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Comparison of two standardisation methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during in vitro growth

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Abstract

By real-time quantitative PCR (RTQ-PCR), two different standardisation methods were used to quantify expression of three target genes (RNAII and RNAIII transcripts of *agr* locus and ica transcript of *icaADBC* locus): (i) a relative quantification, using a transcript of three housekeeping genes (gyrase A, *gyrA*; guanylate kinase, *gmk* and 16S rRNA, *16S*) as internal standard, and (ii) an absolute quantification, using cloned sequences of the target genes in known concentrations as external standards. To determine the efficiency and reliability of these two methods, the gene expressions were studied during the growth of a clinical isolate of *Staphylococcus aureus*. Between 3 and 20 h after inoculation, target gene transcription was analysed using LightCycler Apparatus, LC Data Analysis software and RelQuant software for relative quantification (Roche).

For all target genes, the expression profiles obtained with *gyrA* or *gmk* as internal standards remained almost identical. However, these profiles varied between each other depending on the standard gene. Due to their important expression variations during growth phases, these two housekeeping genes seem inappropriate to be used as internal standards. The absolute quantification of the three transcripts of interest gave results similar to their relative quantification expressed versus 16S rRNA. Therefore, our study suggests the suitable use of 16S rRNA as internal standard in RTQ-PCR quantification of staphylococcal gene expression during the stationary phase of growth.

Keywords: *Staphylococcus*; Transcript; Quantification; RTQ-PCR

1. Introduction

Reverse transcription followed by real-time quantitative PCR (RTQ-PCR) has proven to be very sensitive, more reliable and less laborious than the

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classical methods for the study of gene expression (Gibson et al., 1996; Hein et al., 2001).

However, the results obtained by RTQ-PCR assays may notably vary depending on the choice of the standardisation method. The absolute quantification requires referring to a range of dilutions of the target sequence in known concentrations. Then, results are expressed relatively by dividing the number of cDNA copies by the number of colony forming units (CFU) obtained by enumeration of the bacterial culture (Vandecasteele et al., 2001). Recently, genomic DNA (gDNA) was proposed and proved to be a good semi-internal standard for gene expression studies in *Staphylococcus epidermidis* (Vandecasteele et al., 2002). Alternatively to the absolute quantification, relative quantification analyses gene expression relatively to the transcript amount of another gene as internal reference (Conlon et al., 2002; Goerke et al., 2001). The internal reference is usually a housekeeping gene which expression is expected to be constant. However, it was recently demonstrated for five housekeeping genes of *S. epidermidis* encoding for dihydrofolate reductase (*dhrf*), triosephosphate isomerase (*tpi*), guanylate kinase (*gmk*), 16S rRNA (*16S*) and heat-shock protein 60 (*hsp-60*), using cDNA/CFU ratio, that the level of expression of housekeeping genes highly varied during the different growth states of bacteria (Vandecasteele et al., 2001). Transcription of the housekeeping gene *gyr* (gyrase) has previously been used as internal reference for RTQ-PCR to evaluate the expressions of *RNAIII* (encoding δ-toxin) and *hla* (encoding α-toxin) genes (Conlon et al., 2002; Goerke et al., 2000, 2001). The ribosomique RNA *16S* is also frequently used in quantitative studies, as internal standard to analyse others gene expression (Vaudaux et al., 2002; Yarwood et al., 2002).

In the present study, the expression of three target transcripts, ica transcript of the *icaADBC* locus and RNAII and RNAIII transcripts of the *agr*-QSS locus, was evaluated during the growth of a clinical isolate of *S. aureus*. *icaADBC* locus is required for PIA (Polysaccharide Intercellular Adhesin) synthesis, and plays a pivotal role in adhesion and biofilm formation of *S. epidermidis* and *S. aureus* (Mack et al., 1994, 2000). *agr*-QSS locus is known to be implicated in quorum sensing and transcriptional regulation of adhesion and virulence genes of *S. aureus* (Ji et al., 1995; Novick, 1993; Shenkman et al., 2001; Vuong et al., 2000). Transcription levels of *RNAII*, *RNAIII* and *ica* were analysed using relative and absolute quantification methods. Absolute quantification referred to an external standard, consisting of dilution ranges of cloned target sequences, and to bacterial concentrations. For relative quantification, three internal standards were retained: *gyrA*, *gmk* and *16S* genes.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Sau383 is a clinical strain isolated from infected femoral pin and kindly provided by Dr. Ghnassia, Mignot hospital, Versailles. It was identified as *S. aureus* using conventional laboratory techniques and confirmed by 16S rDNA sequencing using *16S*-1 and *16S*-2 primers (Table 1) and ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

For all experiments, an overnight culture was made from bacterial frozen stock in 5 ml of Bacto™ Tryptic Soy Broth (TSB; Difco, BD, Sparks, USA) medium at 37 °C, and the cultures were grown after 1/100 dilution in TSB with shaking at 37 °C. After 3, 5, 7, 10, 15 and 20 h of incubation, an aliquot (100 μl) of culture was diluted and plated on TSB agar plates for cell counting and cultures (20 ml) were immediately frozen at −70 °C and stored at this temperature until RNA extraction.

2.2. RNA extraction and purification

Total RNA was extracted using the FastRNA™ kit blue (Q-Biogene, Illkirch, France) with a protocol adapted from Dobinsky and Mack, 2001. Frozen cultures were slowly defrosted on ice with 2.5 ml of 0.5 M EDTA (pH 8). Cultures were sonicated in a cool bath for 2 min (Branson 3510, Branson Ultrasonics, Danburg, CT). After 15 min centrifugation at 2200×*g*, pellets were resuspended in lysis mixture (Lysing Matrix B, Q-Biogene). Tubes were placed in a bead homogeniser (Vibrogen-Zellmühle, Edmund Bühler, Hechingen, Germany) for 5 min at 4 °C. Precipitation in DIPS (FastRNA™ kit) was performed

Table 1
Primers used in this study

Name	Sequence (5′ to 3′)	Accession number	Localisation
gyrA-1	TGGCCCAAGACTTTAGTTATCGTTATCC	AP003129.2	7282
gyrA-2	TGGGGAGGAATATTTGTAGCCATACCTAC		7533
gmk-1	TCGTTTTATCAGGACCATCTGGAGTAGGTA	NC_002745.2	1,191,057
gmk-2	CATCTTTAATTAAAGCTTCAAACGCATCCC		1,191,210
16S-1	CGGTCCAGACTCCTACGGGAGGCAGCA	L37597.1	322
16S-2	GCGTGGACTACCAGGGTATCTAATCC		777
RNAIII-1	GAATTTGTTCACTGTGTCGATAATCCATTT	AP003135.2	277,890
RNAIII-2	GAAGGAGTGATTTCAATGGCACAAGATAT		277,943
RNAII-6	TTCTTTTAAAGTTGATAAACCTAAACC	AP003135.2	280,155
RNAII-7	GAAGCTATCAACAACGAAATGCGCAAGTTC		279,661
RNAII-11	TATGAATAAATGCGCTGATGATATACCACG	AP003135.2	280,076
RNAII-12	TTTTAAAGTTGATAGACCTAAACCACGACC		280,149
ica-9	TCGCACTCTTTATTGATAGTCGCTACGAG	AP003138.2	73,745
ica-10	TGCGACAAGAACTACTGCTGCGTTAAT		73,831
ica-15	CGACGTTGGCTACTGGGATACTGATATGA	AP003138.2	73,338
ica-16	AAATGCGACAAGAACTACTGCTGCGTTAAT		73,831
M13S	CGCCAGGTTTTCCCAGTCACGAC	see pT-Adv kit (BD Biosciences)	
M13R	TCACACAGGAAACAGCTATGAC		

Primers were designed with the help of Oligo 6.23 software (Wojciech and Piotr Rychlik, Molecular Biology Insight, USA) and provided by Proligo.

at −20 °C during 18 h. At the end of the extraction, RNA pellets were suspended in 50 μl of DEPC–water (diethyl pyrocarbonate-treated double–distilled water).

RNA integrity was observed after electrophoresis on 1% agarose gel with BET staining. RNA quantification was performed by measuring the absorbance at 260 nm. Measuring $A_{260\ nm}/A_{280\ nm}$ ratio (always >1.8) assessed the nucleic acid purity.

Twenty-five micrograms of total RNA were treated with 20 U of RNase-free DNase (Ambion, Austin, USA) for 2 h at 37 °C, followed by extraction with 1 volume of phenol–chloroform (50:50) and precipitation with 2.5 volumes of ethanol (95%). Genomic DNA contamination was tested by PCR with *gyrA*-1 and *gyrA*-2 primers (40 cycles: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s). In case of a positive result, another RNase-free DNase treatment was performed and genomic DNA contamination tested again, until a negative result was obtained. DNA-free RNA was resuspended in 25 μl of DEPC–water and stored at −70 °C.

2.3. cDNA synthesis

Reverse transcription was performed using 5 μl of hot-denatured DNA-free RNA, 100 pmol of random hexamer primers (pd5(N6), Amersham Pharmacia Biotech, Orsay, France) and 100 U of Superscript II reverse transcriptase (Roche Diagnostics, Mannheim, Germany). The reaction mixture was incubated for 10 min at 20 °C, 30 min at 42 °C and 5 min at 37 °C. Products were precipitated by NaOAc (0.3 M, pH 6) and 2.5 volumes of ethanol (95%). They were then resuspended in 30 μl of DEPC–water and stored at −20 °C.

2.4. Target regions amplification and cloning

Sequences inside RNAII, RNAIII and ica loci were amplified by PCR and cloned in pT-Adv vector using the AdvanTAge™ PCR Cloning Kit (BD Biosciences, San Jose, USA). Primers used were RNAII-7 and RNAII-6 for the *RNAII* locus, RNAIII-1 and RNAIII-2 for the *RNAIII* locus and ica-15 and ica-16 for the *ica* locus (Proligo France SAS) (Table 1). Sau383 gDNA was used as template. *Taq* polymerase (Ependorff, Hamburg, Germany) was used as recommended by supplier (25 cycles: 30 s at 94 °C, 45 s at 51, 53 or 55 °C for RNAII, RNAIII or ica, respectively, and 45 s at 72 °C). PCR products were directly cloned in pT-Adv vector and transformed into Top 10F′ *Escherichia coli* competent cells, according to supplier recommendations

(BD Biosciences). Clones were screened by restriction profile and PCR with M13S and M13R primers (30 cycles: 94 °C for 30 s, 54 °C for 45 s, 72 °C for 80 s). Resulting plasmids, called pTA-RNAII, pTA-RNAIIII and pTA-ica, were then purified with mini Qiagen columns and quantified by $A_{260\ nm}$ measurements. Concentrations were calculated and dilutions made to obtain 10^{10} copies/μl.

2.5. Real-time quantitative PCR conditions

Quantification was performed using a Light Cycler apparatus and the FastStart DNA Master SYBR Green I kit (Roche), using 1 μl of cDNA or plasmid dilution, 3.75 pmol of each primer (Proligo), in a final volume of 10 μl. Magnesium concentration and hybridisation temperature depended on primers (Table 2). RTQ-PCR runs were divided in three phases, denaturation, amplification and fusion. In all cases the first phase of denaturation was performed at 95 °C for 8 min. Amplification phase included cycles of three steps: denaturation (95 °C for 15 s, temperature transition 20 °C/s), hybridisation (time and temperature depended on primers, see Table 2; temperature transition 20 °C/s) and elongation (72 °C for time depending on primers, see Table 2; temperature transition 20 °C/s), except for *ica-9-10* (hybridisation and elongation at 70 °C). Fluorescence acquisition was carried out at 72 °C (or 70 °C for *ica-9-10*) in single mode at the end of the elongation step. Amplification phase was stopped when fluorescence reached its maximum in each capillary or after a maximum of 45 cycles. Then a third phase of fusion was performed at 55 to 90 °C (temperature transition 0.2 °C/s) with stepwise fluorescence acquisition. Product specificity was evaluated by melting-curve analysis and by electrophoresis on 2% agarose gel and BET staining. Prior to 16S quantification, cDNA were diluted 1/100 in order to have results in the confidence interval of the technique.

2.6. Real-time quantitative PCR data analysis

Fluorescence was analysed by Light Cycler Data Analysis software (Roche). Crossing points (Cp) were established using the second derivative method. Target gene expressions were reported to the internal standards expression in the same extract or to concentration of the cloned external standards.

The *relative quantification* analyses the amount of a target transcript relatively to an internal standard (a housekeeping gene) in the same sample. Moreover, samples were normalised through a calibrator introduced in each run, that corrected the result of PCR for different detection sensitivities. Results were expressed as the target/internal standard concentration ratio of the sample divided by the target/internal standard concentration ratio of the calibrator. As target and internal standard gene had different sequences and amplicon lengths, it was probable that they would show different PCR efficiencies. PCR efficiency was first established for each couple of primers (Table 2). To do so, six different dilutions of a pool of cDNA (in arbitrary concentrations) were quantified in triplicate. Expression of Cp versus log of concentration allowed to draw a calibration curve by linear regression. PCR efficiency was equal to $10^{-1/slope}$. These efficiencies were taken into account in relative quantification, performed using the RelQuant software (Roche).

Table 2
Conditions and efficiency of RTQ-PCR

Upper primer	Lower primer	Magnesium concentration (mM)	Hybridisation temperature (°C)	Hybridisation time (s)	Elongation time (s)	PCR efficiency
gyrA-1	*gyrA*-2	3	63	10	15	1.89
gmk-1	*gmk*-2	3.5	55	10	15	1.89
16S-1	*16S*-2	4	59	12	22	1.84
RNAIII-1	RNAIII-2	4.5	58	10	10	1.72
RNAII-11	RNAII-12	4	53	10	15	1.90
ica-9	*ica*-10	4.5	70[a]	15[a]	–	1.87

[a] Hybridisation and elongation occurred at the same temperature.

The *absolute quantification* compares the amplification of a target transcript in the sample against a standard curve prepared with known concentrations of the same target previously cloned. The standard curves were generated by using 10-fold serial dilutions (10^2 to 10^{10} copies/μl) of each plasmid (pTA-RNAII, pTA-RNAIII and pTA-ica) and quantified in duplicate by RTQ-PCR to make standard curves.

3. Results

3.1. Comparison of three internal standards: gyrA, gmk and 16S

RNA extraction was performed using cultures in late-exponential and stationary growth phases, i.e., 3, 5, 7, 10, 15 and 20 h after inoculation. Taking into account the high variation in the housekeeping gene expression during the earlier exponential phase, our measurement started 3 h after inoculation. Cell concentrations ranged from 2×10^5 to 2.8×10^9 CFU/ml. For each growth duration, total RNA samples were extracted from five independent cultures and the corresponding cDNA pools prepared by random priming.

Then, six transcripts (gyrA, gmk, 16S, RNAII, RNAIII and ica) were quantified by RTQ-PCR. PCR efficiency for each couple of primers is given in Table 2. Three housekeeping genes, *gyrA*, *gmk* and *16S* were used as internal standards. Quantification in each sample was done by expressing the ratio of target genes (i.e., RNAII, RNAIII and ica transcripts) versus standard transcripts. Each independent experimental result and average curves are shown in Fig. 1. Some independent results are scattered, but average curves obtained with 3, 4 or 5 points per condition are similar.

Regardless the considered target gene, the appearance of the expression curves is similar for each internal standard, particularly, for *gyrA* and *gmk*. Differences between them were more important between 3 and 7 h, i.e., before stationary phase, suggesting that these two genes are not co-regulated. When 16S was used as internal standard, the appearance of the curves of the RNAII and RNAIII gene expression were similar to each other (Fig. 1g and h), but quite different from *ica* gene expression curve (Fig. 1i). This latter curve decreases rapidly from the beginning of the measurement, 3 h after inoculation, while in all the others an increase is observed from 3 to 5 h, followed by a significant decrease.

The relative quantification of three target gene expressions, using *gmk* as internal standard, showed profiles with a progressive increase until 5–10 h from the beginning of the stationary phase, followed by a slow decrease (Fig. 1d–f). With *gyr*A as internal standard, for all three genes, a peak was observed at 5 h between two lower values at 3 and 7 h (Fig. 1a–c). Then, after 7 h, the evolution was similar to one expressed versus *gmk*. To summarise, the expression of the three target genes was significantly different depending on the use of *gmk* or *gyr*A as internal standards. On the contrary, the three target gene expression profiles versus *16S* presented more differences to each other, particularly from 3 to 7 h.

3.2. Absolute quantification

Since the expression profile of each target gene is different depending on the used internal standard, we have chosen an alternative quantification method to get a mean of comparison. To find the most convenient technique, we analysed the three target transcripts expression by absolute quantification and compared corresponding results.

Amplified sequences of the three target genes were cloned in pT-Adv vector. The resulting plasmids were purified, their concentration calculated using absorbance at 260 nm measurement and ranges of dilutions were made to serve as standards. Each standard curve was made in duplicate, using 10-fold serial dilution of purified plasmids from 10^{10} to 10^2 copies/μl. Using Light Cycler Analysis software, resulting quantification gave regression lines with slopes of −3.206, −3,590 and −3.990 and error of 0.0553, 0.226 and 0.137 for pTA-RNAII, pTA-RNAIII and pTA-ica plasmids, respectively. Previous raw data of RNAII, RNAIII and ica transcript quantifications were reported to those standard curves to determine absolute quantification. Results were then divided by the number of CFU in the same culture. The results are summarised in Fig. 1j–l. Despite the different scales, expression profile of

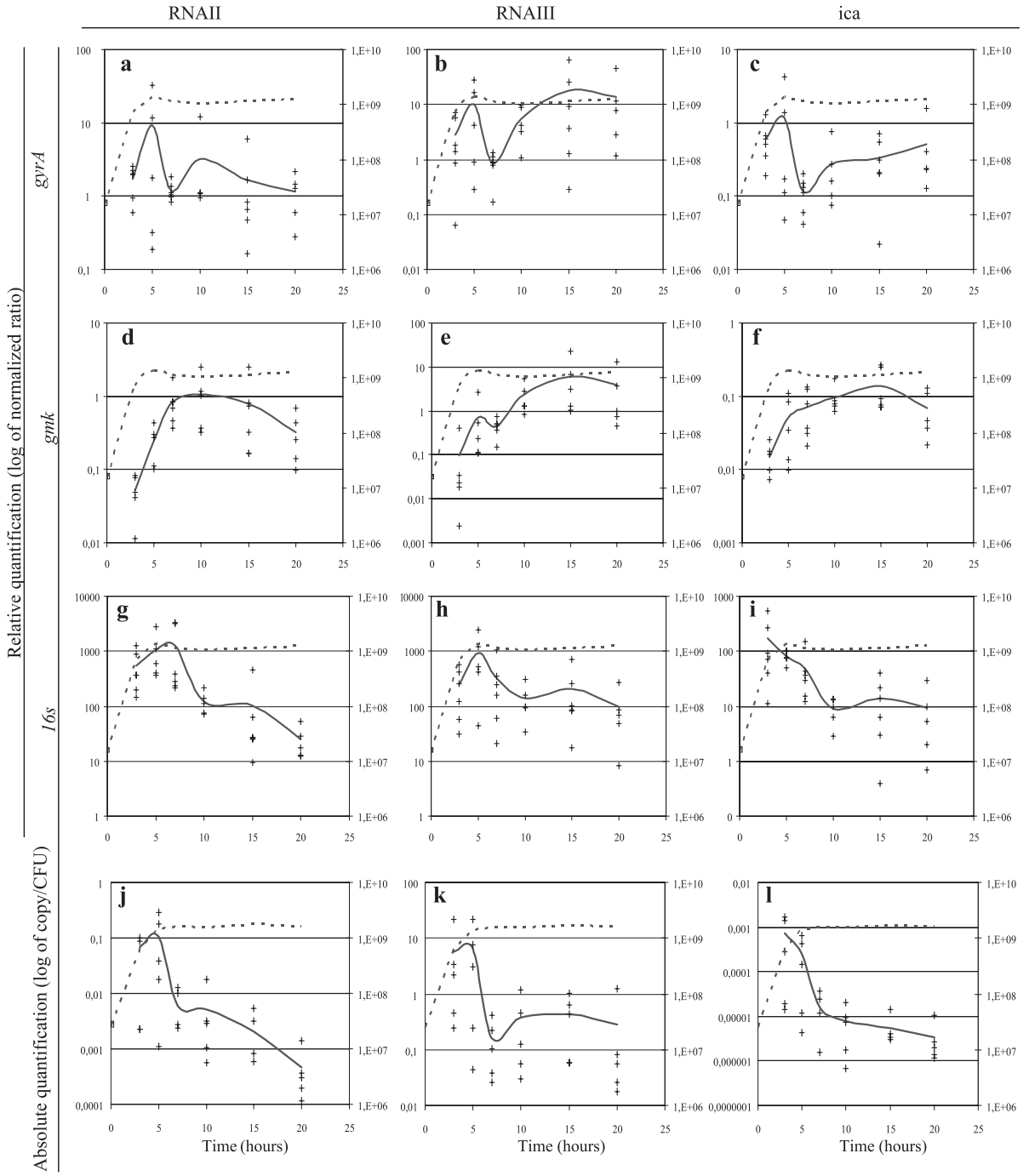

Fig. 1. Expression profiles of RNAII, RNAIII and ica transcripts versus gyrA (a, b, c), gmk (d, e, f) and 16S (g, h, i) housekeeping gene transcripts or versus calibrations curves (j, k, l) during growth. Crosses and solid lines represent the log of RTQ_PCR results and refer to left axis, each cross represents an independent experimental result, solid lines represent average expressions. Dashed lines represent bacterial growth curve and refer to right axis (log of CFU/ml).

each transcript calculated versus external standard is quite similar to one expressed versus 16S in relative quantification.

4. Discussion

The aim of this study was to test the reliability of RTQ-PCR to evaluate the expression of staphylococcal genes during growth in batch culture. The relative quantification using internal standard is mainly used. This very sensitive method minimises differences between samples, which are due to the instability of bacterial RNA molecules and multiplicity of steps in cDNA preparation. Moreover, it avoids the cloning step and the preparation of external standard curves. It also avoids the enumeration step by CFU counts or gDNA quantification by PCR. It has already been shown that the housekeeping metabolism of a *S. epidermidis* 10b strain is highly variable during the growth (Vandecasteele et al., 2001). In fact, the use of housekeeping genes as internal standards in relative quantification can be considered adapted to compare the expressions of two or more genes at the same time and conditions of growth. Otherwise, an internal RNA standard is less suitable in bacterial gene expression experiments in *Staphylococcus* sp. (Vandecasteele et al., 2002). Therefore, the reliability of relative comparison depends largely on the used standards for normalisation of the results.

To appreciate relative expression of three target genes *RNAII*, *RNAIII* and *icaADBC* during the growth of *S. aureus*, three internal standards were separately used: *gmk*, *gyrA* and *16S*. Two of them, *gmk* and *16S* transcripts, have already been quantified by RTQ-PCR in *S. epidermidis* 10b strain during growth, by expressing their amounts versus CFU number (Vandecasteele et al., 2001). Vandecasteele et al. (2001) study showed that *gmk* expression varied up to 7 h from the beginning of the stationary phase, while this of 16S rRNA became stable at the end of the exponential phase (5 h after inoculation). Since *gyrA* and *gmk* are both implicated in nucleic acids replication, it seems interesting to compare them as internal standards. The relative stabilisation of 16S rDNA expression in earlier and prolonged stationary phase should allow to better study the differences in expression profiles of the different genes of interest, when used as internal reference during the stationary phase of growth.

Thus *gmk*, *gyrA* and *16S* of *S. aureus* Sau383, a clinical isolate, were used here. The results obtained by RTQ-PCR quantification using them as internal standards, are shown in Fig. 1.

The quantification profiles of the three target genes expressed versus *gyrA* (Fig. 1a–c) and *gmk* (Fig. 1d–f) are nearly identical. These results confirm that the use of metabolic housekeeping genes, like *gyr* or *gmk* is inappropriate during all the growth stages. The quantification obtained with 16S as standard is different for RNAII, RNAIII and ica transcripts (Fig. 1g–i). These appearances of profiles are close to those obtained with an external standards (Fig. 1j–l).

Methods using cloned target sequences as external standard and CFU counts (Vandecasteele et al., 2001) or genome quantification (Vandecasteele et al., 2002) have the same disadvantage. They do not take into account the variation due to the RNA degradation, which is avoided by the choice of internal transcript as internal standard.

Despite the high transcriptional level of rRNA (90% of total RNA in bacteria), 16S rRNA gave the most reliable results. To reduce the important difference between the quantities of internal standard and the target gene, as it is recommended in RTQ-PCR, mRNA enrichment method could be considered (McGowan et al., 1998). This enrichment improves significantly the sensitivity of transcription analysis methods like micro-arrays and RTQ-PCR. Provided that the process removes the same amount of rRNA, 16S rRNA seems to be the most appropriate internal standard.

In conclusion, in agreement with literature data for *S. epidermidis* (Vandecasteele et al., 2001, 2002), our results confirm that during the exponential growth of bacteria, the use of housekeeping genes as internal standards in relative quantification studies is unsuitable, due to the rapid changes in their expression. However, the transcript of 16S rRNA gene may be used, given the similarity to the results of absolute quantification demonstrated in our study. This finding provides reference to use the 16S rRNA gene as internal standard to follow the expression of a given gene over time during the stationary phase of *S. aureus* growth. Considering the similarity in their

metabolic regulation, this may be extended to all *Staphylococcus* genus.

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