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Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR

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Constantly expressed genes are used as internal controls in relative quantification studies. Suitable internal controls for such studies have not yet been defined for *Pseudomonas aeruginosa*. In this study, the genes *ampC*, *fabD*, *proC*, *pbp-2*, *rpoD* and *rpoS* of *P. aeruginosa* were compared in terms of expression stability by real-time quantitative RT-PCR. A total of 23 strains with diverse resistance phenotypes were studied. Stability of expression among the housekeeping genes was assessed on the basis of correlation coefficients, with the best-correlated pair accepted as being the most stable one. Eventually, *proC* and *rpoD* formed the most stable pair ($r = 0{\cdot}958$; $P < 0{\cdot}001$). Next, in four ciprofloxacin-selected *nfxC*-like mutants, levels of *oprD*, *oprM* and *oprN* mRNA were compared with those of their wild-type counterparts. The comparison was made after correcting the raw values by the geometric mean of the internal control genes *proC* and *rpoD*. The level of *oprN* mRNA was significantly up-regulated, while the *oprD* gene was down-regulated (although this difference was statistically insignificant), in the mutants. This expression pattern was consistent with that of the expected expression profile of *nfxC*-type mutants; this experiment therefore lends further support to the use of *proC* and *rpoD* genes simultaneously as internal controls for such studies.

INTRODUCTION

Pseudomonas aeruginosa is an important nosocomial pathogen, particularly in intensive care units (Yucesoy *et al.*, 2000). Members of this species are inherently resistant to a range of antibiotics. In addition, they are capable of conferring resistance to others by shifting the regulation levels of various innate genes (Livermore, 1992).

Porins (particularly porin D) and efflux pumps of the resistance-nodulation-division (RND) family are the currently recognized genetic systems that are associated significantly with antibiotic resistance (Okamoto *et al.*, 2001). The exact links between the expression patterns of these and other resistance phenotypes have not been fully elucidated, and inconsistent conclusions appear in the literature (Masuda *et al.*, 2000; Morita *et al.*, 2001; Sumita & Fukasawa, 1996; Trias *et al.*, 1989). Therefore, studies focusing on this topic still attract considerable interest.

Abbreviation: MH, Mueller–Hinton.

Levels of expression of these genes have traditionally been studied by Western blotting with the aid of mAbs. Unfortunately, this method is unable to help in studying different proteins simultaneously and so is weak in relative comparisons. Moreover, mAbs are not commercially available. The quantification of mRNAs by real-time RT-PCR has been used with great success in other fields (Johnson *et al.*, 2000; Wang & Brown, 1999). We suggest that this highly sensitive method may also be useful in relative comparison of resistance gene expression.

The reliability of a relative comparison depends largely on the normalization of unwanted variations between samples. Constantly expressed genes, often selected from among housekeeping genes, are used as internal controls for normalization of the results. The proportion of mRNA of constantly expressed genes in the total cellular RNA is assumed to be equal between different samples. Normalization of raw values by means of internal controls therefore serves to eliminate sample-to-sample variation of the RNA isolation and reverse transcription steps and, even more importantly, serves to eliminate variations in total transcriptional activity between cells.

For this purpose, six housekeeping genes were compared in this study. These were pyrroline-5-carboxylate reductase (*proC*), malonyl CoA : acyl carrier protein (ACP) transacylase (*fabD*), sigma factors RpoD (*rpoD*) and RpoS (*rpoS*), penicillin-binding protein 2 (*pbp-2*) and chromosomal beta-lactamase (*ampC*). The expression stability of these housekeeping genes was first investigated as proposed by Vandesompele *et al.* (2002) and, later, the levels of *oprM*, *oprN* and *oprD* mRNA were compared in a set of *P. aeruginosa* strains.

METHODS

Strains, resistance tests and beta-lactamase assays. We selected 17 *P. aeruginosa* strains from a set obtained from university hospitals in four different geographical regions of Turkey. The bacteria had been identified in those universities by various identification systems. The strains were, nevertheless, reidentified in our institute by classical methods and, if required, by the non-fermenter ID panel of the Becton Dickinson system (Diagnostic Instrument Systems).

Of the 17 strains selected, 10 were fully susceptible to antibiotics of the main classes, while the others were of different resistance phenotypes. We challenged the susceptible strains in Mueller–Hinton (MH) broth with ciprofloxacin (0·1 μg ml^{-1}) in order to obtain resistant variants. Consequently, six isogenic mutants with various resistance patterns were selected. These mutants, as well as their counterparts, were included in the study group.

Resistance patterns were determined by the disk diffusion method on MH agar plates. MICs were determined by either E-test strips on MH agar plates or by the agar dilution method as described by the NCCLS. Antibiotic disks and MH agar were obtained from Oxoid while E-test strips were sourced from AB Biodisc. Powder forms of the antibiotics were obtained as gifts from the respective companies.

Beta-lactamases were analysed as described elsewhere (Vahaboglu *et al.*, 1998). Extracts obtained by freezing and thawing of dense bacterial suspensions were applied to polyacrylamide gels with ampholytes ranging from pH 3 to 10. Nitrocefin overlay and migration relative to TEM-1 and SHV-1 standards enabled us to evaluate the pI values of beta-lactamases. Precise identification of the beta-lactamases depended on sequence analysis of PCR products as described previously (Vahaboglu *et al.*, 1998).

Random amplified polymorphic DNA (RAPD) typing. Clonal variability was further ensured by RAPD typing of eight selected strains, two from each region. DNA was isolated from fresh overnight agar cultures. A loopful of bacteria was homogenized in 50 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and lysis was accomplished with 500 μl guanidium thiocyanate (6 M) plus 0·5 % sodium lauroylsarcosine for 10 min at room temperature. DNA was extracted first by phenol/chloroform and then by chloroform/isoamyl alcohol (24 : 1, v/v) and then precipitated with 0·1 vols sodium acetate (3 M, pH 5·4) plus an equal volume of 2-propanol at room temperature. Precipitates were collected by centrifugation (10 min at 12 000 ***g***) and then the pellets were washed twice with 70 % ethanol, air-dried for 2 min and resuspended in 30 μl double-distilled water.

RAPD PCR was performed as described elsewhere (Kerr *et al.*, 1995; Renders *et al.*, 1996). The primers were ERIC-1R (5′-AAGCTCCTGG GGATTCA-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). Master mixtures were prepared as described in the above references. However, the amplification program was modified as follows: one cycle of denaturation for 5 min at 95 °C followed by 25 cycles of 3 min at 39 °C (with an increase of 0·3 °C every cycle), 2 min at 72 °C and 1 min at 94 °C and 30 cycles of 2 min at 44 °C, 3 min at 72 °C and 1 min at 94 °C. A final extension for 1 h at 72 °C completed the procedure.

PCR products were separated on a 2 % agarose gel and visualized by ethidium bromide staining. For better resolution, they were also run on a 6 % acrylamide/bis-acrylamide gel (data not shown). The banding patterns of both gels were analysed by the freely distributed gel analysis software LabImage (version 2.62). Molecular sizes of the bands were calculated by this software relative to the marker DNA.

RNA isolation and reverse transcription. Total RNA was isolated from 5 ml fresh overnight (approx. 18 h) broth culture (MH broth) by using the NucleoSpin RNA II kit (Macherey-Nagel), as described by the manufacturer. Genomic DNA was eliminated by RNase-free DNase I treatment during the isolation procedure. Finally, RNAs were eluted from the silica membranes in a volume of 40 μl diethyl pyrocarbonate-treated double-distilled water. The A_{260} of the resulting RNA solution was between 1 and 10. Reverse transcription was performed at 42 °C for 90 min by using random hexamer primers so as to obtain cDNA copies of mRNAs (2 μl) with 100 IU MMuLV reverse transcriptase (MBI Fermentas) in 20 μl total volume. Concentrations of cDNAs were adjusted on a LightCycler (Roche Diagnostics). For every sample, 1 μl cDNA and 9 μl SYBR Green I (the same concentration as indicated by the manufacturer for the PCR assay) were mixed in capillary tubes. After incubation at 95 °C for 5 min, fluorescence emissions were read at 55 °C with the real-time fluorometry facility of the LightCycler. This enabled us to compare the total cDNA concentrations of the samples with the control transcript, which was approximately 1 mg ml^{-1} at the highest dilution. Concentrations of cDNAs of the samples were adjusted to a level close to the second dilution (10^{-1}) of the control cDNA. This adjustment was critical for performing successful calculations. The aim was to keep the cDNA concentrations of the samples between the concentrations of the controls in order to avoid large variations during calculations by the LightCycler software.

Real-time PCR. The sequences of the genes studied were obtained from GenBank and the primers were designed with the aid of the OLIGO software (version 5.0; Molecular Biology Insights). The sequences of the primers are shown in Table 1.

PCR was performed in the LightCycler in capillary glass tubes with the LightCycler FastStart DNA Master SYBR Green I kit (Roche). Work was always carried out on desktop coolers (pre-cooled to 4 °C). Master mixtures were prepared exactly as recommended by the manufacturer, except for the concentration of Mg^{2+}. The final concentrations of Mg^{2+} and primers were respectively 2·5 mM and 50 pmol per reaction.

The control cDNA was from *P. aeruginosa* ATCC 27853 and the primers of the control reactions were specific for *pbp-2*. An arbitrary concentration value of $1{\cdot}5 \times 10^4$ copies of the *pbp-2* gene was assigned to the control transcript. Tenfold dilutions of this down to 15 copies of the *pbp-2* gene were always included in the reactions.

PCR was accomplished after a 5 min activation and denaturation step at 95 °C, followed by 45 cycles of 15 s at 95 °C, 10 s at 60 °C and 15 s at 72 °C.

Primer dimers and other artefacts were evaluated by melting curve analysis and eventually only dimer- and artefact-free reactions were considered valid. Results were read with the 'second derivative maximum' algorithm of the software provided. The LightCycler software generated a standard curve by plotting 'crossing cycle number' versus logarithms of the given concentrations for each control. Eventually, a regression line was drawn between these points. The software calculated the concentrations of the studied genes with the aid of this standard curve.

Statistical analysis. The stability of mRNA expression was assessed by

Table 1. Primers used in quantification studies

Target	Forward (5′⟶3′)	Reverse (5′⟶3′)
ampC	AGATTCCCCTGCCTGTGC	GGCGGTGAAGGTCTTGCT
fabD	GCATCCCTCGCATTCGTCT	GGCGCTCTTCAGGACCATT
oprD	TCCGCAGGTAGCACTCAGTTC	AAGCCGGATTCATAGGTGGTG
oprM	CCATGAGCCGCCAACTGTC	CCTGGAACGCCGTCTGGAT
oprN	GCGCGAGAAGATTGCCCTGAG	GCGGCGAAAGGTCCACTGTCA
pbp-2	CCGCCACTACCCGCTGAAG	TGCCGTGCAACTCGCTCTC
proC	CAGGCCGGGCAGTTGCTGTC	GGTCAGGCGCGAGGCTGTCT
rpoD	GGGCGAAGAAGGAAATGGTC	CAGGTGGCGTAGGTGGAGAA
rpoS	CTCCCCGGGCAACTCCAAAAG	CGATCATCCGCTTCCGACCAG

calculation of Spearman's correlation coefficients of the raw concentration data with the aid of the statistical package SPSS (version 9.0). The best-correlated pair was considered to be the most stable one.

Stability was further evaluated by a freely distributed MS Excel application (geNorm). Detailed information on this application can be obtained from Vandesompele *et al.* (2002). This approach assumes that minimally regulated, stably expressed genes stay in a constant ratio to each other. In other words, in a given set of genes, it must be the pair of most stable genes that will be able to keep the ratio to each other constant in different samples. Importantly, co-regulated genes are exceptions to this assumption and they are not included.

The applet geNorm helps to calculate the gene expression stability measure (M), which is the mean pair-wise variation for a gene from all other tested control genes (Vandesompele *et al.*, 2002). A higher value of M means greater variation in expression. The stepwise exclusion of genes with the highest M values allows the ranking of the tested genes according to their expression stability. The proposed threshold for eliminating a gene as unstable was an expression stability measure of $\geqslant 0{\cdot}5$.

Raw quantities were corrected by dividing a value by the geometric mean of *proC* and *rpoD* genes of the same sample. Relative comparisons were done between corrected values with the ANOVA test for significance.

RESULTS AND DISCUSSION

A total of 23 strains were included in the study. The group was composed of 17 wild-type strains and six ciprofloxacin-selected, isogenic mutants. Clonal heterogeneity was investigated by DNA typing of eight of these wild-type strains. One sample did not provide a readable pattern, probably because of inhibitors carried over from the DNA isolation step. However, the other seven were amplified successfully and the fingerprints were sufficiently polymorphic to confirm the clonal diversity (Fig. 1).

Of the 17 wild-type strains, seven were already resistant to various antibiotics. It is noteworthy that three of these did not produce a beta-lactamase other than the chromosomal one (Table 2). These strains were probably resistant because of activated porin and/or efflux systems. Of the six ciprofloxacin-selected mutants, two were resistant only to the fluoroquinolones, while the other four expressed *nfxC*-like multiple-resistance phenotypes (Table 3). In addition to fluoroquinolones, the MIC of imipenem increases among

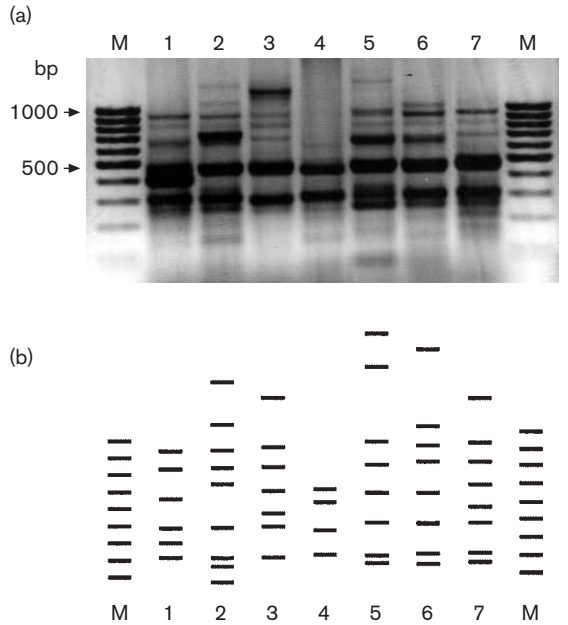

Fig. 1. (a) RAPD fingerprints (negative exposure). Lanes: M, molecular size marker (100 bp DNA ladder); 1–7, selected strains (strains 2 and 5–7 are the parents of *nfxC* mutants). (b) Fingerprints according to the lengths (bp) of fragments calculated by gel analysis software. Lanes are the same as in (a).

nfxC mutants because of the concurrent down-regulation of *oprD* (Maseda *et al.*, 2000; Ochs *et al.*, 1999). The resistance phenotypes of the mutants were in agreement with this.

Interestingly, the MICs of ceftazidime were variable; one was increased and one decreased while the other two were unchanged. One study reported an unexplained decrease in the ceftazidime MICs of *nfxC* mutants (Maseda *et al.*, 2000). However, another study showed that the functional subunit of the *mexEF–oprN* operon is not related to beta-lactam hypersusceptibility (Maseda *et al.*, 2000). These observations suggest the existence of other co-operating systems responsible for beta-lactam susceptibility or resistance in *nfxC* mutants. This issue remains unresolved.

Table 2. Antibiotic-resistance phenotypes and extrinsic beta-lactamases in strains examined

Seventeen strains were examined: strains R1–7, resistant wild-type; S1–10, susceptible wild-type. Strains were classed as resistant (R) or susceptible (S) according to NCCLS criteria.

Strain	Imipenem	Meropenem	Ceftazidime	Ciprofloxacin	Amikacin	Beta-lactamase*
S1–S10	S	S	S	S	S	None
R1	S	R	R	R	R	None
R2	R	S	S	S	S	None
R3	R	S	S	R	S	None
R4	R	R	R	S	S	oxa-17†
R5	R	R	R	R	R	oxa-17
R6	R	R	R	S	R	pI 5·2
R7	R	R	R	S	R	pI 5·2

*Other than chromosomal AmpC beta-lactamase.

†From sequence analysis.

Table 3. MICs of ciprofloxacin-selected (*nfx*C-like) mutants compared with wild-types

MICs are given in μg ml^{-1} for the parent strain/mutant.

Mutant	Meropenem	Imipenem	Ceftazidime	Ciprofloxacin	Levofloxacin
NFX1	0·06/1	0·25/1	0·25/0·25	4/16	8/32
NFX2	0·5/1	0·25/1	0·25/4	4/16	8/64
NFX3	0·12/0·5	0·5/2	4/0·25	4/32	8/64
NFX4	0·5/1	0·5/2	0·25/0·125	4/32	8/64

The housekeeping metabolism of prokaryotes has been shown to be highly variable (Vandecasteele *et al.*, 2001), so genes expressed stably under one condition might not be so under others. Stability in terms of mRNA expression in prokaryotic cells, therefore, should be tested under equivalent conditions with the investigated setting. In other words, internal controls intended for use in resistance gene quantification studies should be tested in strains with changing resistance phenotypes. The diversity in the resistance phenotypes of this study group fulfils this requirement.

Six genes were compared in this study group. In order to avoid co-regulated genes, we carefully selected genes that are distantly related in metabolic function and chromosomal order. The selected genes participate in critical functions. *pbp-2* has a central role in peptidoglycan metabolism, while it has some relation to the rod-shape-determining protein. *ampC* is involved in cell-wall recycling (Jacobs *et al.*, 1995). The sigma factor-encoding *rpoD* is a critical housekeeping gene (Schnider *et al.*, 1995). *proC* is involved in amino acid biosynthesis, while *fabD* is involved in a different class of metabolic function (Kutchma *et al.*, 1999). However, their metabolic importance was not the only reason for selecting these genes. Equally important is that they all were shown in an *Escherichia coli* DNA array study to be expressed at levels comparable to outer-membrane proteins (Wei *et al.*, 2001). These data led us to assume that these genes might also be expressed in sufficient quantities in *P. aeruginosa*, a further advantage in optimization of the PCR test.

The raw quantities of mRNA of the six genes studied obtained by RT-PCR are shown in Table 4. Correlation coefficients indicated *proC* and *rpoD* as the most significant pair ($r = 0{\cdot}958$; $P < 0{\cdot}001$). Similarly, the stepwise exclusion of the genes with the highest M values by geNorm left *proC* (M = 0·36) and *rpoD* (M = 0·36) as the most stable genes.

Next, the levels of *oprD*, *oprM* and *oprN* mRNA of the four *nfxC*-like isogenic mutants were compared with those of their wild-type counterparts. In this experiment, the geometric mean of the levels of *proC* and *rpoD* in a sample was its normalization factor. Comparison of the normalized quantitative values of these genes is shown in Table 5. The mean level of *oprN* was 1·3 times higher in the mutants, while the mean level of *oprD* was 1·28 times lower. Values were comparable for all the mutants. Interestingly, the statistical comparison was significant only for *oprN* concentrations.

Data on expression of these resistance genes in the literature have to date been obtained by immunoblotting. Evaluations were dependent on crude differences and lacked precise numerical values. Therefore, comparison of the results of this study with the literature was not possible. Moreover, immunoblotting indicates differences in the level of mature

Table 4. Raw concentrations of mRNA of six genes from 23 strains obtained by RT-PCR

Concentrations are given as copies μl^{-1} ($\times 10^{-3}$). S1–S10 are susceptible strains; S5 and S6 are the parents of M1 and M2 (ciprofloxacin-selected non-*nfxC*-type mutants) and S7–S10 are the parents of NFX1–NFX4 (ciprofloxacin-selected *nfxC*-type mutants). R1–R7 are wild-type resistant isolates.

Strain	*proC*	*fabD*	*rpoD*	*rpoS*	*pbp-2*	*ampC*
S1	2·17	2·28	3·32	2·56	0·37	2·40
S2	0·92	0·047	1·33	1·32	0·36	1·62
S3	0·64	1·05	0·76	0·77	0·43	0·53
S4	1·49	1·52	2·60	1·92	0·49	1·74
S5	0·35	0·29	0·30	0·51	0·069	0·54
S6	0·74	1·39	1·72	1·25	0·17	1·29
S7	0·24	0·31	0·28	0·87	0·21	0·29
S8	0·27	0·43	0·37	1·14	0·04	0·59
S9	0·42	0·41	0·50	0·70	0·21	0·60
S10	0·14	0·25	0·41	0·45	0·14	0·39
R1	5·91	3·82	8·55	6·89	0·68	7·65
R2	2·42	3·67	4·79	5·20	0·329	5·60
R3	0·48	0·82	0·89	0·41	0·30	1·13
R4	1·92	2·00	2·51	2·41	0·17	3·11
R5	1·88	1·24	2·75	2·87	0·17	2·74
R6	0·96	0·66	1·40	1·18	0·13	1·39
R7	0·38	0·29	0·58	0·46	0·21	0·77
M1	1·50	1·31	2·26	1·98	0·24	2·58
M2	1·60	1·66	2·51	1·94	1·31	1·39
NFX1	0·92	0·95	1·17	1·26	0·48	1·50
NFX2	0·47	0·60	0·80	0·93	0·13	1·04
NFX3	0·87	0·86	1·49	1·22	0·56	1·42
NFX4	1·18	1·11	1·71	1·45	0·92	1·87

Table 5. Comparison of mRNA concentrations of *nfxC*-like mutants and their wild-type counterparts

Property	Wild-type ($n = 4$)	Mutants ($n = 4$)	P*
oprD			
Mean (SD)	0·883 (0·169)	0·689 (0·104)	0·099
Geometric mean	0·872	0·683	
Variance	0·029	0·011	
oprM			
Mean (SD)	1·173 (0·173)	1·169 (0·202)	0·977
Geometric mean	1·163	1·155	
Variance	0·030	0·041	
oprN			
Mean (SD)	0·899 (0·066)	1·208 (0·207)	0·030
Geometric mean	0·897	1·195	
Variance	0·004	0·043	

*Obtained by ANOVA.

protein. It is known that mRNA expression predicts mature protein levels poorly and there may be up to 30-fold differences (Gygi *et al.*, 1999). Transcription and translation are regulated individually to some extent. The levels of mRNA of these genes may, therefore, not be exactly in accord with protein levels. However, the quantification results with RT-PCR in this study were in agreement with the expected expression profile of *nfxC*-type mutants.

Resistance due to the altered regulation of intrinsic genes in *P. aeruginosa* is not well understood. This type of resistance may depend on the regulation of a more composite co-regulated network of multiple operons as well as the 'quorum-sensing systems' of *P. aeruginosa* (Kohler *et al.*, 2001; Poole, 2001). Unfortunately, our understanding of this is extremely limited due to the limited power of the methods used at present. Therefore, further studies using new methods deserve increased scientific interest. We believe that real-time quantification with the selection of suitable internal control genes will facilitate studies and provide new insights into the regulatory alterations of innate genes and the multiple antibiotic resistance problem of *P. aeruginosa*.

This study showed that *proC* and *rpoD* form the most stable pair in a set of clonally unrelated *P. aeruginosa* strains with diverse resistance phenotypes. Thus, this pair may be used as internal controls in relative comparison studies of resistance genes in *P. aeruginosa*.

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