = 0.453$ and $CV = 0.185$ for gyrB, and $M = 0.5$ and $CV = 0.221$ in case of rpoD. According to Hellemans and coworkers\textsuperscript{56}, the recommended threshold stability values depend on the type of analyzed samples. For homogeneous samples, defined in medical studies as such originating from cell cultures of the same cell type, these requirements are more stringent and amount to $M < 0.5$ and $CV < 0.25$. For heterogeneous samples, in medical studies defined as originating from different cell types, clinical biopsies and cancer tissue in general, the criteria are less stringent (M < 1 and CV < 0.5). Transposition of these thresholds to prokaryotic studies is not straightforward. However, recent publications concerning RT-qPCR in prokaryotes suggest that for a given bacterial strain cultured in different conditions, genes with geNorm values $M < 1$ can be applied for normalization of RT-qPCR data\textsuperscript{10,13}. In this study, rho, gyrB, rpoD met both aforementioned thresholds for acceptable stability.

In strain A44, the 16S rRNA-coding gene, a popular RG candidate in bacteria\textsuperscript{8}, has shown poor expression stability and was not considered as a component of NF. In some studies, however, the target may meet the stability criteria (see Gomes et al., 2018\textsuperscript{18}). In such cases it should be considered that factors other than expression stability may also influence the performance of an RG. It was reported that while the turn-over of messenger RNAs in the cells is rather rapid, the ribosomal RNA is more stable and becomes degraded only under certain stress conditions\textsuperscript{57}. These disproportions make a quantitative comparison between the two RNA types more difficult\textsuperscript{13}. Another factor that comes to mind is the high disproportion in the C\textsubscript{q} range observed between the potential genes of interest and the rRNA species, resulting from the high abundance of the latter in the cells. In RT-qPCR, however, as opposed to Northern blotting, it is not crucial for a good reference gene to be expressed at the same level as the gene of interest (Barbara D’haene, The gene expression blog, qbase\textsuperscript{+}; https://blog.qbaseplus.com/four-tips-for-rt-qpcr-data-normalization-using-reference-genes).

A gfp-tagged derivative of the A44 strain\textsuperscript{44} was used throughout this study. Thus, apart from the genome-encoded RG candidates, we also investigated the stability of the heterologously expressed gfp. The gene is unique in terms of its occurrence in the terrestrial environment, therefore its stable expression in A44 GFP would create a low-cost alternative to the probe-based qPCR (i.e. TaqMan) for specific amplification of this RG candidate in complex samples. However, the stability of gfp in the tested setup proved insufficient. A potential cause may be uneven copy number per cell of the pPROBE-GTkan plasmid expressing gfp.

In this work, as a part of a broader study on the role of AiiO in the metabolism and environmental fitness of A44, we used RT-qPCR with the newly-established reference genes to investigate whether the expression of AiiO...
in A44 is altered upon exposure to AHLs and under a set of culture conditions. It is known that the expression of some enzyme-encoding genes may become highly elevated in the presence of cognate substrate(s), providing a solid link between a gene (protein) and a given compound or process. For example, Hommais et al. showed that the expression of pellA, a pectate lyase, can be induced over 10-fold in the stationary phase when polygalacturonate (substrate) is present.

The change in the expression of aiiO was investigated ninety minutes following the addition of synthetic AHLs (C6-HSL or 3OC12-HSL). The particular time point was chosen based on our previous experiments where the rate of exhaustion of AHLs following addition to cell culture was examined. The consideration was to adjust the initial concentration of AHLs and time of sampling to prevent the total exhaustion of AHLs due to the activity of AiiO prior cell harvest.

Depending on the study, a gene is considered differentially regulated between two conditions when the change exceeds a 1-fold or 1.5-fold or 2-fold threshold. Using RT-qPCR, we showed that the expression of aiiO in O. quorumnocens A44 is not upregulated (fold change <1.5) upon supplementation with two synthetic AHLs, C6-HSL or 3OC12-HSL, nor in the presence of 3OC8-HSL and 3OC6-HSL and other compounds secreted to the stationary phase by P. parmentieri SCC3193. No induction after addition of AHLs was reported earlier for aiiB and blc genes encoding two QQ lactonases from A. tumefaciens. Instead of being induced by signal molecules, the expression of those genes was enhanced in the presence of specific plant-derived compounds: succinic semialdehyde, gamma-hydroxybutyrate, gamma-butyrolactone, gamma-amino butyrate and salicylic acid in case of blc, and agrocinopine-enriched plant extracts in case of aiiB. In this study, the expression of aiiO was investigated in the presence of water extract from potato roots. However, no significant up- or down-regulation of aiiO was observed in the applied setup.

Although the expression of aiiO was not distinctly upregulated in any of the conditions included in this study, we cannot exclude that such conditions exist. Some genes may be conserved in bacterial genomes, alike the aiiO homologues seem to be present in among different Ochrobactrum spp. Which may not to be distinctly induced by the presence of any of the AHLs, thereby providing no obvious indication that the primary substrate of AiiO are the AHLs.

### Materials and Methods

#### Strains, chemicals and culture conditions.

GFP-tagged derivative of O. quorumnocens A44 (A44 GFP)46, carrying plasmid pPROBE-GTkan45, was used throughout the experiments. The pVS1/p15a ori present on pPROBE-GTkan enables its stable propagation without selection. The strain was grown under culture conditions listed in Table 1. Three types of basal media were used: Miller’s LB (Novagen, Germany), Miller’s LB agar (Novagen, Germany), and M63 mineral medium. For the growth at pH 5.5, LB medium was buffered with 0.05M potassium hydrogen phthalate and the pH was adjusted to 5.5 with NaOH. To expose A44 GFP to metabolites secreted by the AHL-producing P. parmentieri SCC3931, the two strains were co-cultured in the presence of water extract from potato roots. However, no significant up- or down-regulation of aiiO was reported earlier for aiiB and blc genes encoding two QQ lactonases from A. tumefaciens. Instead of being induced by signal molecules, the expression of those genes was enhanced in the presence of specific plant-derived compounds: succinic semialdehyde, gamma-hydroxybutyrate, gamma-butyrolactone, gamma-amino butyrate and salicylic acid in case of blc, and agrocinopine-enriched plant extracts in case of aiiB. In this study, the expression of aiiO was investigated in the presence of water extract from potato roots. However, no significant up- or down-regulation of aiiO was observed in the applied setup.

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#### Selection of candidate RGs and primer design.

The pool of candidate reference targets comprised 11 bacterial genes. Nine of them (16S rRNA, 23S rRNA, dnaK, groEL, gyrB, recA, rho, rpoB, rpoD) were chosen based...
on literature data. Homologues of these sequences were derived from the complete genome of *O. quorumnocens* A44 (CP022602.1–CP022605.1) (detailed list of loci in Table 2). The two other genes were: *gfp*, carried on the artificially introduced vector pFPROBE-GTkan45 and CES85_4722, a hypothetical A44 gene with no data concerning its stability (a blind control). All PCR primers (Supplementary Table S1) were designed using the Primer3Plus software46–49 (http://primer3plus.com/). Specificity of the primers was verified using real-time PCR followed by melt curve analysis and electrophoresis in 1.2% agarose gel (TopVision, Thermo Scientific).

**Isolation of RNA and cDNA synthesis.** Total RNA from bacterial monocultures was isolated using RNeasy Protect Bacteria Mini Kit according to the manufacturer's protocol (Qiagen, Germany), with the optional on-column DNase digestion. Approximately 4.5 × 10⁸ cells were harvested and used per single isolation (cells harvested from 1 mL of cell suspension of turbidity equal to 5 units in McFarland's scale). For cultures in liquid media, the turbidity was measured prior sampling and the volume of harvested cells was adjusted proportionally. Cells form solid media were suspended in sterile saline prior the turbidity measurement. The harvested cells (8000 RCF, 5°) were suspended in 500 μL of sterile saline to which 1 mL of RNA Protect Bacteria reagent (Qiagen, Germany) was immediately added to prevent RNA decay.

All RNA samples were treated with TURBO DNA-free Kit (Thermo Scientific, USA) to remove any gDNA contamination and stored at −80 °C. The concentration and the quality of RNA for gene expression stability assessment was evaluated using an Agilent 2100 Bioanalyzer Instrument and an Agilent RNA 6000 Nano Kit (Agilent Technologies, USA). All processed samples had RNA Integrity value (RIN) ≥ 8.7. RIN values between 8–10 indicate intact, high quality RNA samples and RIN = 5.0 is the suggested minimum threshold for applicability of the given RNA in downstream RT-qPCR analyses50,51. Next, RNA was reverse-transcribed to cDNA with Transcriptor First Strand cDNA Synthesis Kit (Roche, Poland) using random hexamer primers. The optional denaturation step was included. Total amount of RNA used per reaction was 500 ng.

**qPCR.** The assays were carried out using the CFX96 instrument (Bio-Rad), in a 96-well plate format. Reaction mixtures of 20μL included: 2 × Power SYBR Green Mastermix (Thermo Scientific, USA), forward and reverse primers at a final concentration of 300 nM and 4μL of diluted (1:3) post-RT mixture containing cDNA. The PCR conditions were 95 °C for 10 min, 40 cycles of 95°C for 15S and 60 °C for 1 min, with a final melt curve 55–95 °C at a 0.5 °C/5S increment. Each reaction was performed in duplicate. The difference between duplicates was <0.5 cycle for 98% of reactions. Inter-run calibrator (IRC) was included in each subsequent plate. Parameters of the assay, including sensitivity, linearity and primer efficiency, were validated using a 6-point 10-fold serial dilution of templates, for which the corresponding post-PCR amplicons were applied.

**Expression stability and the optimal number of RGs.** Eleven candidate RGs were ranked according to their expression stability under 10 culture conditions. Initially, the expression stability was evaluated in a geNorm66 pilot assay where a set of 10 cDNA samples (one per condition) was targeted for all RG candidates. geNorm incorporated into the qbase+ software, version 2.3 (Biogazelle NV, Belgium) was applied. In a geNorm pilot study, typically 8–10 candidate RGs are measured in a set of samples (usually 10). It is recommended that no sample type is overrepresented, what could introduce bias in terms of candidate genes being more stable in a given condition65. The resulting values (geNorm M) were compared with RG ranks obtained with NormFinder47 and BestKeeper48. Each of the applied methods is based on a different statistical approach, therefore the obtained results are not identical. To make our decision on the choice of a specific approach less arbitrary, we conducted a juxtaposition of the results obtained using the three algorithms by calculating a geometric mean. Genes with the best overall score were selected for further experiments. The expression stability the RGs pre-selected in the pilot were verified on the final dataset (11 culture conditions, 2–3 biological replicates) – the same one used to investigate the expression of *aiiO*. The validation was conducted using geNorm (M values).

The optimal number of RGs to be included in the normalization factor was established by calculating the Vn/Vn+1 pair-wise variation, where n is the number of RGs in the NF46. In this method, a V value below 0.15 indicates no benefit of going from n to n + 1.

The performance of different normalization factors was compared on CNRQ values where the expression of *aiiO* was normalized to either one (NF1; rho), two (NF2; rho, gyrB) or three (NF3; rho, gyrB, rpoD) RGs. The statistical significance (*α = 0.05*) of pair-wise differences between groups normalized with different NFs was calculated using two-tailed Student's t-test with unequal variation (Welch’s t-test).

**Analysis of gene expression.** Gene expression analysis was conducted using the qbase+ software, version 2.3 (Biogazelle NV, Belgium). Subsequent steps were involved: the relative quantity (RQ) was calculated using ΔΔCt method53, the RQ was normalized with respect to the established RGs (NRQ) and the value was calibrated with respect to the applied inter-run calibrator samples to obtain calibrated NRQ (CNRQ) interpreted as the relative expression. The significance of changes in the expression of *aiiO* between the ‘control’ and the ‘treated’ samples was evaluated using the BootstRatio tool for statistical analysis of fold-change (http://pdo.iconcologia.net/stats/br/index.html)63. To plot the fold change, CNRQ values were log base 2 transformed with respect to median of the respective reference samples for the three tested media types (LB agar, LB, M63 0.4% glucose).

**Data Availability**
All data generated or analysed during this study are included in this published article (and its Supplementary Information files).
References
1. Galis, P. S. et al. Identification and validation of reference genes to study the gene expression in Gluconacetobacter diazotrophicus grown in different carbon sources using RT-qPCR. J. Microbiol. Methods 91, 1–7 (2012).
2. Menna, P., Barcellos, F. G. & Hungria, M. Phylogenoy and taxonomy of a diverse collection of Bradyrhizobium strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and glmI, recA, atpD and dnaK genes. Int. J. Syst. Evol. Microbiol. 59, 2934–50 (2009).
3. Florindo, C. et al. Selection of reference genes for real-time expression studies in Streptococcus agalactiae. J. Microbiol. Methods 90, 220–227 (2012).
4. Bhubhanil, S., Niamyim, P., Sukchawalit, R. & Mongkolsuk, S. Cysteine desulphurase-encoding gene sufS2 is required for the repressor function of RirA and oxidative resistance in Agrobacterium tumefaciens. Microbiology 160, 79–90 (2014).
5. Mirabella, A. et al. Brucella melitensis MuCR, an orthologue of Sinorhizobium meliloti MuCR, is involved in resistance to oxidative, detergent, and saline stresses and cell envelope modifications. J. Bacteriol. 195, 653–65 (2013).
6. Oliveira, L. R., Rodrigues, E. P., Marcelino-Guimarães, F. C., Oliveira, A. L. M. & Hungria, M. Fast induction of biosynthetic polysaccharide genes ipxA, ipxE, and rkp1 of Rhizobium sp. strain PRF 81 by common bean seed exudates is indicative of a key role in symbiosis. Funct. Integr. Genomics 13, 275–83 (2013).
7. Czajkowski, R. et al. Validation of candidate reference genes in Bifidobacterium adolescentis for gene expression normalization. Anaerobe 27, 34–39 (2014).
8. Rocha, D. J. P., Santos, C. S. & Pacheco, L. C. Bacterial reference genes for gene expression studies by RT-qPCR: survey and analysis. Antonie Van Leeuwenhoek 108, 685–693 (2015).
9. Krzyżanowska, D. M. et al. Selection of stable reference genes for RT-qPCR in Rhodococcus opacus PD630. Sci. Rep. 8, 6019 (2018).
10. Papenfort, K. & Bassler, B. L. Quorum sensing signal–response systems in Gram-negative bacteria. Microbiology 166, 29–90 (2014).
11. Dirksen, P. et al. Evaluation of the relatedness of Brucella spp. and Ochrobactrum anthropi and description of Ochrobactrum intermedium sp. nov., a new species with a closer relationship to Brucella spp. Int. J. Syst. Bacteriol. 48, 759–768 (1998).
12. Scholz, H. C. et al. Genetic diversity and phylogenetic relationships of bacteria belonging to the Ochrobactrum–Brucella group by recA and 16S rRNA gene-based comparative sequence analysis. Syst. Appl. Microbiol. 31, 1–16 (2008).
13. Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. Ochrobactrum anthropi sp. nov., isolated from nodules of Glycyrrhiza uralensis. Arch. Microbiol. 198, 171–179 (2016).
14. Brasic, M. s. al. Multilocus sequence analysis of the 16S rRNA gene, ITS region and recA genes. Anaerobe 22, 220–227 (2012).
15. Li, L. et al. Evaluation of the relatedness of Brucella spp. and Ochrobactrum anthropi gen. nov. sp. nov. from Human Clinical Specimens and Previously Known as Group Vd. Int. J. Syst. Bacteriol. 36, 406–416 (1986).
16. Liu, L. et al. Ochrobactrum endophyticum sp. nov., isolated from roots of Glycyrrhiza uralensis. Arch. Microbiol. 198, 171–179 (2016).
17. Tiwari, M. et al. Detection and characterization of bacteria from the potato rhizosphere degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
18. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
19. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
20. Bhubhanil, S., Niamyim, P., Sukchawalit, R. & Mongkolsuk, S. Cysteine desulphurase-encoding gene sufS2 is required for the repressor function of RirA and oxidative resistance in Agrobacterium tumefaciens. Microbiology 160, 79–90 (2014).
21. Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. Ochrobactrum anthropi sp. nov., isolated from nodules of Glycyrrhiza uralensis. Arch. Microbiol. 198, 171–179 (2016).
22. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
23. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
24. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
25. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
26. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
27. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
28. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
29. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
30. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
31. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
32. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
33. Filipets, G., F. et al. Brucella endophyticum strains capable of degrading the nootropic drug—Piracetam. N. Biotechnol. (2017).
34. Sanjeev Kumar, S., Kumar, M. S., Siddavattam, D. & Karegoudar, T. B. Generation of continuous packed bed reactor with Ochrobactrum endophyticum strain KUDC1013 involved in induction of systemic resistance against Pectobacterium carotovorum subsp. carotovorum in tobacco leaves. Plant Pathol. J. 29, 174–181 (2013).
38. Pirhonen, M., Flego, D., Heinikheimo, R., & Palva, E. T. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen Erwinia carotovora. EMBO J. 12, 2467–76 (1993).
39. Czajkowski, R. & Jafra, S. Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. Acta Biochim. Pol. 56, 1–16 (2009).
40. Grandclément, C., Tannières, M., Moreira, S., Dessaux, Y. & Faure, D. Quorum quenching: role in nature and applied developments. FEMS Microbiol. Rev. 40, 86–116 (2016).
41. Roche, D. M. et al. Communications blackout? Do N-acylhomoserine lactone-degrading enzymes have any role in quorum sensing? Microbiology 150, 2023–8 (2004).
42. Khan, S. R. & Farrand, S. K. The BlcC (AttM) lactonase of Agrobacterium tumefaciens does not quench the quorum-sensing system that regulates Ti plasmid conjugal transfer. J. Bacteriol. 191, 1320–9 (2009).
43. Nadjal Jimenez, P. et al. Role of PvdQ in Pseudomonas aeruginosa virulence under iron-limiting conditions. Microbiology 156, 49–59 (2010).
44. Krzyzanowska, D. M., Obuchowski, M., Bikowski, M., Rychłowski, M. & Jafra, S. Colonization of potato rhizosphere by GFP-tagged Bacillus subtilis MB73/2, Pseudomonas sp. P482 and Ochrobactrum sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. Sensors (Basel). 12, 17608–19 (2012).
45. Miller, W. G., Leveau, J. H. & Lindow, S. E. Improved gfp and inlA broad-host-range promoter-probe vectors. Mol. Plant. Microbe. Interaction 13, 1243–59 (2000).
46. Vanesdomepele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, RESEARCH0034 (2002).
47. Andersen, C. L., Jensen, J. L. & Ørntoft, T. F. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250 (2004).
48. Pfaffl, M. W., Tichopad, A., Progemot, C. & Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515 (2004).
49. Taylor, S., Wackem, M., Djikiman, G., Alsarraj, M. & Nguyen, M. A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines. Methods 50, S1–S5 (2010).
50. Supernat, A. et al. Epiplasm-mesenchymal transition and cancer stem cells in endometrial cancer. Anticancer Res. 33, 5461–9 (2013).
51. Martínez-Giner, M., Noguera, J. L., Balcells, I., Fernández-Rodríguez, A. & Pena, R. N. Selection of internal control genes for real-time quantitative PCR in ovary and uterus of sows across pregnancy. PLoS One 8, e66023 (2013).
52. Robledo, D. et al. Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (Scophthalmus maximus) gonad dataset. BMC Genomics 15, 648 (2014).
53. De Spiegelaere, W. et al. Reference gene validation for RT-qPCR, a note on different available software packages. PLoS One 10, e0122515 (2015).
54. Sliver, N., Best, S., Jiang, J. & Thein, S. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol. Biol. 7, 33 (2006).
55. Helleman, J. & Vanesdomepele, J. qPCR data analysis – unlocking the secret to successful results. PCR troubleshooting and optimization: the essential guide, 139–150 (2011).
56. Helleman, J., Mortier, G., De Paepe, A. & Vanesdomepele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19 (2007).
57. Deutscher, M. P. Degradation of RNA in bacteria: comparison of mRNA and stable RNA. Nucleic Acids Res. 34, 659–66 (2006).
58. Hommais, F. et al. lpxC and yafS are the most suitable internal controls to normalize real time RT-qPCR expression in the pathobacterium bacteria Dickeya dianthii. PLoS One 6, e20269 (2011).
59. Zeller, T., Moskvin, O. V., Li, K., Klug, G. & Gomelsky, M. Transcription time and physiological responses to hydrogen peroxide of the facultatively phototrophic bacterium Rhodobacter sphaeroides. J. Bacteriol. 187, 7232–42 (2005).
60. Varano, M. et al. Temperature-dependent regulation of the Ochrobactrum anthropi proteome. Proteomics 16, 3019–3024 (2016).
61. Pulawaska, J., Kahužná, M., Warabieda, W. & Mikiciński, A. Comparative transcription analysis of a lowly virulent strain of Erwinia amylovora in shoots of two apple cultivars – susceptible and resistant to fire blight. BMC Genomics 15, 868 (2017).
62. Fan, B. et al. Transcriptomic profiling of Bacillus amylolyticus FZB42 in response to maize root exudates. BMC Microbiol. 12, 116 (2012).
63. Hauudecoeur, E. et al. Different Regulation and Roles of Lactonases AiiB and AtTM in Agrobacterium tumefaciens C58. Mol. Plant-Microbe Interact. 22, 529–537 (2009).
64. Lang, J. & Faure, D. Functions and regulation of quorum-sensing in Agrobacterium tumefaciens. Front. Plant Sci. 5, 14 (2014).
65. Mei, G.-Y., Yan, X.-X., Turak, A., Luo, Z.-Q. & Zhang, L.-Q. AidH, an alpha/beta-hydrolase fold family member from an Ochrobactrum sp. strain, is a novel N-acylhomoserine lactonease. Appl. Environ. Microbiol. 76, 4933–42 (2010).
66. Rohmer, L., Hocquet, D. & Miller, S. I. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. Trends Microbiol. 19, 341–8 (2011).
67. Whatmore, A. M. Current understanding of the genetic diversity of Brucella, an expanding genus of zoonotic pathogens. Infect. Genet. Evol. 9, 1168–1184 (2009).
68. Gnanamanickam, S. S. Plant-Associated Bacteria. (Springer Netherlands, 2006).
69. Huang, S., Sheng, P. & Zhang, H. Isolation and identification of cellulosytic bacteria from the gut of Holotrichia parallela larvae (Coleoptera: Scarabaeidae). Int. J. Mol. Sci. 13, 2563–2577 (2012).
70. Wu, Z. et al. Mushroom tumor: a new disease on Flammulina velutipes caused by Ochrobactrum pseudogrigonense. FEMS Microbiol. Lett. 363, fmv266 (2016).
71. Elias, M. & Tawfik, D. S. Divergence and convergence in enzyme evolution: parallel evolution of paraoxonases from quorum-quenching lactonases. J. Biol. Chem. 287, 11–20 (2012).
72. Tannières, M. et al. A Metagenomic study highlights phylogenetic proximity of quorum-quenching and xenobiotic-degrading amidasines of the AS-family. PLoS One 8, e54733 (2013).
73. Hallmann, L, Quad-Hallmann, A., Miller, W. G., Sikora, R. A. & Lindow, S. E. Endophytic Colonization of Plants by the Biocontrol Agent Rhizobium etli G12 in Relation to Meloidogyne incognita Infection. Phytopathology 91, 415–422 (2001).
74. Heeb, S. et al. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. Mol. Plant-Microbe Interact. 23, 252–270 (2007).
75. Miller, J. H. Experiments in molecular genetics. (Cold Spring Harbor Laboratory, 1972).
76. Pirhonen, M., Saarlahihti, H., Karlsso, M. B. & Palva, E. T. Identification of pathogenicity determinants of Erwinia carotovora subsp. carotovora by transposon mutagenesis. Mol. Plant Microbe. Interaction 4, 276–283 (1991).
77. Ravn, L., Christensen, A. B., Molin, S., Givskov, M. & Gram, L. Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. J. Microbiol. Methods 44, 239–251 (2001).
78. Koressaar, T. & Remm, M. Enhancements and modifications of primer design program Primer3. Bioinformatics 23, 1289–91 (2007).
79. Untergasser, A. et al. Primer3 – new capabilities and interfaces. Nucleic Acids Res. 43, 15–22 (2015).
80. Feige, S. & Pfaffl, M. W. RNA integrity and the effect on the real-time qRT-PCR performance. Mol. Aspects Med. 27, 126–139 (2006).
81. Pazzaglia, M. et al. SPIDIA-RNA: First external quality assessment for the pre-analytical phase of blood samples used for RNA based analyses. Methods 59, 20–31 (2013).
82. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **25**, 402–408 (2001).

83. Clèries, R. et al. BootstRatio: A web-based statistical analysis of fold-change in qPCR and RT-qPCR data using resampling methods. *Comput. Biol. Med.* **42**, 438–445 (2012).

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Conceptualization: D.M.K. and S.J.; Methodology: A.S. and D.K.; Investigation: A.S., D.M.K., T.M., M.M.; Writing-Original Draft Preparation: D.M.K.; Writing-Review & Editing, S.J., D.M.K.; Visualization, D.M.K.; Supervision, S.J.; Funding Acquisition, S.J.

**Additional Information**

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