

Transcript profiling of the *Pseudomonas aeruginosa* genomic islands PAgI-2 and pKLC102

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The phylogenetically ancient genomic islands of the abundant PAgI-2/pKLC102 family are prone to horizontal gene transfer amongst proteobacteria, and account for most genomic diversity in *Pseudomonas aeruginosa*. The mRNA expression levels of the sequenced PAgI-2 and pKLC102 islands were determined in *P. aeruginosa* clone C strains C and SG17M during exponential and stationary growth in Luria broth or Vogel–Bonner mineral medium. Of the 111 ORFs of PAgI-2, only one gene was significantly expressed at a level of more than 0.0001 % of total RNA. The individual mRNA transcripts of the 103 pKLC102 ORFs, however, were present in the range of 0.001 % to more than 1 % in the bacterial RNA population, and amounted altogether to more than 10% of cellular RNA. Homologous genes were strongly transcribed from pKLC102, but not at all from PAgI-2 under the tested conditions. Thus PAgI-2, which was stably captured by its host chromosome, was transcriptionally silent, whereas the mRNA transcripts derived from the mobile and episomally replicating pKLC102 were constitutively more abundant in the cell than the mRNA pool transcribed from the core genome.

INTRODUCTION

The *Pseudomonas aeruginosa* chromosome consists of three hypervariable regions (Römling *et al.*, 1995, 1997) that harbour the most abundant genomic islands in the *P. aeruginosa* population. The related pKLC102-type and PAgI-2-type islands are present in ~70 and ~40 % of *P. aeruginosa* strains, respectively (Klockgether *et al.*, 2007). pKLC102-type islands (He *et al.*, 2004; Klockgether *et al.*, 2004) integrate into one of the two copies of a tRNA^{Lys} gene at positions PA0976.1 and PA4541.1 of the PAO1 reference genome (Stover *et al.*, 2000), whereas PAgI-2 type islands preferentially target one of the two tRNA^{Gly} genes at position PA2819.1-2 (Larbig *et al.*, 2002). pKLC102-type and PAgI-2-type islands belong to an ancient family of genomic islands in proteobacteria and share a syntenic backbone of 36 homologues that include genes related to DNA replication or genetic mobility, or that encode conserved hypothetical proteins of unknown function (Juhas *et al.*, 2007; Mohd-Zain *et al.*, 2004). The islands differ in genetic mobility. PAgI-2-type islands spread across species barriers to other proteobacteria (Gaillard *et al.*, 2006; Müller *et al.*, 2003), but their spontaneous excision rates are well below 10⁻⁷ (Klockgether *et al.*, 2007), and the expression of the integrase is under stringent regulation (Sentchilo *et al.*, 2003a, b). pKLC102, however, is the most mobile genomic island known to date (Klockgether *et al.*, 2007). Being a

hybrid of phage and plasmid elements, the spontaneous excision rate from the host chromosome is at least 10⁻¹. The copy number of extrachromosomal circular pKLC102 in the cell varies between one and 30 during bacterial growth. Qiu *et al.* (2006) have proven that the pKLC102-type island PAPI-1 can be transferred by conjugation into other *P. aeruginosa* strains, where it integrates into one tRNA^{Lys} gene of the recipient chromosome.

We wanted to know whether the differential mobility of the island types is mirrored by differential utilization of the encoded genes. Taking the completely sequenced islands PAgI-2 and pKLC102 as prototypes, the clone C strains C and SG17M (Römling *et al.*, 1997) were assayed for mRNA expression of the 103 pKLC102 and 111 PAgI-2 ORFs. PAgI-2 ORFs were not transcribed or were weakly transcribed under standard growth conditions, whereas the mRNA transcripts of the mobile pKLC102 made up at least 10 % of the RNA of the bacterial cell. For selected ORFs, the transcript level was determined in detail by semiquantitative RT-PCR.

METHODS

Bacterial strains and growth conditions. *P. aeruginosa* clone C strains C and SG17M had been isolated from the airways of a cystic fibrosis patient and from a river, respectively (Römling *et al.*, 1997). PAO DSM 1707 was used as the *P. aeruginosa* reference strain.

Bacteria were grown at 37 °C in either Luria–Bertani (LB) broth or modified Vogel–Bonner (VB) medium (7.6 mM sodium citrate, 28 mM NaNH₄HPO₄, 37 mM K₂HPO₄, 3.3 mM MgSO₄, 213.4 mM potassium-(D)-gluconate; pH 7.2). Cells were harvested in the exponential phase at OD₅₇₈ 1.0 and in the stationary phase at OD₅₇₈ 3–4.

PAGI-2 and pKLC102 macroarrays. The macroarrays (Klockgether *et al.*, 2007) consisted of Hybond-N⁺ membranes (Amersham), on which 208–805 bp PCR-amplified sequences of pKLC102 or PAGI-2 had been spotted in a 96 (8×12) dot format. For the PAGI macroarrays, 91 PCR products were distributed onto the membrane representing 94 of the 111 predicted ORFs from PAGI-2. ORF C47 was represented by two different products ('C47c' and 'C47d'). C54 and C55, C68 and C69, C76 and C77, and C82 and C83 were represented pair-wise by one product each. In the pKLC102 macroarray, 86 PCR products represented 85 ORFs of pKLC102. Three PCR products represented the 2.9 kb ORF CP94 ('CP94a', 'CP94b' and 'CP94c') and two products represented ORF CP103 ('CP103a' and 'CP103b'). Three PCR products spanned two ORFs each (CP47–CP48, CP52–CP53, and CP73–CP74). Control PCR products were spotted in the lower-left corners. In the case of the PAGI-2 array, the five dots contained partial sequences of (from top to bottom) the *P. aeruginosa* genes *gltA*, *fliC* (type A) and *fliC* (type B) (positive controls), and of an intergenic sequence of *Pseudomonas putida* KT2440 and of the human *ob* gene (negative controls). In addition to these five control dots, the pKLC102 macroarray contained, in the second lane from the left, the five controls, *ori1'* of pKLC102, PA0977 and PA0981 of *P. aeruginosa* PAO1, and two strain-specific ORFs of *P. aeruginosa* TBCF10839.

Copies of the macroarrays were produced in parallel from the same set of PCR products (pooled from four reactions each). Aliquots (50 µl) of these pooled products, 15 µl 3 M NaOH and 85 µl Tris-EDTA buffer were denatured at 65 °C for 30 min and chilled on ice. After adding 100 µl 3 M ammonium acetate, aliquots of 100 µl per dot were transferred to the membranes by a Minifold dot-vacuum-blot apparatus (Schleicher & Schuell), thus generating array copies with an identical amount of PCR product of the same DNA quality at comparable positions. After spotting, the DNA was cross-linked to the membranes by 1 min exposure to UV light. Control stripes for the calibration of expression levels contained two controls also used for the macroarrays (*gltA*, *ob*) and four genes of the *P. aeruginosa* core genome (Stover *et al.*, 2000) with strongly different mRNA transcript expression levels [*gltA*, *rpoN*, *rpsL* and *rrn* (two segments of the 16S rDNA sequence)].

RNA isolation and cDNA synthesis. Bacterial RNA from 10¹⁰ *P. aeruginosa* cells grown in LB broth was isolated by a modified hot phenol method (von Götz *et al.*, 2004). For cDNA synthesis, 10 µg bacterial RNA and 750 ng random primer p(dN₆) (Roche) in 30 µl water were incubated at 70 °C for 10 min and at 25 °C for an additional 10 min. After chilling on ice, 12 µl 5× first strand buffer (Invitrogen), 6 µl 0.1 M DTT, 3 µl dNTP-solution (dATP, dTTP, dCTP and dGTP, 10 mM each), 1.5 µl RNase-inhibitor (SUPERaseIn, 20 U µl⁻¹, Ambion) and 7.5 µl reverse transcriptase (Superscript II, 200 U µl⁻¹, Invitrogen) were added and incubated for 10 min at 25 °C, 60 min at 37 °C, 60 min at 42 °C, and finally 10 min at 70 °C. Afterwards, RNA strands were degraded by the addition of 20 µl 1 M NaOH and 30 min incubation at 65 °C. The solution was neutralized with 20 µl 1 M HCl, and the cDNA was purified by using the QIAquick PCR purification kit (Qiagen). The final cDNA concentration was determined spectrophotometrically.

mRNA transcript profiling on macroarrays. The generated cDNA was cut into fragments of 50–200 bases in length by incubating 12.5 µg cDNA and 6.25 U DNase I (Amersham) in 38 µl OnePhorAll buffer (Amersham) at 37 °C for 10 min, followed by inactivation of

DNase I at 98 °C for 10 min. Fragments were labelled at the 3' end with DIG by using the Terminal Transferase kit (Roche).

Labelled cDNA fragments (5 µg) were hybridized onto macroarrays and control stripes. The hybridization (0.5 M sodium phosphate, 1 mM EDTA, 1% SDS, pH 7.2 for 16 h at 60 °C) was sufficiently stringent so that no cross-hybridization was observed for any of the 36 ORFs that are homologous between the two islands (data not shown). Non-specifically bound probe solution was removed by several washing steps, and hybridization signals were detected by washing the membrane with anti-DIG-alkaline phosphatase (Roche) and CDP Star (Römling *et al.*, 1994). Chemiluminescence signals were detected on X-ray films and quantified by PCBAS, version 2.09f (Raytest Isotopenmeßgeräte).

Semiquantitative RT-PCR kinetics. The mRNA concentration was determined from the kinetics of amplification of cDNA during PCR by titration for the first reaction cycle with detectable fluorescence of gel-separated PCR product with the ethidium bromide stain (Bremer *et al.*, 1992; Hoof *et al.*, 1991). A PCR product becomes visible by ethidium fluorescence during the late-exponential phase of PCR. Using our PCR instrument (Landgraf), the efficiency, *R*, of the exponential phase of PCR, $N = N_0 (1 + R)^n$, was always $R = 0.78 \pm 0.02$, within the range of 10⁵–10¹⁰ transcript molecules per microgram RNA as the initial cDNA template concentration, if the cDNA PCR product was smaller than 800 bp in size [*N*, amount of PCR product; *N*₀, initial amount of template (cDNA); *n*, number of PCR cycles]. The amount of PCR product increased by one order of magnitude within four reaction cycles. The oligonucleotide primer sequences are listed in Table 1.

cDNA was synthesized with the RevertAid First Strand cDNA synthesis kit (Fermentas). A mixture of 2 µl total RNA (2 µg pooled in equal amounts from three separate RNA preparations of bacteria grown in LB broth to mid-exponential or early stationary phase, respectively), 2 µl ORF-specific reverse primer (10 µM) and 8 µl diethyl pyrocarbonate (DEPC)-treated H₂O were covered with mineral oil, heated for 5 min at 70 °C and then chilled on ice. Then, 4 µl 5× reaction buffer, 1 µl RiboLock RNase inhibitor (20 U µl⁻¹) and 2 µl dNTPs (10 µM each of dATP, dGTP, dCTP and dTTP) were added and incubated for 5 min at 37 °C. After addition of 1 µl RevertAid M-MuLV reverse transcriptase (200 U µl⁻¹), cDNA was synthesized for 60 min at 42 °C and then the reaction was stopped (10 min at 70 °C). cDNA was amplified by PCR in a 50 µl reaction volume [5 µl 10× reaction buffer (Invitex), 1.5 µl MgCl₂ (50 mM), 1.5 µl DMSO, 4 µl dNTPs (2 mM each of dATP, dGTP, dCTP and dTTP), 3 µl ORF-specific forward primer, 3 µl ORF-specific reverse primer, 0.2 µl polymerase (Tth-pyrophosphatase, 5 U µl⁻¹), 2 µl reverse-transcription mixture containing cDNA, 29.8 µl H₂O]. After denaturation at 94 °C for 5 min, PCR was run in cycles of 120 s at 94 °C, 45 s at 58 °C, and 45 s (30 s for CP28, 60 s for C81) at 72 °C. Aliquots (6 µl) were taken at regular intervals and separated by agarose gel electrophoresis.

RT-PCR kinetics were performed at least four times for each selected mRNA transcript and each experimental condition. The mean and variance of the lowest cycle number with a visible PCR product with the ethidium bromide stain were determined for each set of RT-PCR kinetics by visual inspection of photos of the agarose gels. Cycle numbers were then converted into concentrations in femtomoles mRNA transcript per microgram bacterial RNA with the aid of calibration curves generated by PCR with an MDR1 cDNA standard on the same thermocycler instrument, as described previously by Hoof *et al.* (1991).

RESULTS AND DISCUSSION

The mRNA expression profiles of pKLC102 and PAGI-2 ORFs in *P. aeruginosa* clone C strains C and SG17M were investigated during growth in LB broth or Vogel–Bonner

Table 1. Oligonucleotide primers used in this study

ORF	Reverse primer	Forward primer	Size of PCR product
CP1	5'-CCTTGACCAGTGCTGCTACC	5'-CTACGACCTGGTGTGATCG	497 bp
CP28	5'-CAGAGCTTGCACCTGCTCGG	5'-GCAACTCGCCAAACTGGTAC	204 bp
CP32	5'-CTTCTTCGCTTGGGTCGTGC	5'-ACGCTCAAACAACCTCCGCCG	395 bp
CP43	5'-AGCAGGTCAGCGGATCAACC	5'-CCACCAGCTTCGAAATCGGC	375 bp
CP46	5'-CTTCGCATCGTAGACGGTGG	5'-GAGCAACAACACCCCAAGCCC	397 bp
CP70	5'-TTGGTAGGCCTTGTGCGTGC	5'-CAGCGCAGAGTTCAATGGCG	587 bp
CP91	5'-CTGAGATCGCTGAGGAGAGC	5'-GGGTAGCCTGGCTTACTTCC	405 bp
C81	5'-TGTGGACCAGCGTCGATTCC	5'-TCGCCTTATCAACCCACCGC	805 bp
C98	5'-GTTGAGAATGCCGCACTCGC	5'-ATGCGGATCGGTGAACCTGGG	375 bp
C108	5'-CCATTGCGGGAACAACCTCGC	5'-ACTACGAACTGACCCAGCGC	659 bp
<i>gltA</i>	5'-GCTTCGTTGCGGCCCCCATG	5'-CATGTCGACCGCCTCCTGCG	672 bp
<i>fliC</i> (A-type)	5'-AGTCGGTGTCTTCGATCCGG	5'-GGTCGACATGAAGGGCAACG	455 bp

minimal medium during mid-exponential and early stationary phase. Both strains were growing at comparable rates. Fig. 1 shows two representative examples of the hybridization of cDNA samples onto pKLC102 and PAGI-2 macroarrays. Hybridization signals of individual

pKLC102 and PAGI-2 ORFs did not differ significantly between the four tested growth conditions, each of which was examined at least in duplicate. Absolute signal intensities, however, were strikingly different between pKLC102 and PAGI-2 ORFs (Fig. 1).

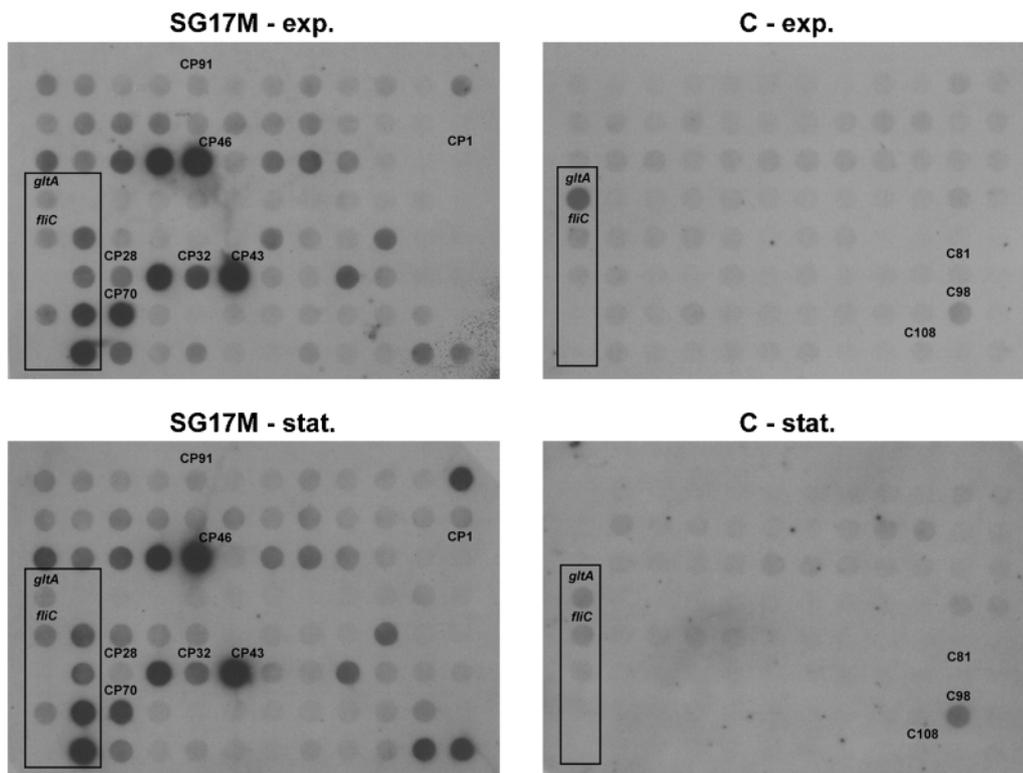


Fig. 1. Macroarray cDNA hybridization patterns of pKLC102 of strain SG17M (left) and of PAGI-2 of strain C (right). *P. aeruginosa* clone C strains SG17M and C were grown in LB broth to mid-exponential (exp., upper panel) or early stationary phase (stat., lower panel). RNA was extracted, and reverse-transcribed cDNAs were hybridized onto the macroarray. PAGI-2 and pKLC102 ORFs whose mRNA expression levels were also determined by RT-PCR (see Table 2) are indicated by ORF number (Klockgether *et al.*, 2007). Ten (left) or five (right) positive or negative control dots were spotted in the boxed lower-left corners (Klockgether *et al.*, 2007).

PAGI-2-reactive hybridization signals were only detected for 31 of the 111 ORFs, of which only that of C98 was clearly discernible within the range of the weakly expressed control gene *gltA*, whereas the remaining signals were just above the threshold of detection (Fig. 1). The majority of the transcribed ORFs belonged to cargo parts of PAGI-2 (most ORFs of C14–C35, C56–C62, C97–C99) that are absent in the related genomic islands PAGI-3 (Larbig *et al.*, 2002), pKLC102 (Klockgether *et al.*, 2004) and PAPI-1 (He *et al.*, 2004). RT-PCR kinetics verified that the transcribed ORFs were each present at less than 1000 copies per microgram of RNA, the exception being C98, with a copy number of about 10^7 mRNA molecules per microgram of RNA (Table 2). C98 encodes a putative transcriptional regulator that shares extensive homology with a family of response regulators in *Pseudomonas* species that control detoxification of heavy metal ions (Lee *et al.*, 2001). In summary, all but one of the PAGI-2 ORFs were transcriptionally silent or very weakly expressed under standard *in vitro* growth conditions.

PAGI-2 encodes a putative mercury-resistance transposon (C84–C88) and a P-type ATPase involved in transport of heavy metal ions (C97–C98). Hence, we hypothesized that exposure to mercury affects the mRNA expression pattern

of PAGI-2. However, when we grew *P. aeruginosa* strain C in Vogel–Bonner medium supplemented with $7.5 \mu\text{M}$ HgCl_2 , which is half the maximally tolerated mercury concentration, no change in the expression profile of the PAGI-2 macroarray compared with standard growth conditions was discernible (data not shown).

In contrast to PAGI-2, ORFs from pKLC102 were expressed significantly under the conditions tested: virtually all ORFs showed moderate to very strong cDNA-reactive hybridization signals (Fig. 1, Table 2). RT-PCR kinetics verified the abundance of pKLC102 mRNA transcripts. Based on an estimate derived from whole-genome sequence data (<http://cmr.tigr.org>) of $3 \text{ pmol RNA molecules } (\mu\text{g RNA})^{-1}$, the amount of pKLC102 mRNA transcripts should be at least 10% of total RNA molecules of the SG17M cell in both exponential and stationary phase. The *soj* and *int* genes, which are required for mobilization from and integration into the chromosome (Qiu *et al.*, 2006), as well as the genes defined as the phage and plasmid modules of pKLC102 (Klockgether *et al.*, 2007), belonged to the weakly or moderately expressed genes. Of the 18 most strongly expressed pKLC102 genes (Fig. 1), 11 reside within the contig CP43–CP53. The most abundant mRNA transcripts,

Table 2. mRNA expression levels of selected PAGI-2 and pKLC102 genes determined by semiquantitative RT-PCR kinetics

ORF	Annotation	Amount mRNA transcript [fmol mRNA ($\mu\text{g RNA})^{-1}$]*†
pKLC102		
CP1	<i>soj</i> , chromosome-partitioning protein	0.4 (0.1–1.3)
CP28	Putative cold-adaptation protein	1 (0.3–4.0)
CP32	Hypothetical protein, specific for pKLC102 subtype	1.6 (0.4–6.0)
CP43	Hypothetical protein, conserved among pKLC102-type islands	26 (9–84)
CP46	Hypothetical protein, conserved among pKLC102-type and PAGI-2-type islands, homologue to C81	63 (15–270)
CP70	Pathogenicity-related protein, putative RND transporter, present in most pKLC102 subtypes	Exponential 3.9 (1–12) Stationary 0.4 (0.2–0.8)
CP91	Hypothetical protein, conserved among pKLC102-type and PAGI-2-type islands	0.2 (0.07–0.6)
PAGI-2		
C81	Hypothetical protein, homologue of CP46	$<10^{-6}$
C98	Putative transcriptional regulator, conserved among PAGI-2-type islands	1.4 (0.7–2.6)
C108	<i>soj</i> , chromosome-partitioning protein	$<10^{-6}$
Internal controls from conserved core genome		
<i>gltA</i>	Citrate synthase	0.5 (0.1–1.4)
<i>fliC</i>	Flagellin	2.5 (1.3–4.7)

*Data are the mean of at least four independent RT-PCR experiments on RNA preparations from cells grown to mid-exponential or early stationary phase. With the exception of CP70, mRNA transcript levels at exponential and stationary phase were indistinguishable by RT-PCR kinetics. The numbers in parentheses indicate the interval for the variance of the mean in fmol mRNA transcript ($\mu\text{g RNA})^{-1}$. Please note that mean and variance were determined from the titration for the first reaction cycle of the exponentially growing PCR that gave a visible product with the ethidium bromide stain. The reaction cycle was then converted into a concentration with the aid of semilogarithmic calibration graphs (see Methods). Consequently, the variance of the experimental parameter reaction cycle corresponds to fold variations of the mean concentration.

†From PAO1 genome data (<http://cmr.tigr.org>), $1 \mu\text{g}$ *P. aeruginosa* total RNA is estimated to contain about 3 pmol RNA.

CP43 and CP46 (Fig. 1, Table 2), each made up more than 1 % of total RNA of the SG17M cell. Homologues of CP43–CP53 are found in the majority of pKLC102-type islands of *P. aeruginosa* (Klockgether *et al.*, 2007), with CP43, CP46–CP49 and CP51–53 homologues present in more than 90 % of the islands. Outside the pKLC102/PAGI-2 family of genomic islands, however, no ORF with significant sequence similarity to any of the 11 ORFs could be retrieved from public databases at the time of writing. Hence, bioinformatic tools could not provide any hint as to why this contig is so strongly expressed, at levels close to those of rRNA genes. Functional screens have also been uninformative: the homologous PAPI-1 ORFs of strain PA14 have been shown to be phenotypically inconspicuous in screens for pathogenicity genes in several infection models (He *et al.*, 2004). Of the strongly expressed genes, only CP70 contributes to virulence. CP70 carries the signature of an RND efflux transporter and was the only pKLC102 ORF that was significantly more strongly expressed in mid-exponential than in stationary phase (Table 2).

Seven of the 18 most strongly expressed pKLC102 genes have homologues in PAGI-2. It is interesting to note that none of the homologues was transcribed from the latter at detectable levels. In other words, expression was silent in the stably captured PAGI-2, but extremely high in the mobile and episomally replicating pKLC102, suggesting that the genetic repertoire is utilized in the episomal state and during mobilization, replication or transfer of the genomic island. Differences in copy number, DNA structure and functional needs probably all account for the huge disparity between mRNA expression levels of PAGI-2 and pKLC102 genes under identical environmental conditions. First, the copy number is one for the stably integrated PAGI-2 and up to 30 for the mobile pKLC102. Second, the host chromosome controls the local chromatin structure of its genomic island, PAGI-2, and thus the accessibility of promoters and other regulatory sequences. The circular pKLC102, on the other hand, is largely autonomous in its control of topology and promoter accessibility. Moreover, *cis* regulatory elements of promoter activity that flank the integration site can cooperate in the circular state, but are physically separated and thus silenced in the chromosomal genomic island (Sentchilo *et al.*, 2003a, b). Third, the genetic information encoded by the genomic island is not essential for the host and hence can be switched off. The selfish episomal form, however, needs its genes to be permanently transcribed for replication and horizontal transfer.

In summary, the mobile gene island pKLC102 is constitutively highly expressed by its host strain SG17M at levels close to those of the most strongly transcribed genes of the core genome, whereas the stably incorporated PAGI-2 is kept transcriptionally silent for most ORFs and probably becomes actively expressed only under exposure to specific environmental cues, as has been described elsewhere for the PAGI-2-type island *clc* (Gaillard *et al.*, 2006; Müller *et al.*, 2003; Sentchilo *et al.*, 2003b).

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