Chapter 4
Molecular Tools to Study *Azospirillum* sp. and Other Related Plant Growth Promoting Rhizobacteria

Lily Pereg and Mary McMillan

Abstract Molecular methods have been used in the study of *Azospirillum* and other related PGPRs to carry out gene functional analysis, create gene knockouts, generate genetically engineered strains, and carry out gene expression studies. Genetic transformation has routinely been carried out using conjugation, while chromosomal modifications have been performed using unstable, suicide plasmids, or more stable, broad host-range vectors. Gene expression studies are often carried out using promoter-bound reporter genes; however, quantitative methods such as reverse transcribed polymerase chain reaction can now be used to directly study gene expression. In this chapter we describe the common types of vectors used in *Azospirillum*, as well as methods for transformation and mutagenesis. We also describe the use of promoter-bound reporter genes and the applications of quantitative RT-PCR for *Azospirillum* gene expression studies. Methods for the isolation of DNA and RNA from *Azospirillum* for use in molecular and gene expression studies are also described.

4.1 Mutagenesis and Genetic Transformation

Genetic transformation has been used in *Azospirillum* for gene functional analysis employing random and site-directed transposon-induced mutagenesis, gene knockout and genetic exchange, gene expression studies using promoterless reporter gene translation cassettes, genetic engineering by introducing new genes/traits, and for genetic labelling by inserting constitutively expressed reporter genes.

Genetic transformation in *Azospirillum* spp. has been mainly performed by conjugation using various groups of plasmid vectors. Generally, chromosomal modifications have been performed using unstable, suicide plasmids, while stable broad host-range vectors have been used in applications requiring the maintenance of plasmids.

L. Pereg (✉) • M. McMillan
Discipline of Molecular and Cellular Biology, School of Sciences and Technology, University of New England, Armidale, NSW 2351, Australia
e-mail: lily.pereg@une.edu.au

F.D. Cassán et al. (eds.), *Handbook for Azospirillum*,
DOI 10.1007/978-3-319-06542-7_4

In this section we discuss the main vectors used with *Azospirillum*, transformation by conjugation, gene replacement, and transposon mutagenesis. The analysis that follows mutagenesis requires the extraction of genomic DNA, and while nowadays there are commercial kits suitable for this purpose, a cost-effective method for the isolation of large amounts of *Azospirillum* DNA is described.

4.1.1 Vectors

Among other vehicles, such as the cosmid pLAFR3, used for the construction of *Azospirillum* genomic libraries (Revers et al. 2000), DNA elements can be transformed into recipient cells on plasmid vectors. The selection of a vector depends on the intended use, method of genetic transformation, and user preference/tool availability. Vectors available for *Azospirillum* can be divided into stable, broad host-range vectors, and unstable suicide vectors.

4.1.1.1 Broad Host-Range Vectors in *Azospirillum*

Until the early 1980s genetic tools for *Azospirillum* chromosome mobilization included the IncP1 plasmid R68-45 (Haas and Holloway 1976), adopted from use with *P. aeruginosa* (Elmerich and Franche 1982). Michiels et al. (1985) tested plasmids belonging to the incompatibility groups (Inc) P1, Q, and W, but only IncP1 plasmids pRK290, pRK252, and BIN19 were stable in *Azospirillum* (all derivatives of RP4). The most widely used stable vectors in *Azospirillum* have been pRK290 (Ditta et al. 1980) and its derivatives the cosmid pVK100 (Fig. 4.1) (Knauf and Nester 1982) and pLA2917 (Fig. 4.2) (Allen and Hanson 1985). These low copy, broad-host range vectors, from the IncP1 group with RK2 replication factors, are not self-transmissible but can be mobilized if supplied with the plasmid transfer elements in trans (see Sect. 4.1.2). They can be transferred to *Azospirillum* recipients by conjugation and are stably maintained. These vectors contain a number of unique restriction sites (Figs. 4.1 and 4.2) to enable selection and analysis following cloning. Such restriction sites exist within either the kanamycin or tetracycline resistance markers, which will be inactivated if disrupted with cloned DNA. Other vectors stable in *Azospirillum* include the cosmid pLAFR1, originally used with *Rhizobium*, and its derivative pLAFR3 (Milcamps et al. 1996; Kadouri et al. 2002); a pRAJ275 derivative, namely, pFAJ21 (Revers et al. 2000) as well as pBBR1MCS-2 vector (Kovach et al. 1995), which was shown to be stable even without selective pressure (Rothballer et al. 2003).

Stable vectors can be used to clone genes for complementation (Pedrosa and Yates 1984; Pereg Gerk et al. 1998), create genomic libraries (Fogher et al. 1985), study gene expression (Fani et al. 1988; Liang et al. 1991; Vieille and Elmerich 1992; Pereg Gerk et al. 2000; Pereg Gerk 2004; Revers et al. 2000), and carry reporter genes for cell visualization (Arsène et al. 1994; Pereg Gerk et al. 1998).

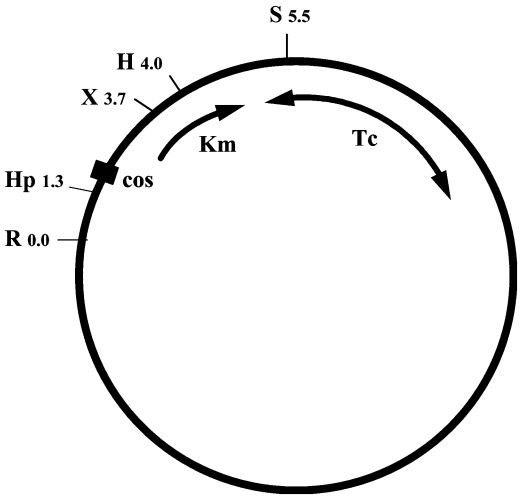

Fig. 4.1 Map of the plasmid vector pVK100. This plasmid is a derivative of pRK290 with a *Sal*I-*Eco*RI fragment of the cosmid pHK17. It contains the *cos* site of phage λ and it is a broad host vector, stable in *Azospirillum*. Unique restriction sites: (H) *Hind*III (S) *Sal*I (R) *Eco*RI (Hp) *Hpa*I (X) *Xho*I. Numbers are given in kb. There are neither *Pst*I nor *Bam*HI sites in the vector. The plasmid codes for tetracycline (Tc) and kanamycin (Km) resistance (Knauf and Nester 1982)

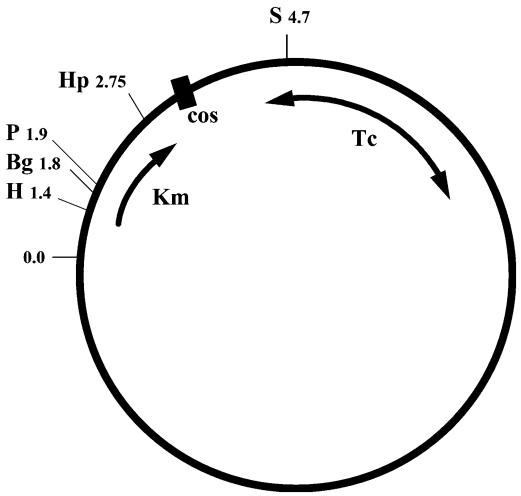

Fig. 4.2 Map of the plasmid vector pLA29.17. It contains the *cos* site of phage λ and it is a broad host vector, stable in azospirilla. Unique restriction sites: (H) *Hind*III (S) *Sal*I (Bg) *Bgl*II (P) *Pst*I (Hp) *Hpa*I. Numbers are given in kb. There are neither *Xho*I nor *Bam*HI sites in the vector. The plasmid codes for tetracycline (Tc) and kanamycin (Km) resistance (Allen and Hanson 1985)

They have been used in gene discovery, analysis of genetic regulation, and in studying *Azospirillum*–plant association. Table 4.1 summarizes the most common stable vectors used in *Azospirillum* transformation.

Table 4.1 Stable vectors and suicide vectors used in *Azospirillum*

Vectors	References	Examples of use in *Azospirillum*
Stable vectors		
pR68-45	Haas and Holloway (1976)	Cloning of *ntrB&C* by Pedrosa and Yates (1984) for gene complementation
pRK290	Ditta et al. (1980), Michiels et al. (1985)	Regulation of *nif* gene expression by Liang et al. (1991); promoter identification by Fani et al. (1988)
pVK100	Knauf and Nester (1982)	Gnomic library and cloning of *glnA* by Fogher et al. (1985); *flcA* cloning for complementation by Pereg Gerk et al. (1998)
pLA2917	Allen and Hanson (1985)	A constitutively expressed pLA-*lacZ* fusion by Arsène et al. (1994), for direct observation and quantitative measure of wheat root colonization Pereg Gerk et al. (1998); *flcA-lacZ* for gene expression studies by Pereg Gerk (2004)
pGD926 (pRK290 derivative, lacYZ)	Liang et al. (1991)	*nif-lacZ* gene expression cassettes by Liang et al. (1991) also used for gene expression in association with plants by Arsène et al. (1994), Katupitiya et al. (1995), Pereg Gerk et al. (2000); *nodG-lacZ* cassette by Vieille and Elmerich (1992)
pLAFR1	Milcamps et al. (1996)	Construction of genomic DNA libraries
pLAFR3	Milcamps et al. (1996)	Cloning and analysis of the *rpoN* gene (Milcamps et al. 1996) and *phbC* (Kadouri et al. 2002)
pFAJ21 (pRAJ275 derivative)	Revers et al. (2000)	*nif-gusA* cassette to study *nif* gene expression
pBBR1MCS-2	Kovach et al. (1995)	*ipdC* translational promoter fusions with *gfp* by Rothballer et al. (2005) to study gene expression; labelling *Azospirillum* for plant interaction assays by Rothballer et al. (2003)
Suicide vectors		
pGS9	Selvaraj and Iyer (1983)	Random Tn5 mutagenesis in *A. brasilense* and *lipoferum* by Vanstockem et al. (1987)
pSUP2021	Simon et al. (1983)	Random Tn5 mutagenesis in *A. brasilense* and *lipoferum* by Vanstockem et al. (1987)
pSUP202	Simon et al. (1983)	Identification of nif regulatory genes by Liang et al. (1991); Tn5-induced, site-directed mutagenesis of *flcA* by Pereg Gerk et al. (1998) and *mreB* by Biondi et al. (2004)
pCIB100	Van Rhijn et al. (1990)	Tn5-*lacZ* random mutagenesis for gene discovery and expression study

4.1.1.2 Suicide Vectors in *Azospirillum*

Suicide vectors are useful for transposon mutagenesis, gene knockout, and chromosomal exchanges since they allow mobilization of DNA into *Azospirillum* without stable integration of the whole vector. Instead, double recombination events replace host DNA with vector-borne DNA. Suicide vectors for gene replacement in Gram-negative bacteria may carry a conditional lethal gene that can discriminate between the integration of the entire vector and double recombination events. The suicide vectors pJQ200 and pJQ210, carrying P15A origin of replication, have been used successfully with *Rhizobium* (Quandt and Hynes 1993). The plasmid pGS9, originally developed as a suicide plasmid for insertional mutagenesis in *R. meliloti* (Selvaraj and Iyer 1983), is composed of p15A-type replicon and N-type bacterial mating system.

The suicide vector pSUP2021 is a derivative of pSUP202 carrying a Tn5 mobilizable transposon (Simon et al. 1983). The plasmid pSUP202 (Fig. 4.3) is derived from the commonly used *E. coli* vector pBR325, with a ColE1 replicon and a IncP-type Mob region, which is unable to replicate outside the enteric bacteria—it can be mobilized into, but not stably maintained, in *Azospirillum*. Therefore pSUP202 and pSUP2021 are good transposon carriers for random or site-directed transposon insertion. However, note that not all other derivatives of pBR325 can be used

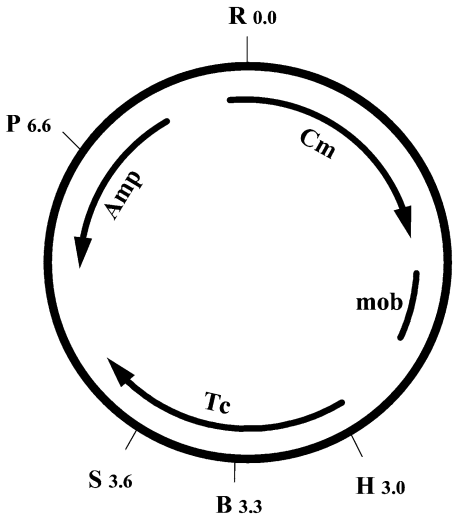

Fig. 4.3 Map of the vector pSUP202. It is a pBR325 (E. coli vector) derivative carrying the IncP-type transfer genes (Mob site from plasmid RP4) and can be mobilised with high frequency from the donor strains. It is unable to replicate in strains outside the enteric bacterial group thus, it is not stable in *Azospirillum* and can be used as transposon carrier replicon for random transposon insertion mutagenesis. This vector and members of its family, such as pSUP101, 201, 203, are especially useful for site-directed transposon mutagenesis and for site-specific gene transfer in a wide variety of Gram-negative organisms in any strain into which they can be mobilised but not maintained (Simon et al. 1983). Unique restriction sites shown: (H) *Hind*III (S) *Sal*I (B) *Bam*HI (P) *Pst*I (R) *Eco*RI. Numbers are given in kb. The plasmid codes for tetracycline (Tc), chloramphenicol (Cm) and ampicillin (Amp) resistance

effectively for this purpose in *Azospirillum*, as is the case with pSUP5011 (Vanstockem et al. 1987).

The plasmid pCIB100 (ColE1 replicon) has also been used as a donor for Tn5 random mutagenesis in the isolation of motility and chemotaxis mutants. The transposable element in this case included a promoterless *lacZ* reporter gene (Tn5-*lacZ*) enabling gene expression studies (Van Rhijn et al. 1990). Table 4.1 summarizes the most common suicide vectors used with *Azospirillum*.

4.1.2 Transformation Techniques: Electroporation and Conjugation

Genetic transformation techniques including the use of heat shock competent *A. brasilense* cells (Fani et al. 1986) and electroporation have been published. Vande Broek et al. (1989) reported on electroporation of *A. brasilense* but not *A. lipoferum* cells with plasmid pRK290 and concluded that optimal conditions for electroporation probably vary with the *Azospirillum* strain. Nevertheless, the most common procedure for the transformation of *Azospirillum* with plasmid DNA over the last three decades has been conjugation.

Transformation by biparental conjugation requires donor and recipient strains. To mobilize plasmids that are not self-transmissible, the donor strain, often *E. coli*, has to carry the transfer functions of the broad host range IncP-type plasmid RP4 integrated in its chromosome. Examples of plasmid-mobilizing *E. coli* donors that can utilize many Gram-negative bacteria as recipients include the strains SM10 and S17-1 (Simon et al. 1983).

In triparental conjugation, when plasmid DNA is mobilized into *Azospirillum* from another *E. coli* strain which does not contain the RP4 transfer elements, a third parent strain is required as a helper to supply the transfer gene in trans. *E. coli* HB101 containing the helper plasmid pRK2013 (*tra*$^+$) (Ditta et al. 1980) is often used as a helper strain.

4.1.2.1 Conjugation Protocol

In a typical conjugation protocol, overnight liquid cultures of donor and recipient strains (and helper strain if required) are mixed on nonselective nutrient agar (NA) plates. These can be mixed prior to applying, or applied to plates in stages, beginning with the donor, then the recipient on top, with drying in between. The mixture is allowed to dry and incubated overnight at 28–30 °C. A quantity of the mating mix can be spread on selective medium and incubated for 48 h or longer at 30 °C to observe for transformants (Pereg Gerk et al. 1998). In some cases 10 mM $MgSO_4$ is used in the donor and recipient cell cultures and added to the selective medium (Vanstockem et al. 1987).

The selective medium is a medium on which only the transformed or mutated recipients should grow; control cultures of the parent donor, recipient, and helper

strains should not grow. Other selective pressure in addition to antibiotics can be applied for the elimination of some parent strains. For example, the *E. coli* S17.1 donor is an auxotroph for proline and thus cannot grow in proline-free minimal lactate medium (Table 4.2, Dreyfus et al. 1983) or Nitrogen-free medium (Nfb, Table 4.2, Katupitiya et al. 1995). While proline-free minimal media can be used to select against

Table 4.2 Proline-free minimal medium for *Azospirillum* transformation

Minimal lactate medium[a]	Nitrogen-free medium (Nfb)[a]
Prepare minimal medium supplemented with 6.3 mL/L of sodium lactate	Into 800 mL of DW add:
Before use add 10 mL of $CaCl_2$ solution (7 g/L), 10 mL of trace element solution, 1 mL of $FeCl_3 \cdot 6H_2O$ solution (10 g/L), 1 mL of $Na_2MoO_4 \cdot 2H_2O$ solution (0.8 g/L), and 1 mL of vitamin solution	$CaCl_2$ 0.02 g (always add $CaCl_2$ first and mix!)
Each of the solutions should be autoclaved separately	Malic acid 5 g
Minimal medium supplemented with 6.3 mL/L of sodium lactate	$K_2HPO_4 \cdot 3H_2O$ 0.5 g
100 mL phosphate solution	$MgSO_4 \cdot 7H_2O$ 0.2 g
10 mL $MgSO_4$/NaCl solution	NaCl 0.1 g
500 mL DW	4 mL Fe-EDTA (1.64 % aqueous, w/v); 2 mL of trace element solution
6.3 mL sodium lactate	Adjust pH to 6.8 with KOH and make up to 1 L with DW
Complete with DW to 1 L	Sterilized by autoclaving at 120 °C for 20 min
Autoclave at 121 °C for 20 min	Add 1 mL vitamin solution per liter medium (after autoclaving)
Phosphate solution pH 6.8–7	*Trace element solution*
K_2HPO_4 16.7 g/L	In 1 L:
KH_2PO_4 8.7 g/L	$MnSO_4 \cdot H_2O$ 250 mg
MgSO4/NaCl solution	$ZnSO_4 \cdot 7H_2O$ 70 mg
$MgSO_4$ 29 g/L	$CoSO_4 \cdot 7H_2O$ 14 mg
NaCl 48 g/L	$CuSO_4 \cdot 5H_2O$ 12.5 mg
Trace element solution	H_3BO_3 3 mg
In 1 L:	$Na_2MoO_4 \cdot 2H_2O$ 200 mg
$MnSO_4 \cdot H_2O$ 250 mg	*Vitamin solution*
$ZnSO_4 \cdot 7H_2O$ 70 mg	In 100 mL:
$CoSO_4 \cdot 7H_2O$ 14 mg	Biotin 1 mg
$CuSO_4 \cdot 5H_2O$ 12.5 mg	Pyridoxine 2 mg
H_3BO_3 3 mg	Filter sterilize
Vitamin solution	
In 100 mL:	
Biotin 1 mg	
Pyridoxine 2 mg	
Filter sterilize	

[a]For solid media add 16 g/L Agar. For aerobic growth add also 2.5 mL of 20 % NH_4Cl as nitrogen source

the donor and helper strains, antibiotics are often used to select against the parent *Azospirillum* recipient strain.

When selecting for recipients transformed with a stable plasmid antibiotic resistance encoded by the plasmid, and not by the recipient parent, will be used for selection. In the case of suicide vectors the selection pressure will be dependent on the marker integrated into the recipient chromosome; for example, Tn5 often encodes for antibiotic resistance (often kanamycin), GFP for green fluorescence, or *lacZ* for β-galactosidase activity (blue-white selection on X-gal). Other morphological traits can be used in the selection of transformants, for example, Pereg Gerk et al. (1998) selected for white, nonencapsulating Tn5-induced mutants, against a background of red colonies on minimal medium containing kanamycin and Congo-Red. In the case of suicide vectors, it is important to check that a double and not single recombination event has occurred, to avoid the integration of the entire plasmid (Simon et al. 1983).

4.1.3 Transposon Mutagenesis and Gene Knockout

Classical methods of bacterial mutagenesis such as chemical treatment or UV irradiation have been successfully employed in *Azospirillum* (examples are given in Elmerich 1983; Del Gallo et al. 1985; Holguin et al. 1999). However, mutated genes are more easily and confidentially analyzed in genetically defined transposon-induced mutants or those produced by chromosomal site-specific exchanges.

Tn5 is a DNA transposable element, which carries an antibiotic resistance gene, often encoding kanamycin resistance. Similarly to Tn10 it is bracketed by the same insertion sequence IS50 (Reznikoff 1982). Vanstockem et al. (1987) performed transposon mutagenesis and generated Tn5-induced mutants of *Azospirillum brasilense* Sp7 and *A. lipoferum* Br17 by mating with *E. coli* strains carrying suicide plasmid vectors pSUP2021 and pGS9. These Tn5-carrier plasmids were developed for use with any Gram-negative bacteria not closely related to *E. coli* (Simon et al. 1983; Selvaraj and Iyer 1983). This random mutagenesis system is based on the following: (1) the vector plasmids are mobilized with high frequency into non-*E. coli* hosts by the broad host range transfer functions of the donor strain, (2) the vector plasmids are unable to replicate in these hosts, since their basic replicon displays a very narrow host range, and (3) transposition events can be isolated simply by selecting for transposon-mediated drug resistance while the initial transposon carrier plasmid is eliminated (Simon et al. 1983).

For random mutagenesis, the suicide plasmid containing the self-mobilized transposon is inserted into the recipient cells with the expectation that the transposon will be randomly mobilized into the host genome and that the suicide vector will not be maintained in the next generation. The final selection step is therefore critical and there are two main strategies that can be applied: (1) collect a large number of [transposon$^+$/vector$^-$] cells and screen them for different traits and (2) if direct screening for specific trait is possible, use selective media to identify [transposon$^+$/vector$^-$/mutation$^+$] mutant strains.

The development of the pSUP family of suicide plasmids (Simon et al. 1983) also promoted the possibility of site-directed Tn5 mutagenesis. This is based on homologous recombination between vector-borne and specific genomic DNA sequences. Site-directed mutagenesis is achieved by cloning the gene of interest, disrupted by a transposon (e.g., Tn5 derivative), onto a suicide plasmid and inserting the plasmid by conjugation into *Azospirillum*. To increase the chance of double homologous recombination between the chromosome and the plasmid it is important to ensure that there are sufficiently long *Azospirillum* gene sequences bracketing the Tn5 carried on the plasmid (Pereg Gerk et al. 1998). It is recommended to include at least 500 bp of chromosome-homologous sequences on each side of the plasmid-borne Tn5 for optimal results in *Azospirillum* (Pereg, unpublished). Similarly to random transposon mutagenesis, the selection stage is important in the generation of specific mutants. Pereg Gerk et al. (1998) directly selected for mutants that cannot undergo morphological transformation to cyst-like cells by identifying white colonies on selective medium containing kanamycin (selected for Tn5) and Congo-red (binds to exopolysaccharides, wild-type appears red). White, kanamycin resistant colonies were then tested for chloramphenicol sensitivity, indicative of double homologous recombination and the elimination of the pSUP202 vector (Pereg Gerk et al. 1998). Kadouri et al. (2003) used a derivative of pSUP202 for site-directed mutagenesis and characterization of *Azospirillum phaZ*.

De Lorenzo et al. (1990) and Herrero et al. (1990) constructed a selection of mini-Tn5 and Tn10 transposon delivery, R6K-based, suicide plasmids with antibiotic resistance and nonantibiotic selection markers for chromosomal insertion of DNA into Gram-negative bacteria. The system was used successfully with *Rhizobium* for insertion mutagenesis and gene expression studies using the *gusA* and *lacZ* reporter genes (Reeve et al. 1999). Elements from these plasmids have been used in mutagenesis of specific *Azospirillum* genes in IAA synthesis (Carreno-Lopez et al. 2000) and genetic labelling of *Azospirillum* with a fluorescence marker (Rodriguez et al. 2006).

Gene knockout and other chromosomal gene replacements can be achieved by double homologous recombination in a similar manner to site-directed Tn5 mutagenesis. In these cases no transposon is used, and either a truncated gene or gene sequences bracketing a marker gene, such as antibiotic resistance, are cloned into the suicide plasmid (Hou et al. 2014). Other *Azospirillum* mutants isolated globally using gene replacement with a marker include *nif*, *nodPQ*, *glnB*, *DraT*, *DraG*, *rpoN*, *NtrBC*, *recA* gene mutants, and others (summarized in Holguin et al. 1999).

4.1.4 Genomic DNA Extraction

Genetically transformed *Azospirillum* strains are often analyzed by techniques such as Southern blotting and PCR amplification. PCR amplification requires only a small amount of genomic DNA and extraction methods such as the "Freeze-boil" method can be used to obtain a sufficient amount of template. However, Southern blotting analysis requires a large amount of genomic DNA which can be purified using a commercial DNA extraction kit, or the more cost-effective protocol outlined below.

4.1.4.1 Freeze-Boil Method

A loop-full of fresh cells is resuspended in 50 μL of sterile milli-Q water. The cell suspension is frozen at −70 °C for 30 min; then boiled at 100 °C for 2 min; spun down at high speed for 3–4 min; and the debris-free supernatant used immediately (preferably) or kept frozen at −20 °C.

4.1.4.2 Large Scale Azospirillum Genomic DNA Extraction Protocol

(As used by Pereg Gerk et al. (1998) The protocol was provided to L Pereg by C. Elmerich.)

Prepare 5 mL of a late logarithmic phase culture of *A. brasilense* in nutrient broth. Centrifuge for 10 min and wash the pellet twice with 1.5 mL of $T_{50}E_{20}$ buffer (Tris 50 mM, EDTA 20 mM, pH 8). Resuspend in 400 μL of $T_{50}E_{20}$ buffer. Lyse cells by adding 7 μL of Pronase E (50 mg/mL) and 50 μL of 10 % SDS, and incubate at 37 °C for 1 h. Gently pump the clear lysate several times up and down with a 1-mL syringe equipped with a wide needle (18G1.5, 1.2×40) to physically disrupt the DNA. Extract the DNA by adding 300 μL of phenol and 300 μL of chloroform. Repeat the phenol–chloroform extraction until the supernatant is clear. RNA can be eliminated from the solution by the addition of 3 μL of RNase (0.5 μg/μL) and incubation at 37 °C for 30–60 min. Perform a final extraction with one volume of chloroform and transfer the supernatant containing the DNA into a clean tube. Solubilize the DNA by adding 1:10 volumes of sodium acetate (3 M, pH 5.5) and 2 volumes of 100 % ethanol. Incubate the solution at −20 °C for 2 h or overnight. Precipitate the DNA by centrifugation for 15 min at 4 °C, then wash the pellet with cold 70 % ethanol. Allow pellet to dry then dissolve in 200 μL of TE buffer. The DNA can be examined by gel electrophoresis (0.8 % agarose mini gel) and stored at −20 °C for further use.

4.2 Gene Expression in *Azospirillum*

Regulation of gene expression in *Azospirillum* has been studied largely using expression systems consisting of promoterless reporter genes. With the availability of the genomic sequences, the study of gene expression using more direct methods, such as quantitative reverse transcribed polymerase chain reaction (qRT-PCR), is becoming more feasible. This technique eliminates the need for cloning the gene promoter and overcomes the problems associated with studying the expression of genes in the presence of foreign vectors.

In this section we describe the use of promoter-bound reporter genes for studying gene expression in *Azospirillum*. We also present a protocol for the extraction of total RNA from *Azospirillum* for use in gene expression studies. Considerations for the use of qRT-PCR are also described.

4.2.1 Using Promoter-Bound Reporter Genes

Promoter-bound reporter genes are constructed by fusing promoterless reporter genes, such as *lacZ*, *gusA*, and *gfp*, to gene regulatory elements (promoters). These gene expression cassettes may be plasmidborne, or integrated into the host genome, and can be introduced into *Azospirillum* by genetic transformation (see Sect. 4.1.2).

Fani et al. (1988) used the promoterless gene encoding for the enzyme chloramphenicol acetyl transferase and conferring Cm resistance as a reporter gene to test for active promoters in *Azospirillum*. Vande Broek et al. (1992) used the activity of β-glucuronidase encoded by the *gusA* gene to measure gene expression. The regulation and induction of *nifH* have been analyzed using a *nifH-gusA* fusion (Vande Brock et al. 1996). Liang et al. (1991) constructed plasmid-borne fusions of promoterless *lacZ* gene with several gene promoters, such as *nifA-lacZ*, *nifH-lacZ*, *nifB-lacZ*, and *ntrC-lacZ,* that were widely used in the analysis of nitrogen fixation regulation. De Zamaroczy et al. (1993) constructed a *gln-lacZ* and Arsène et al. (1994) have used these constructs to study the expression of *nif* genes in association with plants and further constructed pSUP202 derivatives of these fusions for recombination into the chromosome. Pereg Gerk (2004) constructed a *flcA-lacZ* fusion using pKOK5 (Kokotek and Lotz 1989) as the source of the *lacZ*-Km cassette and pVK100 as the carrier of the fusion. The constitutively expressed *lacZ* fusion on pLA-*lacZ* (Arsène et al. 1994) has been used as a control when studying gene expression using *lacZ* fusions.

The *lacZ* gene, encoding β-galactosidase, is widely used in reporter gene constructs in *Azospirillum*. A typical protocol for β-galactosidase assay is shown below. This assay is based on the ability of the enzyme to hydrolyze the β-galactoside bond of the *o*-nitrophenol-β-D-galactoside (ONPG) substrate to yield a yellow product, orthonitrophenol, which can be quantified using absorption spectrometry.

4.2.1.1 Typical Protocol for β-Galactosidase Assay in Culture or on Plants

Harvest cells from liquid culture by centrifugation. Resuspend cell pellet in 0.5 mL Z buffer supplemented with 5 µL of β-mercaptoethanol. Add 20 µL of 0.1 % sodium dodecyl sulphate (SDS) and 40 µL of chloroform and mix vigorously to lyse cells. Preincubate tubes in a water bath at 28 °C for 5–10 min, then add 100 µL of fresh ONPG solution (Table 4.3) and mix well ("start time"). Continue incubation at 28 °C until the samples start turning yellow. Stop the reaction by adding 250 µL of 1 M

Table 4.3 Phosphate buffer and ONPG solution

Phosphate buffer	ONPG solution
In 100 mL: K_2HPO_4, 1.05 g; KH_2PO_4, 0.45 g $(NH_4)_2SO_4$, 0.1 g; Tris sodium citrate, 0.05 g	In 10 mL phosphate buffer or in 10 mL Z buffer (pH 7.0) dissolve 40 mg of ONPG

Na_2CO_3. Record the start and stop times. Centrifuge for 5–10 min at 14,000 rpm and measure the absorbance of the supernatant at 420 and 550 nm, against a Z buffer blank (treated in the same way as bacterial samples). Express the results as Miller units/min/mg bacterial protein (Miller 1972). This assay can also be carried out on *Azospirillum* growing in association with plant roots, as described in Chap. 10.

4.2.2 RNA Extraction

Extraction of total RNA for downstream applications such as qRT-PCR can be carried out using a Trizol extraction protocol. Up to 1×10^8 cells for RNA extraction should be harvested from liquid culture by centrifugation at $6{,}000 \times g$ for 5 min at 4 °C. Cells may be used immediately or stored in an RNA preservation solution for later use. Resuspend the cell pellet in 1 mL Trizol reagent and incubate for 5 min at room temperature. Add 0.2 mL cold chloroform and shake vigorously, then incubate for 2–3 min at room temperature. Centrifuge at $12{,}000 \times g$ for 15 min. Transfer the colorless upper aqueous phase (~0.4 mL) to a fresh tube. Precipitate RNA by adding 0.5 mL cold isopropanol and mixing. Incubate for 10 min at room temperature. Centrifuge at $15{,}000 \times g$ for 10 min and carefully remove the supernatant. Resuspend the RNA pellet in 1 mL 75 % ethanol and vortex. Centrifuge at $7{,}500 \times g$ for 5 min. Discard the supernatant and allow RNA pellet to air-dry. Resuspend RNA pellet in 50 µL RNase-free water. Agarose gel electrophoresis of RNA should show clear 16S and 23S ribosomal bands. RNA concentration may be determined by spectrophotometry. Extracted RNA should be stored at −80 °C in aliquots to avoid repeated freeze-thawing.

4.2.3 qRT-PCR

Quantitative reverse transcribed polymerase chain reaction (qRT-PCR) has become the preferred method for the study of differential mRNA expression. Semiquantitative RT-PCR has been used in *Azospirillum* to analyze, for example, changes in expression of genes involved in CO_2 fixation (Kaur et al. 2009), quorum-sensing (Vial et al. 2006), and cell aggregation (Valverde et al. 2006). Quantitative RT-PCR has been less widely used in *Azospirillum* studies (see, for example, Kumar et al. 2012; Hou et al. 2014) but presents a much more sensitive system to detect changes in mRNA levels.

4.2.3.1 Reference Gene Selection

One important consideration in the application of qRT-PCR is the selection of internal reference (housekeeping) genes for the normalization of data. As no standard set of reference genes has been determined for prokaryotic cells such as *Azospirillum*, it is important to identify stable reference genes prior to undertaking qRT-PCR

Table 4.4 Reference genes identified in different bacterial species for qRT-PCR data normalization

Bacterial species	Reference genes	Reference
Azospirillum brasilense	*gyrA*, *glyA*	McMillan and Pereg (2014)
Clostridium ljungdahlii	*gyrA*, *rho*, *fotl*	Liu et al. (2013)
Lactobacillus casei	*gyrB*, *GAPB*	Zhao et al. (2011)
Xanthomonas citri	*atpD*, *rpoB*, *gyrA*, *gyrB*	Jacob et al. (2010)
Pectobacterium atrosepticum	*recA*, *ffh*	Takle et al. (2007)
Staphylococcus aureus	*Pyk*, *proC*	Theis et al. (2007)
Actinobacillus pleuropneumoniae	*glyA*, *recF*	Nielsen and Boye (2005)
Pseudomonas aeruginosa	*proC*, *rpoD*	Savli et al. (2003)

analysis (McMillan and Pereg 2014). Appropriate reference genes can be selected from a set of potential reference genes by analyzing expression of each gene in the target species under all different experimental treatments. Free software packages such as BestKeeper (Pfaffl et al. 2004), Normfinder (Andersen et al. 2004), and GeNorm (Vandesompele et al. 2002) can then be used to identify the most stable reference genes. Some reference genes identified for different bacterial species, including *A. brasilense*, are shown in Table 4.4.

4.2.3.2 Primer Design

qRT-PCR primers should be designed against the specific sequence of the gene of interest. For *Azospirillum* spp. these sequences may not always be available; however, the complete genome sequences of *Azospirillum* sp. B510 (Kaneko et al. 2010), *A. brasilense* Sp245 and *A. lipoferum* 4B (Wisniewski-Dye et al. 2011), are available and may be used to design specific primers for closely related species. Ideally, for optimal PCR efficiency, the amplicon length should be between 50 and 150 bases. Longer amplicons can lead to poor amplification efficiency. Primers should be between 18 and 25 bases (20 bases is standard). The primer melting temperature (T_m) of each PCR primer should be between 58 and 60 °C, and the T_m of both primers should be within 4 °C of each other. The GC content of primers should be within 40–60 %. To avoid the formation of primer dimers in the PCR reaction complementarities between primers should be avoided. Primer design software can be used to simplify the primer design process and select the optimal primer pair for a given sequence.

4.2.3.3 One-Step qRT-PCR

In one-step PCR the reverse transcription step is carried out in the same reaction tube as the PCR reaction. This has the advantage of being quicker, involving less pipetting than a two-step protocol, and eliminates the possibility of contamination between reverse transcriptase and PCR steps. Commercial one-step RT-PCR kits

Table 4.5 Typical two-step cycling protocol for qRT-PCR

Step	Temperature (°C)	Time
Reverse transcription	50	10 min
Enzyme activation	95	5 min
Two-step cycling (35–40 cycles)		
Denaturation	95	10–15 s
Annealing/extension	60[a]	30 s

Table 4.6 Typical three-step cycling conditions for qRT-PCR

Step	Temperature (°C)	Time
Reverse transcription	50	10 min
Enzyme activation	95	5 min
Three-step cycling (35–40 cycles)		
Denaturation	95	10–15 s
Annealing	55–60[a]	30 s
Extension	72	30 s

[a]Annealing temperature can be altered based on the T_m of primers used

include a mastermix containing Taq DNA polymerase, fluorescent dye (most commonly SYBR), dNTPs, and $MgCl_2$, to which reverse transcriptase and template RNA is added. A typical 25 μL reaction mix consists of 12.5 μL RT-PCR master mix, 1 μM each forward and reverse primer, 0.25 μL reverse transcriptase, 10–100 ng template RNA.

Most commercial qRT-PCR kits have been optimized for use in a two-step cycling protocol, with a combined annealing/extension step. Typical two-step cycling conditions are shown in Table 4.5. A no-template control (to test for primer dimer formation or contamination of reagents), a positive control, and a minus reverse transcriptase control (to test for genomic DNA contamination) should always be included.

A three-step cycling protocol may be used as an alternative to the two-step cycling protocol. Typical three-step cycling conditions are shown in Table 4.6.

4.2.3.4 Two-Step qRT-PCR

Two-step qRT-PCR is carried out with separate reverse transcriptase and PCR cycling steps. A two-step protocol may be more sensitive and allows for individual optimization of the reverse transcriptase and PCR steps. cDNA synthesis can be carried out using a commercial kit, and the composition of buffers and amounts of reagents used will vary with supplier. For *Azospirillum*, a typical reaction combines 0.1 ng–5 μg total RNA with 50 ng random hexamers in annealing buffer and is incubated at 65–70 °C for 5 min to denature RNA. The reaction is then chilled for 2–5 min to allow annealing of primers. Other components are added to the reaction including dNTPs (0.5–1 mM), reverse transcriptase (15–200 U/reaction depending on enzyme used), RNase inhibitor (40–50 U/μL), and $MgCl_2$ (5 mM). The reaction

is incubated for 5–10 min at 25 °C, followed by 50 min at 50 °C to allow for extension. The reaction is terminated by heating to 85 °C for 5 min. The cDNA can then be used as template for a qPCR assay.

The reaction mix and cycling conditions for the qPCR reaction are similar to those described for one-step qRT-PCR, with the omission of reverse transcriptase from the reaction mix, and the initial 50 °C reverse transcriptase step is eliminated from the PCR cycle.

References

Allen NL, Hanson RS (1985) Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J Bacteriol 161:955–962

Andersen CL, Jensen JL, Orntoft TF (2004) 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

Arsène F, Katupitiya S, Kennedy IR, Elmerich C (1994) Use of *lac*Z fusions to study the expression of *nif* genes of *Azospirillum brasilense* in association with plants. Mol Plant Microbe Interact 7:748–757

Biondi EG, Marini F, Altieri F, Bonzi L, Bazzicalupo M, del Gallo M (2004) Extended phenotype of an *mreB*-like mutant in *Azospirillum brasilense*. Microbiology 150:2465–2474

Carreno-Lopez R, Campos-Reales N, Elmerich C, Baca BE (2000) Physiological evidence for differentially regulated tryptophan-dependent pathways for indole-3-acetic acid synthesis in Azospirillum brasilense. Mol Gen Genet 264:521–530

de Lorenzo V, Herrero M, Jakubzik U, Timmis KN (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J Bacteriol 172:6568–6572

De Zamaroczy M, Paquelin A, Elmerich C (1993) Functional organization of the *glnB-glnA* cluster of *Azospirillum brasilense*. J Bacteriol 175:2507–2515

Del Gallo MM, Gratani L, Morpurgo G (1985) Mutation in *Azospirillum brasilense*. In: Klingmuller W (ed) Azospirillum III: genetics, physiology, ecology. Springer, Berlin, pp 85–97

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci U S A 77:7347–7351

Dreyfus BL, Elmerich C, Dommergues YR (1983) Free living Rhizobium strain able to grow on N2 as the sole nitrogen source. Appl Environ Microbiol 45:711–713

Elmerich C (1983) *Azospirillum* genetics. In: Puhler A (ed) Molecular genetics of the bacteria-plant interaction. Springer, Berlin, pp 367–372

Elmerich C, Franche C (1982) *Azospirillum* genetics: plasmids, bacteriophages and chromosome mobilization. In: Klingmuller W (ed) Azospirillum genetics, physiology, ecology. Birkhäuser, Basel, pp 9–17

Fani R, Bazzicalupo M, Coianiz P, Polsinelli M (1986) Plasmid transformation of *Azospirillum brasilense*. FEMS Microbiol Lett 35:23–27

Fani R, Bazzicalupo M, Ricci F, Schipani C, Polsinelli M (1988) A plasmid vector for the selection and study of transcription promoters in *Azospirillum brasilense*. FEMS Microbiol Lett 50:271–276

Fogher C, Bozouklian H, Bandhari SK, Elmerich C (1985) Constructing of a genomic library of *Azospirillum brasilense* Sp7 and cloning of the glutamine synthetase gene. In: Klingmuller W (ed) Azospirillum III: genetics, physiology, ecology. Springer, Berlin

Haas D, Holloway BW (1976) R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol Gen Genet 144:243–251

Herrero M, de Lorenzo V, Timmis KN (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J Bacteriol 172:6557–6567

Holguin G, Patten CL, Glick BR (1999) Genetics and molecular biology of Azospirillum. Biol Fertil Soils 29:10–23

Hou X, McMillan M, Coumans JVF, Poljak A, Raftery MJ, Pereg L (2014) Cellular responses during morphological transformation in *Azospirillum brasilense* and its *flcA* knockout mutant. PLoS One 9(12):e114435. doi:10.1371/journal.pone.0114435

Jacob T, Laia M, Ferro J, Ferro MT (2010) Selection and validation of reference genes for gene expression studies by reverse transcription quantitative PCR in *Xanthomonas citri* subsp. citri during infection of *Citrus sinensis*. Biotechnol Lett 33:1177–1184

Kadouri D, Burdman S, Jurkevitch E, Okon Y (2002) Identification and isolation of genes involved in poly(beta-Hydroxybutyrate) biosynthesis in Azospirillum brasilense and characterization of a *phbC* mutant. Appl Environ Microbiol 68:2943–2949

Kadouri D, Jurkevitch E, Okon Y (2003) Poly β-hydroxybutyrate depolymerase (PhaZ) in Azospirillum brasilense and characterization of a *phaZ* mutant. Arch Microbiol 180:309–318

Kaneko T, Minamisawa K, Isawa T et al (2010) Complete genomic structure of the cultivated rice endophyte *Azospirillum* sp. B510. DNA Res 17(1):37–50

Katupitiya S, New PB, Elmerich C, Kennedy IR (1995) Improved N_2 fixation in 2,4-D treated wheat roots associated with *Azospirillum lipoferum*: studies of colonization using reporter genes. Soil Biol Biochem 27:447–452

Kaur S, Mishra M, Tripathi A (2009) Regulation of expression and biochemical characterization of a b-class carbonic anhydrase from the plant growth promoting rhizobacterium, *Azospirillum brasilense* Sp7. FEMS Microbiol Lett 299:149–158

Knauf VC, Nester EW (1982) Wide host range cloning vectors: a cosmid clone bank of an Agrobacterium Ti plasmid. Plasmid 8:45–54

Kokotek W, Lotz W (1989) Construction of a *lacZ*-kanamycin-resistance cassette, useful for site-directed mutagenesis and as a promoter probe. Gene 84:467–471

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM (1995) Four new derivatives of the broad-host-range vector pBBR1MCS carrying different antibiotic-resistance cassettes. Gene 166:175–176

Kumar S, Rai A, Mishra M, Shukla M, Singh P, Tripathi A (2012) RpoH2 sigma factor controls the photooxidative stress response in a non-photosynthetic rhizobacterium, *Azospirillum brasilense* Sp7. Microbiology 158:2891–2902

Liang YY, Kaminski PA, Elmerich C (1991) Identification of a *nifA*-like regulatory gene of *Azospirillum brasilense* Sp7 expressed under conditions of nitrogen fixation and in the presence of air and ammonia. Mol Microbiol 5:2735–2744

Liu J, Tan Y, Yang X, Chen X, Li F (2013) Evaluation of *Clostridium ljungdahlii* DSM 13528 reference genes in gene expression studies by qRT-PCR. J Biosci Bioeng 116(4):460–464

McMillan M, Pereg L (2014) Evaluation of reference genes for gene expression analysis using quantitative RT-PCR in *Azospirillum brasilense*. PLoS One 9(5):e98162. doi:10.1371/journal.pone.0098162

Michiels K, Vanstockem M, Vanderleyden J, Van Gool A (1985) Stability of broad host range plasmids in Azospirillum. Cloning of a 5.9 KBP plasmid from *A. brasilense* RO7. In: Klingmüller W (ed) Azospirillum III: genetics, physiology, ecology. Springer, Berlin, pp 63–73

Milcamps A, Van Dommelen A, Stigter J, Vanderleyden J, de Bruijn FJ (1996) The *Azospirillum brasilense rpo*N gene is involved in nitrogen fixation, nitrate assimilation, ammonium uptake, and flagellar biosynthesis. Can J Microbiol 42:467–478

Miller J (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, New York

Nielsen K, Boye M (2005) Real-time quantitative reverse transcription-PCR analysis of expression stability of *Actinobacillus pleuropneumoniae* housekeeping genes during in vitro growth under iron-depleted conditions. Appl Environ Microbiol 71:2949–2954

Pedrosa FO, Yates MG (1984) Regulation of nitrogen fixation (*nif*) genes of *Azospirillum brasilense* by *nifA* and *ntr* (*gln*) type gene products. FEMS Microbiol Lett 23:95–101

Pereg Gerk L (2004) Expression of *flcA*, a gene regulating differentiation and plant interaction in *Azospirillum*. Soil Biol Biochem 36:1245–1252

Pereg Gerk L, Paquelin A, Gounon P, Kennedy IR, Elmerich C (1998) A transcriptional regulator of the LuxR-UhpA family, FlcA, controls flocculation and wheat root surface colonisation by *Azospirillum brasilense* Sp7. Mol Plant Microbe Interact 11:177–187

Pereg Gerk L, Gilchrist K, Kennedy IR (2000) Mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense*-wheat associations. Appl Environ Microbiol 66: 2175–2184

Pfaffl M, Tichopad A, Prgomet C, Neuvians T (2004) 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

Quandt J, Hynes MF (1993) Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene 127:15–21

Reeve WG, Tiwari RP, Worsley PS, Dilworth MJ, Glenn AR, Howieson JG (1999) Constructs for insertional mutagenesis, transcriptional signal localization and gene regulation studies in root nodule and other bacteria. Microbiology 145:1307–1316

Revers LF, Pereira Passaglia LM, Marchal K, Frazzon J, Blaha CG, Vanderleyden J, Schrank IS (2000) Characterization of an Azospirillum brasilense Tn5 mutant with enhanced N_2 fixation: the effect of ORF280 on *nifH* expression. FEMS Microbiol Lett 183:23–29

Reznikoff WS (1982) Tn5 transposition and its regulation. Cell 31:307–308

Rodriguez H, Mendoza A, Cruz MA, Holguin G, Glick BR, Bashan Y (2006) Pleiotropic physiological effects in the plant growth-promoting bacterium *Azospirillum brasilense* following chromosomal labeling in the *clpX* gene. FEMS Microbiol Ecol 57:217–225

Rothballer M, Schmid M, Hartmann A (2003) In situ localization and PGPR-effect of Azospirillum brasilense strains colonizing roots of different wheat varieties. Symbiosis 34:261–279

Rothballer M, Schmid M, Fekete A, Hartmann A (2005) Comparative in situ analysis of ipdC–gfp mut3 promoter fusions of Azospirillum brasilense strains Sp7 and Sp245. Environ Microbiol 7:1839–1846

Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H (2003) Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. J Med Microbiol 52:403–408

Selvaraj G, Iyer VN (1983) Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. J Bacteriol 156:1292–1300

Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat Biotechnol 1:784–791

Takle G, Toth I, Brurberg M (2007) Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. BMC Plant Biol 7:50

Theis T, Skurray RA, Brown MH (2007) Identification of suitable internal controls to study expression of a *Staphylococcus aureus* multidrug resistance system by quantitative real-time PCR. J Microbiol Methods 70:355–362

Valverde A, Okon Y, Burdman S (2006) cDNA-AFLP reveals differentially expressed genes related to cell aggregation of *Azospirillum brasilense*. FEMS Microbiol Lett 265:186–194

Van Rhijn P, Vanstockem M, Vanderleyden J, De Mot R (1990) Isolation of behavioural mutants of *Azospirillum brasilense* by using Tn5 *lacZ*. Appl Environ Microbiol 56:990–996

Vande Brock A, Keijers V, Vanderleyden J (1996) Effect of oxygen on the free-living nitrogen fixation activity and expression of the *Azospirillum brasilense nifH* gene in various plant-associated diazotrophs. Symbiosis 21:25–40

Vande Broek A, van Gool A, Vanderleyden J (1989) Electroporation of Azospirillum brasilense with plasmid DNA. FEMS Microbiol Lett 61:177–182

Vande Broek A, Michiels J, DeFaria SM, Milcamps A, Vanderleyden J (1992) Transcription of the *Azospirillum brasilense nifH* gene is positively regulated by NifA and NtrA and is negatively controlled by the cellular nitrogen status. Mol Gen Genet 232(2):279–283

Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034

Vanstockem M, Michiels K, Vanderleyden J, Van Gool A (1987) Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn5 and Tn5-Mob insertion mutants. Appl Environ Microbiol 53:410–415

Vial L, Cuny C, Gluchoff-Fiasson K, Comte G et al (2006) N-acyl-homoserine lactone-mediated quorum-sensing in *Azospirillum*: an exception rather than a rule. FEMS Microbiol Ecol 58:155–168

Vieille C, Elmerich C (1992) Characterization of an *Azospirillum brasilense* Sp7 gene homologous to *Alcaligenes eutrophus phb*B and to *Rhizobium meliloti nod*G. Mol Gen Genet 231:375–384

Wisniewski-Dye F, Borziak K, Khalsa-Moyers G et al (2011) *Azospirillum* genomes reveal transition of bacteria from aquatic to terrestrial environments. PLoS Genet 7:e1002430

Zhao W, Li Y, Gao P et al (2011) Validation of reference genes for real-time quantitative PCR studies in gene expression levels of *Lactobacillus casei* Zhang. J Ind Microbiol Biotechnol 38:1279–1286