A cyanobacterial gene encoding an ortholog of Pirin is induced under stress conditions.

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Abstract

Pirin is a recently identified protein in eukaryotes as a transcription cofactor or as an apoptosis-related protein. Although Pirin is highly conserved from bacteria to human, there have been no reports on prokaryotic Pirin orthologs. We show here that *pirA* (sll1773) encoding an ortholog of Pirin together with an adjacent gene, *pirB* (ssl3389), was up-regulated under high salinity and some other stress conditions in a cyanobacterium *Synechocystis* sp. PCC 6803. Induction of the *pirAB* genes was not related to cell death and disruption of *pirA* did not affect the gene expression profile. Expression of the *pirAB* genes was negatively regulated by a LysR family transcriptional regulator encoded by *pirR* (slr1871) located immediately upstream of *pirAB* in the divergent direction. DNA microarray analysis indicated that PirR repressed expression of closely located ORFs, slr1870 and *mutS* (sll1772), in addition to *pirAB* and *pirR* itself.

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1. Introduction

Pirin is a recently defined protein initially isolated in human by a yeast two-hybrid

screen as an interactor of the nuclear factor I/CCAAT box transcription factor (NF1/CTF1) [1].

Subsequently, it was revealed that Pirin formed quaternary complexes with Bcl-3 and two

NF-κBs on the target sequences of NF-κB to promote the transcription of anti-apoptotic genes

[2]. These observations indicated that Pirin worked as a transcription cofactor in human. Pirin

did not have any known functional domains. However, it has been considered to belong to the

cupin superfamily that is among the most functionally diverse of any protein family identified

to date [3], since it contained two β-barrel domains conserved among the cupin superfamily.

Like other members in the cupin superfamily, Pirin had a metal binding motif. Very recently,

the crystal structure of human Pirin was determined at 2.1-Å resolution [4]. It was revealed

that a single Fe^{2+} ion was located in the N-terminal cupin domain where it was coordinated by

2

three histidine residues and one glutamic acid residue. Pirin may require the participation of the metal ion for its interaction with Bcl-3 to co-regulate the NF-κB transcription pathway.

Orthologous proteins of Pirin were found in many organisms ranging from Archae bacteria to mammals [1]. Particularly, the N-terminal cupin domain including the four metal-coordinating residues was strictly conserved among them. As for Pirin orthologs in plants, there have been two reports. In tomato, it was revealed that mRNA level of *Le-pirin* encoding a Pirin ortholog dramatically increased during camptothecin-induced programmed cell death [5]. In *Arabidopsis*, on the other hand, a Pirin ortholog (AtPirin1) was isolated as a protein interacting with α-subunit of G protein by a yeast two-hybrid screen [6]. T-DNA insertion to *atpirin1* caused decreased germination levels in the absence of stratification. In addition, abscisic acid-imposed delay of germination and of early seedling development was observed in the mutant. These data indicate that AtPirin1 is involved in the regulation of seed germination and early seedling development in *Arabidopsis*. There are five genes encoding Pirin orthologs in the genome of *Arabidopsis*. Pirin orthologs may be functionally diverse and it is possible that each of five genes in *Arabidopsis* has different role.

So far, there is no report on characterization of Pirin orthologs in prokaryotes, although Pirin orthologs are highly conserved among many bacterial species. In this report, we showed that a gene encoding a Pirin ortholog in a cyanobacterium *Synechocystis* sp. PCC 6803 was highly induced under severe salt stress and some other stress conditions. Furthermore, we found that expression of this gene, *pirA*, was negatively regulated by a LysR-type transcriptional regulator encoded by a gene, *pirR*, located immediately upstream of *pirA* in the divergent direction.

2. Materials and methods

2.1. Strains and culture conditions

A glucose-tolerant wild-type strain of *Synechocystis* sp. PCC 6803 was grown at 32° C in BG-11 medium with 20 mM Hepes-NaOH, pH7.0, under continuous illumination of 50 μmol photons $m^{-2} s^{-1}$ with bubbling of air. The *pirA*, *pirB* and *pirR* disrupted mutants were grown under the same condition, except that kanamycin sulfate at 20 μg/ml was added to the medium. Cells were grown to a cell density equivalent to an OD_{730} of 0.2-0.3 and various stress treatments were applied.

2.2 Construction of the pirA*,* pirB *and* pirR *disrupted mutants*

To generate constructs for disruption of *pirA* and *pirB*, gene-flanking segments of *pirA* (800 bp) and *pirB* (1000 bp) were amplified by PCR using the chromosomal DNA of *Synechocystis* as the template and cloned into pT7Blue T-vector (Novagen). Primers used for PCR were as follows: Δ*pirA* forward: 5'-TGCAATCTGTGCTTAAAA-3'and reverse: 5'-TAAGGTAGGTCAAATAGT-3', Δ*pirB* forward: 5'-AGTTTACTTAGGAGTGCC-3'and reverse: 5'-GGATCACTCCCAATCAAT-3'. A kanamycin-resistance cartridge, which had been excised from the plasmid pRL161 by digestion with *Hin*cII, was inserted in the *Bsm*FI site of the *pirA* fragment and in the *Sty*I site of the *pirB* fragment. A construct for disruption of *pirR* was a kind gift from Prof. M. Ikeuchi in University of Tokyo. In this construct, the central part of *pirR* coding region was deleted with *Hpa*I and replaced with the kanamycin-resistance cartridge. Wild-type cells were transformed with these constructs and the resulting transformants were selected by 20 μg/ml kanamycin sulfate. The complete segregation of the mutant chromosomes was verified by PCR.

2.3. Northern blot analysis

Isolation of RNA by the hot-phenol method and Northern blot analysis using DIG RNA Labeling and Detection kit (Roche) were performed as previously described [7]. When

4

RNA probes for *pirA*, *pirB* and *pirR* genes were generated, the following primers were used

for amplification: *pirA* forward: 5'-TGCAATCTGTGCTTAAAA-3'and reverse:

5'-TAAGGTAGGTCAAATAGT-3', *pirB* forward:

5'-AACATATGACTGAACCAACCATT-3'and reverse:

5'-AAGGATCCTAAAGTTTGGCGTGGGC-3' and *pirR* forward:

5'-AACATATGGACAAACTTGAAAGT-3'and reverse:

5'-AAGGATCCTTATTTGCGGTAATGGTC-3'. PCR products were cloned into pT7Blue

T-vector and the resulting plasmids were used as templates for in vitro transcription.

2.4. DNA microarray analysis

Total RNA used for DNA microarray analysis was isolated using the RNeasy Midi kit

(Qiagen) as previously described [8]. After the removal of trace amounts of contaminating

genomic DNA by treatment with DNase I (Takara), RNA was labeled with Cy3-dUTP or

Cy5-dUTP (Amersham Pharmacia) using RNA fluorescence labeling core kit Ver. 2.0

(M-MLV version; Takara). Hybridization of labeled probes with CyanoCHIP (ver. 1.6;

Takara) was performed according to the manufacturer's instruction. Image acquisition with a

ScanArray 4000 (GSI Lumonics) and data analysis by a QuantArray version 2.0 software

(GSI Lumonics) were performed as previously described [9].

2.5. Preparation of the PirR protein

A 0.9 kb PCR fragment that covered the putative ribosome binding site and the entire

reading frame of *pirR* was amplified from the chromosomal DNA of *Synechocystis* using a

pair of primers, 5'-AAGAATTCAGGGACAAT<u>AGGA</u>ATATC-3' and

5'-AAGGATCCTTATTTGCGGTAATGGTC-3'. A putative ribosome binding site in the

forward primer is underlined. The resulting PCR fragment was cloned into pT7Blue T-vector

so that PirR protein could be induced under the control of T7 promoter. After the confirmation

of the nucleotide sequence and the direction of the insert DNA, the plasmid, named

pRBS1871, was introduced into *E. coli* strain XL1-Blue MRF' (Stratagene). PirR protein was

mildly induced without IPTG during the overnight culture at 37ºC. Induction by IPTG was

failed because cells could not grow after the addition of IPTG. As a negative control, *E. coli*

transformed with pT7Blue vector without insert DNA was cultured in the same way. The

overnight cultures of *E. coli* cells were harvested by centrifugation, resuspended in buffer (20

mM Tris-HCl, pH 8.0, 0.1 M NaCl), and disrupted by sonication for 30 sec for 10 times at 4ºC.

After the removal of unbroken cells and insoluble materials by centrifugation, the soluble

protein fraction was used for gel mobility shift assays.

2.6. Gel mobility shift assay

For preparation of the probe and the specific competitor DNA for gel mobility shift

assays, DNA fragment including a 129 bp intergenic region of *pirAB* and *pirR* was amplified

by PCR, using the primers 5'-AACGTACGATGACCTCCGTTATTTT-3'and

5'-AACGTACGTGATATTCCTATTGTCCC-3'. The 3' end of DNA fragment for the probe

was labeled with DIG-ddUTP by the terminal transferase according to manufacturer's

instructions (DIG Gel shift Kit; Roche). Assays were performed using DIG Gel shift Kit

according to manufacturer's instructions. Cell extract from *E. coli* harboring pRBS1871 or

pT7Blue vector was incubated with 30 fmol DIG-labeled DNA fragment in a 20 μl reaction

mixture containing 1 μg poly d[I-C], 0.1 μg poly $_L$-lysine, 20 mM Hepes-KOH (pH 7.6), 1

mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% (w/v) Tween 20, and 30 mM KCl. After

incubation for 15 min at room temperature, 5 μl of gel loading buffer consisting of 60% (v/v)

of 1×TBE and 40% (v/v) glycerol was added to the reaction mixture. Samples were then

applied onto a 6% polyacrylamide gel and subjected to electrophoresis at 95 V for 2.5 h at

4°C. DNA and protein were transferred to a nylon membrane (Hybond N+; Amersham Biosciences) by capillary transfer method and fixed by baking at 80°C for 2 h. Detection of DIG-labeled probe was performed according to the standard protocol for DIG Luminescent Detection kit (Roche).

3. Results and discussion

3.1. Expression of the pirAB *genes in* Synechocystis *sp. PCC 6803*

Pirin orthologs were found in almost all cyanobacterial species whose genomic sequences had been determined. In *Synechocystis* sp. PCC 6803, sll1773 encoded a Pirin ortholog that was 37% identical to Pirins of human and tomato. First, we examined if sll1773 was transcribed under normal growth conditions by Northern blot analysis. A faint band of 1.0 kb in length was detected irrespective of growth phase, indicating that sll1773 and small ORF, ssl3389, located immediately downstream of sll1773 were co-transcribed (Fig. 1A, lane for 0 M and Fig. 1C, lane for 0 h). ssl3389 encoded a putative protein having two zinc finger motifs. Hereafter we called sll1773 and ssl3389, *pirA* and *pirB*, respectively. Co-transcription of *pirA* and *pirB* was verified by the fact that 1.0 kb transcript was also detected by Northern blot analysis performed using *pirB* probe (data not shown).

We searched for the conditions where expression of the *pirAB* genes was up-regulated. As shown in Fig. 1A, *pirAB* transcript was highly accumulated when cells were cultured in the presence of 0.8 M or 1.0 M NaCl for 6 h. When considering the growth property of cells with various concentration of NaCl, it seems that *pirAB* genes are expressed only under severe salt stress conditions inducing the inhibition of growth (Fig. 1B). Next, we examined the time course of the induction of *pirAB* after the addition of 1 M NaCl (Fig. 1C). The amount of *pirAB* transcript increased after 3 h of incubation and reached its maximum after 6 h. The high transcript level was maintained even after 30 h (data not shown). Kanesaki

et al. examined the effect of salt stress on the gene expression in *Synechocystis* sp. PCC 6803 using DNA microarray technique [10]. *pirAB* was not included in their list of genes whose expression was enhanced by salt. This is probably because the salt stress condition they employed, that is, incubation with 0.5 M NaCl for 30 min, was too mild to induce the expression of *pirAB*. Apparently, compared with other salt-stress responsive genes, *pirAB* genes are induced by higher concentration of salt with slower kinetics.

Accumulation of the *pirAB* transcript was also observed by the addition of sorbitol (Fig. 2A), ethanol (Fig. 2B) and methyl viologen (Fig. 2C) with smaller extent of induction than that attained by NaCl. Although both NaCl and sorbitol are thought to cause the osmotic stress, the dependence of expression level of *pirAB* on the concentration of these reagents was quite different. The expression was more induced by sorbitol than by NaCl at low concentrations while NaCl was more effective than sorbitol at high concentrations (Figs. 1A and 2A). High concentration of NaCl may be perceived by cells as ionic stress rather than osmotic stress as suggested by Kanesaki et al. [10].

pirAB could be induced by 0.4 M sorbitol or 5% ethanol that did not affect the growth property of cells so much (Figs. 2D and 2E). On the other hand, 8 μM methyl viologen that was sufficient for growth inhibition could not induce *pirAB* (Fig. 2F). These results suggest that there is no correlation between the induction of *pirAB* and growth property of the cells. Although *pirA* encodes an ortholog of Pirin characterized as an apoptosis-related protein in human and tomato [2, 5] and is up-regulated under stress conditions, its induction is not likely to be directly related to cell death. We also examined the transcript level of *pirAB* under low temperature, high temperature and high light conditions. However, we failed to detect the up-regulation of *pirAB* under these conditions so far.

In order to know the physiological significance of the induction of *pirAB* under stress conditions, we made gene disrupted mutants of *pirA* and *pirB* and examined their growth

properties. No difference in growth properties and absorption spectra of cultures was observed between the wild type and mutant strains under normal growth conditions, salt stress conditions with 0.8 M or 1.0 M NaCl and hyperosmotic stress conditions with 0.4 M sorbitol (data not shown). Since PirA is an ortholog of Pirin shown to work as a transcription cofactor in human [2] and PirB has two zinc finger motifs that may be associated with DNA binding, it is possible that both PirA and PirB are involved in transcriptional regulation. Thus, we examined the effect of disruption of *pirA* or *pirB* on the gene expression profile under salt stress conditions by using DNA microarray technique. No significant difference in gene expression profile was observed between the wild type and mutant strains before and after the incubation with 1 M NaCl for 6 h (data not shown). PirA and PirB may be related to cellular function other than transcriptional regulation. A phylogenetic analysis of cupins placed Pirins in the same clade as cysteine dioxygenase [3], although no eukaryotic Pirins have been reported to have enzymatic activities. The structural analysis of human Pirin revealed that the Fe^{2+} binding site in Pirin closely resembled that found in germin, a manganese-containing plant protein belonging to the cupin superfamily [4]. Germin-like proteins have activities of oxalate oxidase and superoxide dismutase [11] and their transcripts were reported to be induced by the addition of NaCl in some cases [12, 13]. It may be possible that Pirins initially worked as oxidases, and eukaryotic Pirins lost the enzymatic activities and gained other functions in the course of evolution. Further investigation will be required to test this possibility.

3.2. Transcriptional regulation of the pirAB *genes by a LysR family transcriptional regulator, PirR*

There is a gene, slr1871, encoding a LysR family transcriptional regulator located immediately upstream of *pirAB* in the divergent direction. Many of the LysR family proteins

are known to regulate the divergently transcribed operons located upstream of themselves [14]. slr1871 was up-regulated by higher concentration of NaCl just like *pirAB*. The induction of slr1871 was detectable after 1 h of incubation with 1 M NaCl (data not shown).

A gene disrupted mutant of slr1871 showed similar growth property to the wild type cells under both normal and stress conditions (data not shown). It was observed in the mutant that *pirAB* was highly expressed either in the presence or absence of NaCl (Fig. 3A). This indicates that slr1871 is a negative regulator of *pirAB*. Therefore, we named slr1871 *pirR*. PirR repressed its own transcription as well as that of *pirAB*, judging from the results of reporter analysis using a bacterial luciferase gene, *luxAB*, fused to the intergenic region of the *pirAB-R* genes. The reporter construct was prepared as described previously [7]. When the reporter construct was introduced into both the wild type and the *pirR*-disrupted mutant, luminescence level from the mutant was 20-25 times higher than that of the wild type under the normal growth conditions irrespective of the direction of the *pirAB-R* promoter fragment fused to *luxAB* (data not shown). To examine whether PirR directly regulates transcription of *pirAB* and *pirR* as a repressor, gel mobility shift assays were performed with an extract of *E. coli* cells expressing PirR, using the *pirAB-pirR* intergenic region as a probe (Fig. 3B). A single shifted band representing a DNA-protein complex was clearly observed by the addition of the extract from *E. coli* cells expressing PirR (lane 3), while the addition of the extract from *E. coli* cells carrying the vector pT7Blue did not yield the shifted band (lane 2). Addition of a 200-fold excess amount of specific competitor DNA inhibited binding of the protein to the probe (lane 4). From these results, it can be concluded that PirR directly binds to the *pirAB-pirR* intergenic region to repress the expression of both *pirAB* and *pirR*.

In order to identify all the genes regulated by PirR, we performed DNA microarray analysis on the wild type and the *pirR*-disrupted mutant (Fig. 4). Besides *pirAB* and *pirR*, only two ORFs, slr1870 encoding an unknown protein and sll1772 encoding DNA mismatch repair

protein, MutS, were identified as putative target genes of PirR under normal growth conditions (Fig. 4, upper panel and Fig. 5). Interestingly, these genes were located very close to *pirAB* and *pirR* genes. Occurrence of the polar effect caused by the insertion of kanamycin-resistance cartridge into *pirR* cannot be ruled out. However, we think such a possibility is rather low since transcription of slr1870 and *mutS* was independent of that of *pirR* and their promoter elements were not modified by the disruption of *pirR*. There were two ORFs, sll1771 and sll1770, immediately upstream of *mutS*. Transcript levels of these two ORFs were not affected by the disruption of *pirR*, indicating that *mutS* was transcribed independently of these ORFs. Up-regulation of *pirA*, *pirR*, *mutS* and slr1870 was not observed after the incubation with 1 M NaCl for 6 h (Fig. 4, lower panel). This result clearly shows that PirR works as a repressor under the normal growth conditions but not under salt stress conditions. This type of transcriptional regulation was also observed with NdhR, the other LysR family transcriptional regulator in *Synechocystis* sp. PCC 6803. NdhR was shown to be important for acclimation to inorganic carbon starvation and salt stress [15]. Under normal growth conditions, it negatively regulates expression of *ndhR* itself, divergently transcribed sll1727 and several genes responsible for both CO_2 and HCO_3^- uptake [15, 16]. How these LysR family transcriptional regulators sense the environmental changes remains to be elucidated.

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Figure legends

Fig. 1. Effects of the addition of NaCl on the expression level of the *pirAB* genes and growth property of the wild-type cells. A: Northern blot analysis of the *pirAB* genes. Total RNA was isolated from wild-type cells incubated with various concentration of NaCl for 6 h. Two μg of RNA was loaded in each lane. B: Growth curves under salt stress conditions. NaCl was added to cultures at the final concentration indicated in the figure. C: Northern blot analysis of the *pirAB* genes. Total RNA was isolated from wild-type cells incubated with 1 M NaCl for indicated period. Two μg of RNA was loaded in each lane.

Fig. 2. Effects of various stress treatments on the expression level of the *pirAB* genes and growth property of the wild-type cells. A, B, C: Northern blot analysis of the *pirAB* genes. Total RNA was isolated from wild-type cells incubated with various concentration of sorbitol (A), ethanol (B) and methyl viologen (C) for 6 h. Two μg of RNA was loaded in each lane. D,E,F: Growth curves under various stress conditions. Sorbitol (D), ethanol (E) and methyl viologen (F) were added to cultures at the indicated final concentration.

Fig. 3. Interaction of PirR with the *pirAB* genes. A: Northern blot analysis of the *pirAB* genes. Total RNA was isolated from wild-type cells and the *pirR* disrupted mutant before and after incubation with 1 M NaCl for 6 h. Two μg of RNA was loaded in each lane. B: Gel mobility shift assays of PirR binding to the *pirAB-pirR* intergenic region. Lane 1 contained no crude protein. Lanes 2 and 3 contained 100 μg of crude protein from *E. coli* cells without and with expression of PirR, respectively. Lane 4 contained 100 μg of crude protein from *E. coli* cells

13

expressing PirR and 200-fold excess amount of the specific competitor DNA.

Fig. 4. Effect of disruption of the *pirR* gene on the global gene expression profile shown by scatter plots. Gene expression profiles before (upper panel) and after (lower panel) the incubation with 1 M NaCl for 6 h were compared between the wild-type cells and the *pirR*-disrupted mutant. Signal intensities of 6148 spots (3074 ORFs in duplicate) were plotted. Solid lines indicate the limit of experimental deviations (50% deviation). Open symbols show the signal intensities of duplicate spots of putative target genes of PirR. open cycle: *pirA*, open square: *pirR*, open diamond: *mutS*, open triangle: slr1870.

Fig. 5. Effect of the disruption of *pirR* on the adjacent ORFs to *pir* genes.
The map of the *pir* region of the chromosome of *Synechocystis* sp. PCC 6803 and induction ratio of each ORF by disruption of the *pirR* gene are shown. Values are averages ± SD obtained from four independent and each duplicate results of DNA microarray experiments (n = 8). Induction ratio of ssr3159 and ssl3389 could not be obtained since these small ORFs were not spotted onto the microarray. Putative target genes of PirR were shown in the hatched boxes in the map.
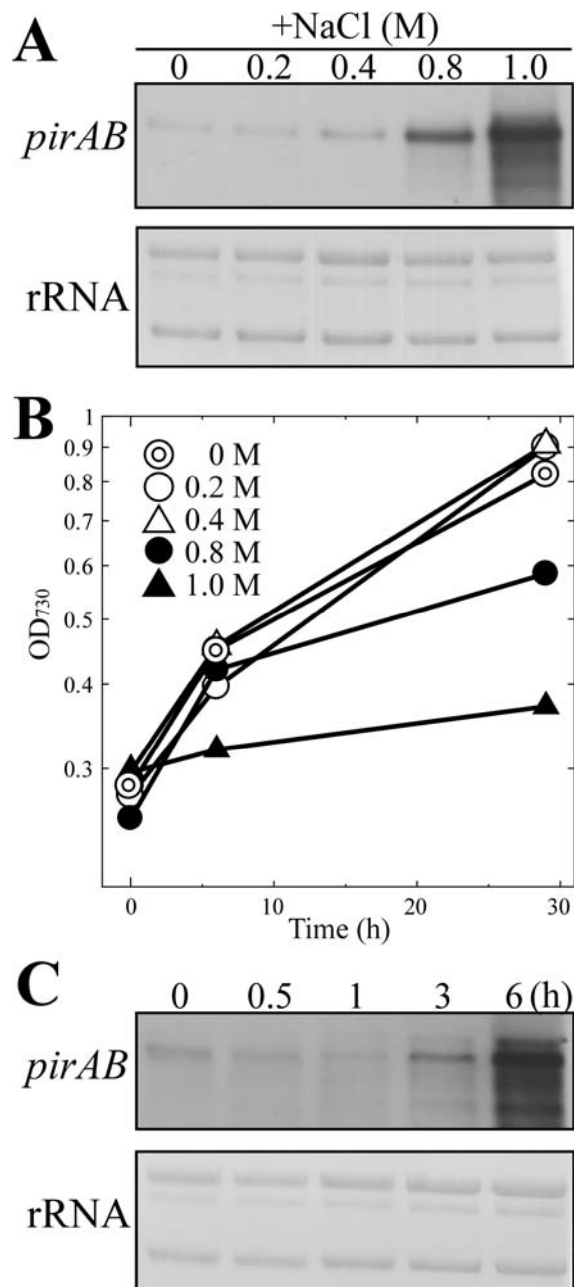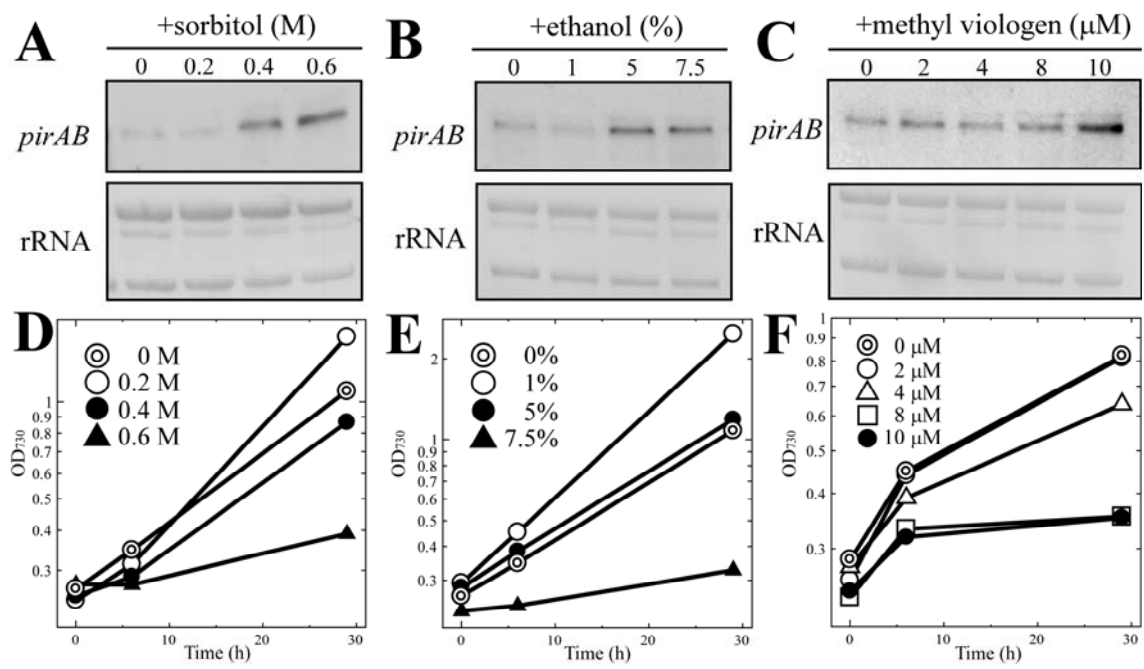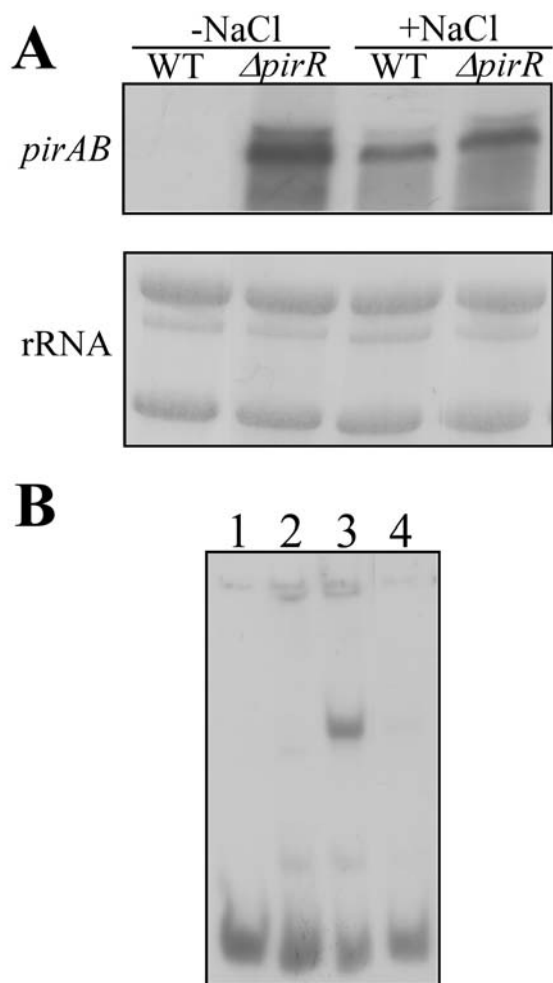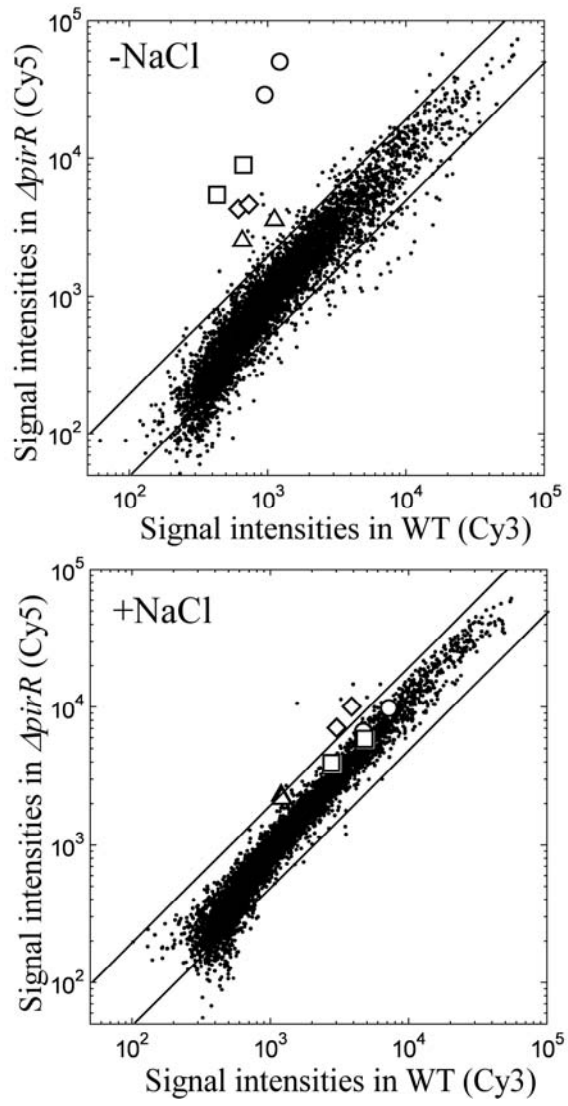
Fig. 1

Fig. 2

A

-NaCl   +NaCl
WT   *ΔpirR*   WT   *ΔpirR*

pirAB

rRNA

B

1   2   3   4

Fig. 3

Fig. 4
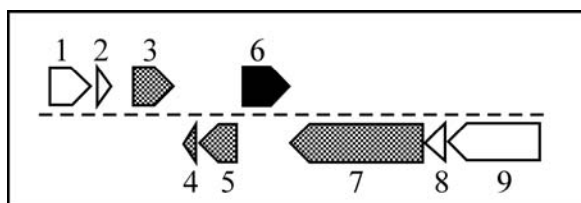
	ORF No.	Gene Name	Induction by disruption of *pirR* (fold)
1	slr1869		1.04 ± 0.09
2	ssr3159		n.d.
3	slr1870		4.83 ± 1.74
4	ssl3389	*pirB*	n.d.
5	sll1773	*pirA*	31.18 ± 6.39
6	slr1871	*pirR*	9.40 ± 3.25
7	sll1772	*mutS*	5.85 ± 1.10
8	sll1771	*pphA*	0.94 ± 0.16
9	sll1770		1.29 ± 0.08

Fig. 5