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Potassium and sodium transporters of *Pseudomonas aeruginosa* regulate virulence to barley

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Abstract We investigated the role of three uncharacterized cation transporters of *Pseudomonas aeruginosa* PAO1 as virulence factors for barley: PA1207, PA5021, and PA2647. PAO1 displayed reduced barley virulence with inactivated PA1207, PA5021, and PA2647 as well as with one known Na^+/H^+ antiporter, PA1820. Using the *Escherichia coli* LB2003 mutant lacking three K^+ uptake systems, the expression of the PA5021 gene repressed LB2003 growth with low K^+, but the strain acquired tolerance to high K^+. In contrast, the expression of the PA1207 gene enhanced growth of LB2003 with low K^+ but repressed its growth with high K^+; therefore, the PA5021 protein exports K^+, while the PA1207 protein imports K^+. The PA5021 mutant of *P. aeruginosa* also showed impaired growth at 400 mM KCl and at 400 mM NaCl; therefore, the PA5021 protein may also export Na^+. The loss of the PA5021 protein also decreased production of the virulence factor pyocyanin; corroborating this result, pyocyanin production decreased in wild-type PAO1 under high salinity. Whole-genome transcriptome analysis showed that PAO1 induced more genes in barley upon infection compared to the PA5021 mutant. Additionally, PAO1 infection induced water stress-related genes in barley, which suggests that barley may undergo water deficit upon infection by this pathogen.

Keywords Antiporter · Barley · Virulence

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Introduction

Pathogenic bacteria like *Pseudomonas aeruginosa* cause disease by forming biofilms (Yahr and Parsek 2006), and biofilms form by initial attachment, production of exopolysaccharides, and community development on the surface of the host (Stoodley et al. 2002). Since biofilm formation is important for pathogenesis, the mechanism of biofilm formation has been studied extensively using *P. aeruginosa.* This ubiquitous opportunistic pathogen forms persistent biofilms on medical devices, in lungs, and on plant surfaces (Zegans et al. 2002). Although some bacteria establish host-specific relationships as seen in symbiosis between leguminous plants and *Rhizobium* sp. (Galibert et al. 2001) or antagonism between rice and *P. avenae* H8201 (Che et al. 2002), *P. aeruginosa* is able to infect a variety of hosts including human (Yahr and Parsek 2006), mice (Rumbaugh et al. 1999), nematode (Tan et al. 1999), insects (D'Argenio et al. 2001), bacteria (Park et al. 2005), and plants (Prithiviraj et al. 2005a, b). Therefore, *P. aeruginosa* is frequently used as a model bacterium for pathological research because of its well-established genomic resources (Stover et al. 2000), deoxyribonucleic acid (DNA) microarrays, and libraries of mutants (Jacobs et al. 2003; Lewenza et al. 2005; Liberati et al. 2006).

Plants and animals are thought to share common bacterial virulence factors (cf., *Arabidopsis* and mice) because pathogenic bacteria often cause disease in both

animals and plants (Rahme et al. 2000). For example, of eight *P. aeruginosa* PA14 mutations that reduced killing in *Caenorhabditis elegans*, six of these mutations decreased virulence to *Arabidopsis* and five mutations decreased virulence to mice (Tan et al. 1999). However, research using animals as infection models is time consuming and tedious compared to plant infection models (Prithiviraj et al. 2005a, b). Hence, plant models may be an effective and inexpensive initial step to identify virulence factors. Additionally, because the genome sequence is highly conserved among pseudomonads (Buell et al. 2003), plant pathological studies using *P. aeruginosa* yield insights for research with plant pathogenic strains such as *P. syringae*, a well-studied model pseudomonad for agricultural research (Buell et al. 2003).

Barley is a major world-wide crop that is used for a variety of products such as human and animal foods and alcohol and is used for its genomic resources such as expressed sequence tags and genetic DNA markers (Pickering and Johnston 2005). The barley 22K GeneChip, carrying 22,840 probe sets (P/N 511012) is a powerful tool for comprehensive gene expression analysis (Close et al. 2004). Barley is often used to study pathological fungi in agriculture, and several bacteria are known barley pathogens such as *Xanthomonas transluscens* and *P. syringae* (Molina et al. 1996); however, information is limited on *P. aeruginosa* infection of barley (Attila et al. 2008). Hence, identification of virulence factors in *P. aeruginosa* will provide insights for barley pathogenesis, and these insights should translate to other hosts.

Pathogenic bacteria have developed diverse virulence factors including quorum-sensing systems, toxin-secretion systems, proteases, and phospholipases (Yahr and Parsek 2006). Additionally, involvement of monovalent cations in pathogenicity is common. Virulence of *Vibrio vulnificus* toward mice is regulated by K^+ uptake (Chen et al. 2004), and activity of listeriolysin O, a virulence factor in *Listeria monocytogenes* for mice, is increased in the presence of high K^+ (Myers et al. 1993). In *P. aeruginosa*, the loss of the Na^+ exporter Sha decreases virulence for mice (Kosono et al. 2005). These results suggest that virulence is partly influenced by K^+ or Na^+ for some pathogens.

In this study, to investigate further whether several putative monovalent cation transport systems function as virulence factors in PAO1, we used a rapid assay for pathogenicity based on barley seed germination. Germination is the initial stage of plant life cycle and is initiated 2 to 3 days after imbibition for barley; hence, the germination assay is rapid compared to animal models. In this paper, we studied four cation-transporting systems including one novel protein for K^+ import (PA1207), one novel protein for export of both K^+ and Na^+ (PA5021), one putative Na^+ exporter (PA2647), and one known Na^+ exporter (PA1820); these four proteins are found to be novel virulence factors for barley. In addition, whole-genome transcriptome analysis of barley revealed that PAO1 infection induces more disease- and stress-related genes than the less virulent PA5021 mutant. This is the first report of the identification of a K^+ transport system in *P. aeruginosa*, and the first whole-transcriptome determination of how barley responds to pathogenic attack by *P. aeruginosa*.

Materials and methods

Bacterial strains and growth conditions Strains and plasmids are listed in Table 1. Wild-type *P. aeruginosa* PAO1 was obtained both from the University of Washington (UW) with isogenic mutants based on transposons IS*phoA*/hah or IS*lacZ*/hah (Jacobs et al. 2003) as well as from the University of British Columbia (UBC) with isogenic mutants based on mini-Tn*5-luxCDABE* (Lewenza et al. 2005). Wild-type *P. aeruginosa* PA14 was obtained from the Harvard Medical School with isogenic mutants based on *MAR2xT7* (Liberati et al. 2006). To examine K^+ transport for the PA1207 and PA5021 proteins, the *E. coli* LB2003 mutant lacking three K^+ uptake systems (*kup1*, *kdp*, and *trk*) and the KNabc mutant lacking two Na^+/H^+ antiporters (*nhaA* and *nhaB*) and one Na^+ and K^+/H^+ antiporter (*chaA*) were obtained from Prof. Bakker (Universität Osnabrück; Stumpe and Bakker 1997) and Prof. Kuroda (University of Okayama; Nozaki et al. 1996), respectively. Luria–Bertani (LB) medium was used for culturing *P. aeruginosa* at 30°C and for culturing *E. coli* at 37°C unless otherwise noted. Tetracycline (50 μg/mL) was used for preculturing the *P. aeruginosa* transposon mutants. Growth of the *P. aeruginosa* PA5021 mutant was examined at 30°C and 250 rpm in M9 minimal medium supplemented with 0.2% (*w*/*v*) glucose and further amended with 400 mM KCl, 400 mM NaCl, 400 mM KNO_3, 400 mM $NaNO_3$, or 800 mM sorbitol. TY buffer (7.5 g/L tryptone, 3.5 g/L yeast extract) was used to investigate the sensitivity to hypo-osmotic stress (Verkhovskaya et al. 2001).

Verification of transposon insertion of P. aeruginosa mutant To confirm the transposon insertions for gene inactivation of all of the *P. aeruginosa* mutants, gene-specific primers were designed for the flanking region of the transposon insertion in the chromosome. For example, to verify the PA1054 mutant, gene-specific primers PA1054-F and PA1054-R were used to amplify the PA1054 gene. As a control, the PA1054 gene was amplified with PAO1 chromosomal DNA, but a polymerase chain reaction (PCR) product was not obtained from chromosomal DNA of the PA1054 mutant due to the transposon insertion. Then, as a second check of the transposon

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
Pseudomonas aeruginosa		
PAO1	Wild-type strain from UW or from UBC	Jacobs et al. (2003), Lewenza et al. (2005)
PA1054 (*sha*)	PA1054 Ω mini-*Tn5-lux*CDABE (PAO1_lux_27_F6), Tet^R	Lewenza et al. (2005)
PA1207	PA1207 Ω IS*lacZ*/hah (lacZwp08q2E05), Tet^R	Jacobs et al. (2003)
PA1496	PA1496 Ω IS*lacZ*/hah (lacZwp02q4D07), Tet^R	Jacobs et al. (2003)
PA1820 (*nhaB*)	PA1820 Ω IS*phoA*/hah (phoAwp05q1C06), Tet^R	Jacobs et al. (2003)
PA2647	PA2647 Ω IS*phoA*/hah (phoAbp01q4F02), Tet^R	Jacobs et al. (2003)
PA3660	PA3660 Ω mini-Tn*5-lux*CDABE (PAO1_lux_15_F2), Tet^R	Lewenza et al. (2005)
PA3739	PA3739 Ω mini-Tn*5-lux*CDABE (PAO1_lux_41_F12), Tet^R	Lewenza et al. (2005)
PA3887 (*nhaP*)	PA3887 Ω IS*lacZ*/hah (lacZwp07q3G05), Tet^R	Jacobs et al. (2003)
PA5021	PA5021 Ω IS*phoA*/hah (phoAwp03q2A07), Tet^R	Jacobs et al. (2003)
PA5518	PA5518 Ω IS*phoA*/hah (PAO1_lux_3_G11), Tet^R	Lewenza et al. (2005)
PA5529	PA5529 Ω IS*phoA*/hah (phoAbp02q3D10), Tet^R	Jacobs et al. (2003)
PA14	Wild-type strain	Liberati et al. (2006)
PA14_09490 (*phzM*)	PA14_09490 Ω *MAR2xT7* (PAMr_nr_mas_09_2_C8), Gm^R	Liberati et al. (2006)
PA14_09400 (*phzS*)	PA14_09400 Ω *MAR2xT7* (PAMr_nr_mas_11_1_D7), Gm^R	Liberati et al. (2006)
PA14_09270 (*pchE*)	PA14_09270 Ω *MAR2xT7* (PAMr_nr_mas_08_3_E3), Gm^R	Liberati et al. (2006)
PA14_33700 (*pvdF*)	PA14_33700 Ω *MAR2xT7* (PAMr_nr_mas_07_2_C6), Gm^R	Liberati et al. (2006)
Escherichia coli		
LB2003	F^- *kup1* (*trkD1*) Δ*kdpABC5* Δ*trkA rpsL metE thi rha gal*	Stumpe and Bakker (1997)
KNabc	Δ*nhaA* Ω Km^R Δ*nhaB* Ω Em^R Δ*chaA* Ω Cm^R	Nozaki et al. (1996)
Plasmid		
pMMB207	broad host range, *lacI*^{q}/*Ptac*, Cm^R	de Lorenzo et al. (1982)
pMMB207-PA1207	pMMB207 with PA1207 gene	This study
pMMB207-PA5021	pMMB207 with PA5021 gene	This study
pVLT31	broad host range, *lacI*^{q}/*Ptac*, Tet^R	de Lorenzo et al. (1982)
pVLT31-PA1207	pVLT31 with PA1207 gene	This study
pVLT31-PA5021	pVLT31 with PA5021 gene	This study

Tet^R, Km^R, Em^R, Cm^R, and Gm^R indicated the resistant genes for tetracycline, kanamycin, erythromycin, chloramphenicol, and gentamicin, respectively

inactivation, PA1054-R and Tn5-out2 primers were used to amplify the DNA fragment corresponding to the end of transposon and flanking the PA1054 gene with the PA1054 mutant (chromosomal DNA from the PAO1 did not yield a PCR product, as expected). Similarly, all of the other mutants used in this study (PA1207, PA1496, PA1820, PA2647, PA3660, PA3739, PA3887, PA5021, PA5518, and PA5529) were verified by this two-step PCR procedure (Table 2 lists the primers). The transposon-specific primers, Hah minus 138, LacZ 148, and Tn5-out2, were used for the transposon mutants created by IS*phoA*/hah (UW), by IS*lacZ*/hah (UW), and by mini-Tn*5-luxCDABE* (UBC), respectively.

Complementation plasmids Plasmid pMMB207 (Cm^R) and pVLT31 (Tet^R) have tight regulation of the inserted genes via a *laqI*^{q} repressor (de Lorenzo et al. 1982) and were used to express the PA1207 and PA5021 genes in *E. coli* LB2003 (pMMB207) or in the *E. coli* KNabc mutant (pVLT31) to examine the sensitivity to changes in K^+ or Na^+ concentration. The PA1207 gene was cloned from a PAO1 chromosomal DNA using Pfu DNA polymerase with the 1207-F and 1207-R primers (Table 2). After digesting both the PA1207 PCR fragment and pMMB207 with *Eco*RI/*Hin*dIII, the digested fragments were ligated into pMMB207. Similarly, the PA5021 gene was cloned using Pfu DNA polymerase with the 5021-F and 5021-R primers. The PA5021 fragment was digested with *Eco*RI and ligated into pMMB207. The resulting plasmids, pMMB207-PA1207 and pMMB207-PA5021, were digested by the restriction enzymes *Apa*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Sph*I, and *Stu*I to confirm correct construction. pVLT31-PA1207 and pVLT31-PA5021 were constructed in a similar manner.

Barley germination assay Seeds of barley (*Hordeum vulgare* L., cv. Belford) were purchased from Stover Seeds (Los Angels, CA, USA) and were surface sterilized in 1% sodium hypochlorite solution by stirring for 30 min followed by washing with sterilized distilled water ten times. *P. aeruginosa* and its mutants were grown in LB medium at 30°C, harvested when the turbidity at 600 nm reached 1.00±0.03, washed with sterilized distilled water twice, washed with Hoagland solution (Hoagland and Arnon 1950) once, and resuspended to a turbidity at

Table 2 Primers used in this study

Name	Sequence (5′ to 3′)
Primers for mutant verification	
PA1054-F	CCGGCAGCTTCGAACTCA
PA1054-R	GGTTTCGCCGAAGAACATCT
PA1207-F	AGTACCGCCACGAACTGGAG
PA1207-R	CTTCAGGTGCGGATACAGCTT
PA1496-F	GTCAAGACTTCCCATGGCTG
PA1496-R	GCTAGTCTGGTAAGGTGCCG
PA1820-F	GCGGAATTCCTCGAGATG
PA1820-R	TCCCGCAACTATAACAAGGG
PA2647-F	CTGATCCACCTGTTTGCCTC
PA2647-R	TGAAGATGTTCTGCTCGTGG
PA3660-F	CCTGGAGAGCGAGGAATTG
PA3660-R	CGGACGGCAGTTTCTACCTG
PA3739-F	GTGCTGCTGCTGATCCAGTT
PA3739-R	GAACTGCGCGTAGGTCAGC
PA3887-F	CAGACCAGGATGCGGATG
PA3887-R	AGTGCTGATGACCTGGTTCC
PA5021-F	GCCGTCACCGTCAATAATTT
PA5021-R	AGCAGTGAGACCAGGACGAT
PA5518-F	AGACGATGACCAGGAAGGTG
PA5518-R	ATGCACCATACGCCGTTACT
PA5529-F	AAACCGGCGAGGTGTTCT
PA5529-R	ATCAATGCCTTGTGTAGCCC
HaH minus 138	CGGGTGCAGTAATATCGCCCT
LacZ 148	GGGTAACGCCAGGGTTTTCC
Tn5-out2	CAGAACATATCCATCGCGTCCGCC
Primers for plasmid construction	
1207-F	GCCCCGAATTCGTGGAACACGGAAGCAGTTTTCTC
1207-R	GCCCCAAGCTTGTATCCCAACCTTCGTACTGCGA
5021-F	GCCCCGAATTCGCATGATCGCCGGCAACGACGG
5021-R	GCCCCAAGCTTTCGAAGAAGGGTGAATGCAGGTT

600 nm of 1.00±0.03. Fifteen barley seeds were germinated in 10 mL of Hoagland solution without PAO1 (control) and with PAO1 at 25°C with gentle shaking. Germination was examined after 3 days, and each experiment was repeated at least three times. Significant differences were estimated by Fisher's least significant difference (LSD) test (Miller 1981).

Biofilm formation Biofilm formation was measured using 96-well polystyrene plates as described previously (Pratt and Kolter 1998). Briefly, overnight cultures of *P. aeruginosa* cells were diluted in LB or LB with 0.2% (*v*/*w*) glucose to the turbidity at 600 nm of 0.05, and then cells were grown in each well at 30°C without shaking for 24 h. After measuring the growth using the turbidity at 620 nm, plates were washed with distilled water three times, and the biofilm formed was stained with 0.1% crystal violet for 20 min. After washing with distilled water three times, the biofilm was eluted in 95% EtOH and then quantified at an absorbance at 540 nm using a Sunrise^{TM} plate reader (Tecan US, Durham, NC, USA). Ten replicate wells were used for each experiment, and the experiment was repeated three times.

Motility assay Swimming motility was measured using 0.3% agar plates with 1% tryptone and 0.25% NaCl as described previously (Sperandio et al. 2002). Halos were measured at 18 h after incubation at 30°C without shaking. Six plates were used for each experiment, and three independent experiments were performed.

Functional analysis of PA1207 and PA5021 E. coli LB2003 lacking three K^+ uptake systems was transformed with pMMB207 (vector control), pMMB207-PA1207, and pMMB207-PA5021, and the transformants were grown at 37°C in 25 mL LBK medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 25 mM Tris–HCl, pH 6.5; Radchenko et al. 2006a, b). Chloramphenicol (50 μg/mL) and isopropyl-β-D(−)-thiogalactopyranoside (IPTG, 1 mM) were routinely added to the medium. Overnight cultures (25 μL) were reinoculated into 25 mL KML medium (46 mM Na_2HPO_4, 23 mM NaH_2PO_4, 8 mM $(NH_4)_2SO_4$, 1 mM sodium citrate, 0.4 mM $MgSO_4$, 6 μM $FeSO_4$, 1 mg/L thiamine, 10 mM glucose) with 50 mM KCl for high K^+ stress conditions or with 200 mM KCl for low K^+ stress conditions. After 24 h, cells were transferred to 25 mL

KML medium with 25, 40, 50, and 60 mM KCl (low K^+ stress) or with 400, 450, 500, and 600 mM KCl (high K^+ stress). The turbidity of the cell suspension at 600 nm was adjusted to 0.05. *E. coli* KNabc lacking two Na^+/H^+ antiporters and one Na^+ and K^+/H^+ antiporter was transformed with pVLT31 (vector control), pVLT31-PA1207, and pVLT31-PA5021 and grown in the LBK medium. Tetracycline (10 μg/mL) and IPTG (1 mM) were routinely added to the medium. To examine the growth of KNabc under 200 mM NaCl, 1/100 volume of KNabc cells grown in LBK medium were transferred to the LBK medium with 200 mM NaCl. Growth was measured after 24 h.

Barley RNA extraction and GeneChip analysis Barley seeds were surface sterilized, and the PAO1 wild-type and the PA5021 mutant cells were grown in LB medium at 30°C and were harvested at a turbidity at 600 nm of 1. Cells were washed with sterilized distilled water twice, washed with Hoagland solution once, and then resuspended to the turbidity at 600 nm of 1.00±0.03. Fifteen barley seeds were germinated in 20 mL of Hoagland solution, Hoagland solution with PAO1, or Hoagland solution with the PA5021 mutant at 30°C for 12 h with gentle shaking. Seeds were washed briefly with sterilized distilled water and stored at −80°C. Total ribonucleic acid (RNA) was extracted by a modified sodium dodecyl sulfate (SDS)–phenol method (Shirzadegan et al. 1991) by homogenizing seeds using a mortar and pestle with liquid nitrogen to a powder, and 20 mL of extraction buffer (100 mM Tris–HCl, 100 mM NaCl, 1% SDS, pH 9.0) was added. After centrifugation at 10,000×*g* at 4°C for 5 min, the 18 mL supernatant was transferred to 45-mL centrifuge tubes. The same volume of phenol/chloroform/isoamylalcohol (PCI) was added to the supernatant. After vigorous shaking for 2 min, the sample was centrifuged at 15,000×*g* at 4°C for 5 min. Supernatants were purified by extracting with PCI twice, 1/3 volume of 10 M LiCl was added, and they were stored at −20°C for 2 h. After centrifugation at 15,000×*g* for 15 min at 4°C, precipitated RNA was resuspended in 500 μL RNase-free water. Following the PCI extraction, RNA was precipitated by 2.5 volumes of EtOH overnight. Extracted RNA was purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Quality and quantity of RNA was estimated by monitoring the absorbance at 260/280 nm and by using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Subsequently, complementary DNA synthesis, biotin labeling, fragmentation, hybridization, washing, and scanning were carried out at the Center for Functional Genomics at State University of New York, University at Albany. The GeneChip Barley Genome Array (P/N 511012, Affymetrix, Santa Clara, CA, USA) was used for transcriptome analysis. Global scaling was applied so the average signal intensity was 500. For the comparisons of each microarray to determine differentially regulated genes, we did not use genes with larger transcription rates whose rate was not consistent based on 11–15 probe pairs (p value less than 0.05). A gene was considered differentially regulated when the p value and the corrected p value based on the false discovery rate method (Benjamini and Hochberg 1995) for comparing two chips were lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher than the standard deviation for all of the barley genes (3.7-fold for both; Ren et al. 2004). BLAST-X search was performed with the sequences that are available from the website of the NetAffyx Analysis Center (http://www.affymetrix.com/analysis/index.affx). The raw data were deposited in the Gene Expression Omnibus (GSE8616) at the National Center for Biotechnology Information.

Bioinformatics All analyses were performed with default parameters. To examine the similarity of proteins, BLAST-X and BLAST-P searches were performed (http://blast.ddbj.nig.ac.jp/top-e.html). Protein hydrophobicity was predicted with default parameters using SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuiframe0.html), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Protein motifs were explored using MOTIF (http://motif.genome.jp).

Pyocyanin assay Pyocyanin concentrations were determined as described previously (Essar et al. 1990). Each strain was grown in LB at 30°C for 24 h, and 1 mL of culture was centrifuged at 10,000×*g* for 2 min, and 800 μL of supernatant was extracted with 480 μL of chloroform. After separating the phases by centrifugation at 10,000×*g* for 5 min, the pyocyanin was extracted into the aqueous phase by adding 800 μL of 0.2 N HCl. Pyocyanin content was measured using the absorbance at 520 nm and normalized by cell growth using the turbidity at 600 nm. Two independent cultures were used.

Results

Identification of monovalent cation efflux transport proteins Since bacterial virulence factors are often regulated by monovalent cations (Chen et al. 2004; Kosono et al. 2005; Myers et al. 1993), we tested whether cation transport systems are involved in *P. aeruginosa* virulence. The *P. aeruginosa* genome encodes 5,570 predicted open reading frames, and 555 (10%) of them may participate in the transport of small molecules (Stover et al. 2000). According to the annotation by the *Pseudomonas* Genome Database (ver.2; Winsor et al. 2005), 12 putative efflux-type trans-

porters for monovalent cations were identified by us in the *P. aeruginosa* genome, including Na^+/H^+ antiporters (PA1054, PA1820, PA2135, PA2647, PA3660, PA3739, PA3887, PA5021, and PA5529), K^+ efflux systems (PA1207 and PA5518), and a K^+ channel (PA1496; Table 3). The PA1054 and PA2647 genes may encode the large subunit of a NADH dehydrogenase because they have a sequence similarity to the NADH dehydrogenase NuoL in *E. coli* that possesses Na^+ antiporter activity (Steuber 2003). The other proteins may encode single gene-type, transporter/channel proteins. These 12 putative efflux transporters/channels have 6 to 25 hydrophobic transmembrane regions (Table 3). To initiate our analysis, the isogenic PAO1 transposon mutants were verified by PCR. All the mutants were correct except for PA2135 (which was not studied further).

PA1207, PA1820, PA2647, and PA5021 genes encode virulence factors We have established a novel barley germination assay to investigate virulence factors in *P. aeruginosa* (Attila et al. 2008). Barley seeds germinated well (80±4%) in the absence of *P. aeruginosa* after 3 days; however, the presence of PAO1-UW and PAO1-UBC severely reduced germination to 12±6% and 20±8%, respectively. Thus, these two PAO1 wild-type strains showed slightly different virulence; therefore, the data from the *P. aeruginosa* transposon mutants from the two different sources, UW and UBC, were always normalized to the parental isolate.

In the barley germination assay, the PA1207 and PA5021 mutants allowed 4.5-fold and 3.8-fold more germination compared to wild-type PAO1, respectively (Fig. 1). Additionally, the PA1820 mutant allowed 2.7-fold more germination, and the PA2647 mutant allowed 2.5-fold more germination, so these four mutants were less virulent to barley. In contrast, the inactivation of PA3739 caused enhanced virulence.

To confirm whether inhibition of germination was due to *P. aeruginosa* pathogenicity, we tested barley germination with *Pseudomonas* sp. strain Pb3-1 that was isolated from the poplar tree rhizosphere (Shim et al. 2000). No significant difference was found in the germination ratio between the no-bacterial control and infection with Pb3-1 (73±9%), which shows that the inhibition of barley seed germination was caused by the virulence factors present in *P. aeruginosa*.

Biofilm formation and swimming motility Biofilm formation (Yahr and Parsek 2006) and swimming motility (Liu et al. 1988) are often involved in the virulence of pathogenic bacteria. Therefore, we examined biofilm formation in 96-well polystyrene plates in LB medium without or with 0.2% glucose at 30°C for 24 h. Both the PA1054 and PA2647 mutants formed 2.3-fold and 1.6-fold more biofilm in comparison to wild-type PAO1, whereas the PA1820 and PA3887 mutants formed 0.7-fold less biofilm (Fig. 2). The two other less-virulent mutants, PA1207 and PA5021, showed less biofilm formation in both media.

Table 3 List of predicted and known monovalent cation transporters in *P. aeruginosa*

Gene ID	Annotation[a]	Amino acid residues	Transmembrane regions[b]	*E. coli* homolog[c] (similarity, %)	References
Multigene-type Na^+ transporter					
PA1054	Na^+/H^+ antiporter (Sha)	933	24–25	NuoL (50%)	Steuber (2003)
PA2647	Probable NADH dehydrogenase I (Nuol)	615	15–16	NuoL (81%)	Steuber (2003)
Single gene-type Na^+ transporter					
PA1820	Na^+/H^+antiporter (NhaB)	500	10–12	NhaB (76%)	Pinner et al. (1992)
PA2135	Probable transporter	454	11–12	YcgO/CvrA (44%)	Verkhovskaya et al. (2001)
PA3660	Probable Na^+/H^+ antiporter	581	11	YjcE (63%)	Verkhovskaya et al. (2001)
PA3739	Probable Na^+/H^+ antiporter	611	12–13	YcgO/CvrA (44%)	Verkhovskaya et al. (2001)
PA3887	Na^+/H^+ antiporter (NhaP)	424	11–13	YjcE (44%)	Verkhovskaya et al. (2001)
PA5021	Probable Na^+/H^+ antiporter (KnaA)[d]	538	9	YcgO/CvrA (81%)	Verkhovskaya et al. (2001)
PA5529	Probable Na^+/H^+ antiporter	585	15–16	YbaL (79%)	N/A
Single gene-type K^+ channel/transporter					
PA1207	Probable glutathione-regulated K^+ efflux system (KchA)[d]	613	12–16	KefB (64%)	Elmore et al. (1990)
PA1496	Probable K^+ channel	283	6–7	Kch (45%)	Milkman (1994)
PA5518	Probable K^+ efflux transporter	567	12–13	YbaL (79%)	N/A

[a] Annotation is based on the description of *Pseudomonas* Genome Database (Winsor et al. 2005).
[b] Transmembrane regions were predicted by the algorithm of SOSUI, TMpred, and TMHMM.
[c] *E. coli* homologs and sequence similarity were searched by EcoBLAST.
[d] New names proposed in this work.

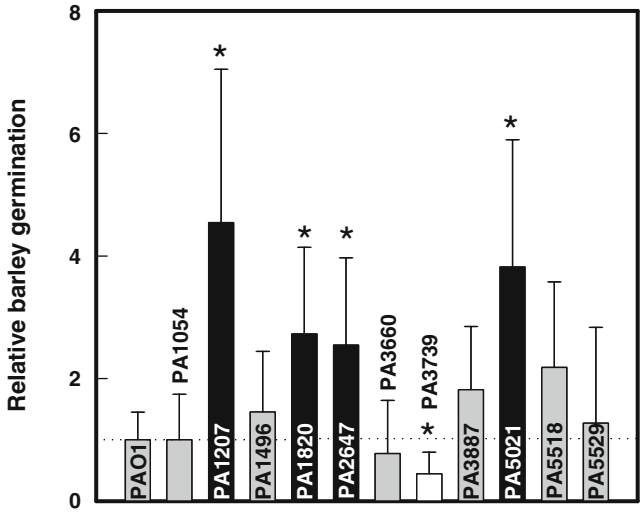

Fig. 1 Barley seed germination with putative antiporter *P. aeruginosa* mutants. Relative germination was calculated using either wild-type parent PAO1-UW or PAO1-UBC. Data are the average of three independent experiments, and one standard deviation is shown. *Asterisks* indicate significant differences as estimated by Fisher's LSD test ($p<0.05$)

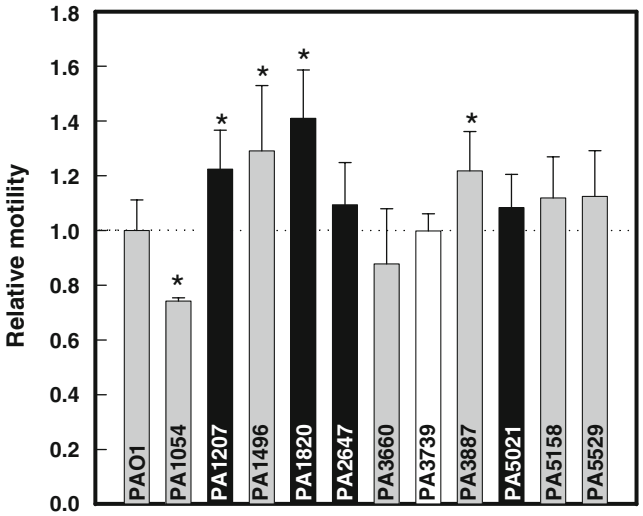

Fig. 3 Motility of with putative antiporter *P. aeruginosa* mutants. Relative motility was calculated using either wild-type parent PAO1-UW or PAO1-UBC. Data are the average of three independent experiments, and one standard deviation is shown. *Asterisks* indicate significant differences as estimated by Fisher's LSD test ($p<0.05$)

Motility was increased for the PA1820 (1.4-fold), PA1496 (1.3-fold), PA1207 (1.2-fold), and PA3887 (1.2-fold) mutants, whereas the PA1054 mutant showed less motility (0.7-fold; Fig. 3). No significant difference was found in motility for the PA2647 and PA5021 mutants; hence, there were no obvious trends in motility, biofilm formation, and virulence.

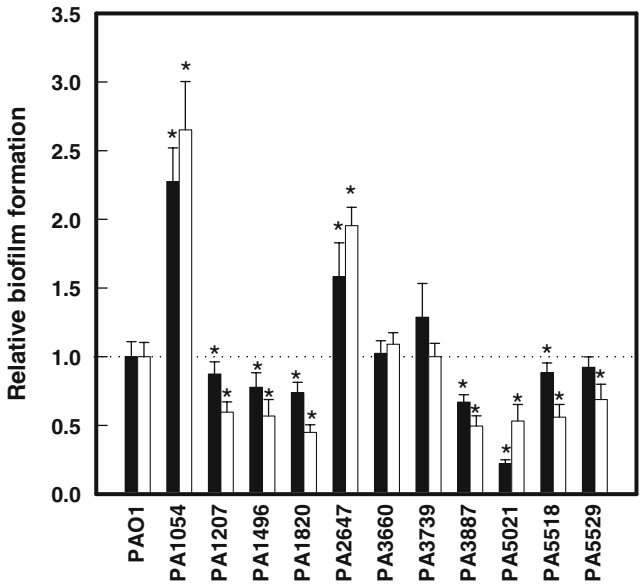

Fig. 2 Biofilm formation with putative antiporter *P. aeruginosa* mutants in 96-well polystyrene plates at 30°C in LB without (*black bars*) or with 0.2% glucose (*white bars*) for 24 h. Relative biofilm formation was calculated using either wild-type parent PAO1-UW or PAO1-UBC. Data are the average of three independent experiments, and one standard deviation is shown. *Asterisks* indicate significant differences as estimated by Fisher's LSD test ($p<0.05$)

The PA5021 mutant is more sensitive to high salinity but not osmotic stress Since the inactivation of PA5021 caused a large reduction in virulence and biofilm formation, it was investigated further. The *Pseudomonas* Genome Database annotates the PA5021 gene as encoding a Na^+/H^+ antiporter that consists of 538 amino acids. According to a Clusters of Orthologous Groups (COG) search, PA5021 homologous proteins are found in only ten species of bacteria, for example, in *E. coli* (YcgO/CvrA), *V. cholerae* (VC2703), and *Agrobacterium tumefaciens* (AGL2194).

To verify that the PA5021 gene encodes an antiporter, the growth of the PA5021 mutant was examined in various salt media since mutations in antiporters cause impaired growth in the presence of high salinity (Kosono et al. 2005; Radchenko et al. 2006a, b). The PA5021 mutation decreased growth in M9 glucose medium (Fig. 4a), so this mutation affects cell growth. In addition, the PA5021 mutant was not able to grow in 400 mM KCl (Fig. 4b) and 400 mM NaCl (Fig. 4c); hence, the inactivation of this putative transporter makes the cell sensitive to K^+ and Na^+. To eliminate the effect of osmotic stress by adding higher concentrations of KCl or NaCl, the growth of the PA5021 mutant was measured in the presence of 800 mM sorbitol. As seen in Fig. 4d, the PA5021 mutation did not prevent growth under high osmotic stress made by sorbitol. To confirm that the salt inhibition was due to the cation and not the Cl^- anion, we measured growth with different anions with K^+ and Na^+; similar results were obtained with 400 mM KNO_3 and 400 mM $NaNO_3$ (Fig. 4e, f); therefore, the impaired growth in the PA5021 mutant is due to ionic stress caused by K^+ or Na^+ but not due to osmotic stress.

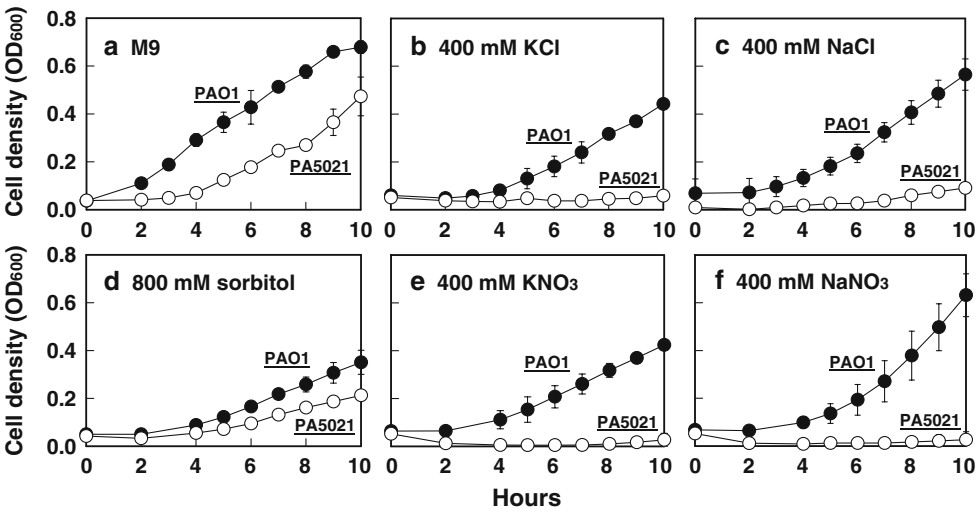

Fig. 4 Growth of wild-type PAO1 and the PA5021 mutant in M9 minimal medium **a** without salts, **b** with 400 mM KCl, **c** with 400 mM NaCl, **d** 800 mM sorbitol, **e** 400 mM KNO_3, or **f** 400 mM $NaNO_3$. Data are the average of two independent experiments, and one standard deviation is shown

We also tested growth of the PA5021 mutant under hypoosmotic conditions because the *E. coli ycgO* mutant, a homolog of the PA5021 protein, was identified as a hypoosmotic stress-susceptible strain (Verkhovskaya et al. 2001). However, no difference was found between PAO1 and the PA5021 mutant in the TY medium that is used as a medium for hypoosmotic stress (Verkhovskaya et al. 2001). These results indicate that the PA5021 protein may function as a K^+ and Na^+ transporter in *P. aeruginosa* and has a different function from *E. coli* YcgO.

PA5021 protein functions as a K^+ antiporter To test whether the PA5021 protein functions as a K^+ transporter, growth of the *E. coli* LB2003 mutant was investigated with pMMB207-PA5021 using low and high K^+ stresses. Because the LB2003 mutant lacks three K^+ uptake systems (*kup1*, Δ*kdp*, Δ*trkA*), the K^+ supplement is necessary for its growth. Since K^+ is the most important monovalent cation in bacterial cells and is a significant part of cellular osmotic potential (Epstein 2003), bacterial growth is poor under K^+ deficiency. Similarly, high concentrations of K^+ are toxic due to high osmotic stress (Radchenko et al. 2006a,b). The growth of the LB2003 cells with the PA5021 gene was repressed compared to the control vector with low concentrations of K^+ (40–50 mM KCl; Fig. 5a), whereas the LB2003 cells expressing the PA5021 gene were more tolerant to high concentrations of K^+ (450–500 mM; Fig. 5b). Together, these results suggested that the PA5021 protein facilitates K^+ export in the LB2003 cells (at low K^+ concentrations, export by the PA5021 protein decreases K^+ too much causing stress and at high K^+ concentrations, stress is diminished by export by the PA5021 protein).

Differentially regulated barley genes by PA5021 To determine the impact of inactivating PA5021 (less virulent mutant) on barley gene expression, a whole-genome transcriptome analysis of gene expression in barley germinating seeds was performed with the GeneChip Barley Genome Array; this microarray contains more than 22,500 probe sets. Barley transcriptomes with wild-type PAO1 and the PA5021 mutant after a 12-h infection were compared to the

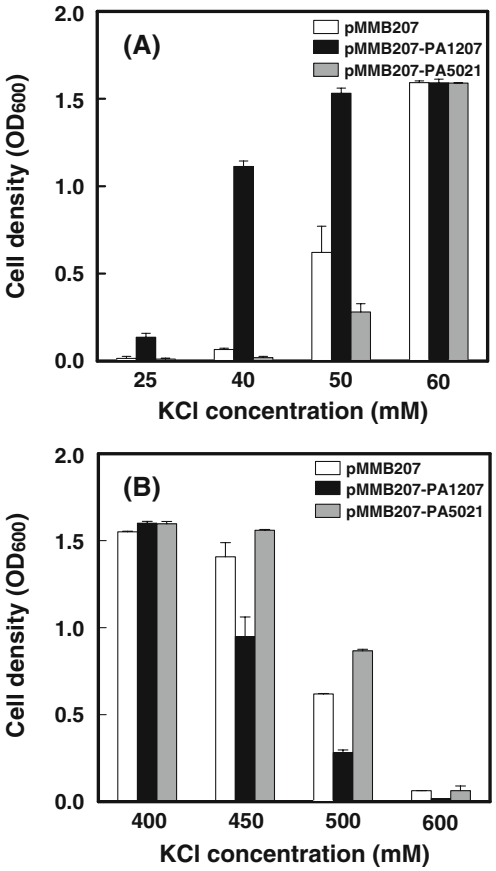

Fig. 5 Effect of PA1207 and PA5021 gene overexpression on the growth of *E. coli* LB2003 mutant lacking three K^+ uptake systems (*kup1*, Δ*kdp*, Δ*trkA*) to examine the K^+-related phenotype in KML medium under low concentrations of K^+ (**a**) or high concentrations of K^+ (**b**) stress conditions. Data are the average of two independent experiments, and one standard deviation is shown

baseline chip without *P. aeruginosa*. Upon infection with PAO1 and the PA5021 mutant, the expression of 43 to 53 genes was significantly induced in barley with 23 common genes (Table 4). The expression of 38 to 41 genes was repressed upon infection by PAO1 and the PA5021 mutant with 26 common genes (Table 5).

Barley transcriptome analysis upon *P. aeruginosa* infection has not been examined yet; hence, these data provide useful information concerning the response of barley to this pathogen. *P. aeruginosa* infection of barley induced some disease-responsive transcriptional regulators, such as the ethylene response factor and the zinc finger transcription factor, which were induced in rice upon *P. avenae* infection (Fujiwara et al. 2004) and induced in *Arabidopsis* treated with flg22, a peptide corresponding to the most conserved domain of flagellin (Navarro et al. 2004; Table 4). Additionally, wild-type PAO1 infection caused the induction of the genes encoding the heat stress transcription factor Spl7, cinnamoyl CoA reductase, glutathione *S*-transferase, and two zinc finger proteins (C2H2 zinc finger protein and ZPT2–13); however, these genes were not induced by the less virulent PA5021 mutant. In addition, transcripts for water stress-responsive genes, such as late embryogenesis abundant protein, dehydrin 11, and dehydrin 3, were increased by PAO1 infection but not by the PA5021 mutant. The expression of the genes involved in proteolysis (26S proteasome regulatory subunit and ubiquitin) and the expression of LysM domain-containing protein that is the determinant of Nod factor-induced infection in pea (Limpens et al. 2003) were induced by the PA5021 mutant infection but not with wild-type PAO1. Hence, PAO1 infection triggered induction of more disease and water stress-related genes in comparison to infection by the PA5021 mutant.

The PA1207 protein functions as a K^+ uptake system The PA1207 mutation caused less virulence to barley seeds in *P. aeruginosa* (Fig. 1), and BLAST-P search indicated that the PA1207 gene encodes a putative K^+ efflux system consisting of 613 amino acids. PA1207 homologs are found in at least 50 species of microorganisms by COG search; for example, *E. coli* (KefB), *V. cholerae* (VC2606), *R. solanacearum* (RSc3096), and *S. cerevisiae* (YJL093c), and also other pseudomonads have homologs, which indicates that the PA1207 protein is a well-conserved protein in bacteria.

To test whether the PA1207 protein functions as a K^+ transporter, growth of the *E. coli* LB2003 strain (deficient in K^+ uptake) while expressing the PA1207 gene was also investigated with low and high K^+ concentrations. Expressing the PA1207 gene in *E. coli* caused the opposite behavior seen with that of the PA5021 gene; that is, the expression of the PA1207 gene increased the growth of the LB2003 cells with low concentrations of K^+ (25 to 50 mM KCl) but decreased its growth with high concentrations of K^+ (450 to 600 mM KCl) (Fig. 5). Together, these results indicate that the PA1207 protein facilitates K^+ import in the LB2003 cells.

PA5021 and PA1207 proteins lack Na^+/H^+ antiporter activity in E. coli The *E. coli* KNabc mutant was used to examine whether the PA5021 and PA1207 proteins have Na^+/H^+ antiporter activity. The KNabc cells are susceptible to higher concentrations of NaCl because they lack two Na^+/H^+ antiporters (NhaA and NhaB) and one K^+ and Na^+/H^+ antiporter (ChaA); hence, the KNabc is often used to examine whether a gene product possesses Na^+ export activity. Growth of the KNabc cells was improved by expressing either the *nhaB* or *nhaP* Na^+/H^+ antiporters from *P. aeruginosa* (Kuroda et al. 2004; Utsugi et al. 1998). However, both the PA5021 and PA1207 proteins were not able to influence growth of the KNabc with NaCl (data not shown). Hence, both the PA5021 and PA1207 proteins facilitate the K^+ transport system in *E. coli* but not Na^+ transport.

PA5021 protein regulates pyocyanin production *P. aeruginosa* produces several pigments including blue-green pyocyanin, fluorescent yellow-green pyoverdin, and rusty-brown pyorubin (Yahr and Parsek 2006). Pyocyanin is a virulence factor and a redox-active compound (Lau et al. 2004; Yahr and Parsek 2006). Since the loss of some of the transport systems caused a noticeable change in cell culture color, we determined whether the loss of one of the cation transport systems (PA1207, PA1820, PA2647, or PA5021) affects pyocyanin production. The PA5021 mutant produced only 40% pyocyanin in comparison with that of PAO1, whereas the other three mutants showed normal pyocyanin production (Fig. 6a). This suggests the PA5021 protein K^+ and Na^+ antiporter system regulates its virulence through pyocyanin production in *P. aeruginosa*. To confirm that the assay is specific to pyocyanin, we also used the pyocyanin-defective *phzM* and *phzS* mutants, the pyoverdin-defective *pvdF* mutant, and a pyochelin-defective *pchE* mutant as controls. Among these mutants, only the *phzM* and *phzS* mutants produced negligible pyocyanin; hence, the pyocyanin assay is specific (data not shown). We also investigated whether high salinity inhibits pyocyanin production in PAO1. Pyocyanin production was clearly inhibited by more than 250 mM KCl or 150 mM NaCl in comparison to that without salts (Fig. 6b). Note that growth inhibition by high salinity was not observed at 600 mM KCl or 600 mM NaCl. To examine whether pyocyanin production controls *P. aeruginosa* virulence to barley, mutants lacking pyocyanin production were used with the barley germination assay. Two mutants that lacked pyocyanin production, *phzM* and *phzS*, showed significantly decreased virulence to barley (3±3-fold and 4±4-fold, respectively).

Table 4 Genes induced more than 3.7-fold in barley upon infection with wild-type *P. aeruginosa* PAO1 and with the PA5021 mutant (K^+ and Na^+ exporter)

Probe set name	Fold change		Description
	PAO1	PA5021	
Common induced genes			
Contig378_s_at	39.4	21.1	rRNA intron-encoded homing endonuclease
Contig2471_at	27.9	9.8	ERF subfamily B-3 of ERF/AP2 transcription factor
Contig376_s_at	24.3	22.6	Putative senescence-associated protein
Contig376_at	16.0	13.0	Putative senescence-associated protein
EBro02_SQ006_N05_at	13.0	13.9	No homolog
Contig377_s_at	10.6	8.6	Putative senescence-associated protein
HS08O16u_s_at	9.2	18.4	10-kDa putative secreted protein
Contig16924_at	9.2	5.3	Putative auxin-responsive protein IAA9
Contig711_s_at	8.6	19.7	Cytochrome P450
Contig8220_at	8.0	6.5	F-box family protein
HU12P09u_s_at	8.0	4.3	No homolog
HU10 M16u_at	7.5	4.9	Knox3
EBro02_SQ005_B08_at	6.1	4.3	Putative Cys2/His2 zinc-finger protein
Contig382_s_at	5.7	5.3	Putative protein
Contig7967_at	5.7	4.3	Putative lipid transfer protein
Contig16135_at	5.7	3.7	No homolog
Contig484_s_at	4.9	8.0	Probable cytochrome P450 monooxygenase
Contig21171_at	4.9	4.0	Homeodomain-leucine zipper transcription factor
Contig787_at	4.9	3.7	Histone H3
HV11C08u_x_at	4.6	9.2	rRNA promoter binding protein
Contig2416_at	4.3	4.0	Early embryogenesis protein
Contig3892_at	4.0	3.7	Quinone reductase
Contig2258_at	3.7	4.3	Histone H1-like protein HON101
Induced genes upon wild-type PAO1 infection			
Contig19553_at	13.9		Unknown protein
Contig23845_at	13.9		GAST-like gene product
EBpi05_SQ004_B11_at	13.9		Plastid–lipid associated protein PAP/fibrillin family-like
Contig19504_at	8.6		Xylogen like protein, Protease inhibitor/seed storage/LTP family protein
HV_CEa0010K11f_x_at	8.6		No homolog
HVSMEn0018J02f_x_at	6.1		No homolog
Contig21899_at	5.7		No homolog
Contig11531_at	5.3		Late embryogenesis abundant protein
Contig18037_at	5.3		Hypothetical protein
Contig14114_at	4.9		C2H2 zinc finger protein
HW01N23u_x_at	4.9		No homolog
Contig3426_at	4.6		High molecular mass early light-inducible protein HV58
Contig19522_at	4.6		Hypothetical protein
Contig16327_at	4.6		Little protein 1
Contig15351_s_at	4.3		Hypothetical protein
EBro02_SQ005_B08_s_at	4.3		ZPT2-13
Contig94_at	4.0		Histone H3
Contig11818_at	4.0		Putative cytochrome P450
Contig10207_s_at	4.0		Dehydrin 11
Contig12484_at	4.0		Uncharacterized plant-specific domain protein
Contig1724_s_at	4.0		Dehydrin 3
HVSMEg0018C11r2_at	4.0		Putative protein
HT11B22u_s_at	3.7		No homolog
Contig18961_at	3.7		Heat stress transcription factor Spl7
Contig10192_at	3.7		Putative protein
Contig13248_at	3.7		Putative UDP-glucose/salicylic acid glucosyltransferase
Contig6123_at	3.7		Cinnamoyl CoA reductase
Contig13706_at	3.7		Putative protein

Table 4 (continued)

Probe set name	Fold change		Description
	PAO1	PA5021	
Contig15264_at	3.7		Glutathione *S*-transferase GST 24
Contig10841_s_at	3.7		Hypothetical protein
Induced genes upon PA5021 infection			
Contig3660_at		55.7	Putative ABC transporter
HA28M24r_at		39.4	26S proteasome regulatory particle triple-A ATPase subunit 4b
Contig9177_at		32.0	Shikimate kinase, chloroplast precursor
HVSMEf0019G16r2_at		26.0	Putative copper chaperone
HW03F12u_s_at		21.1	No homolog
Contig1381_at		16.0	Metallothionein
Contig17916_at		12.1	Neoxanthin cleavage enzyme
HVSMEb0001J09r2_at		12.1	No homolog
Contig24141_at		9.2	Peptidoglycan-binding LysM domain-containing protein
HVSMEl0023K24r2_at		9.2	PAP2 superfamily protein
HVSMEc0014A20r2_at		6.5	No homolog
Contig22928_at		6.5	Nonclathrin coat protein zeta1-COP
Contig25222_at		6.5	Ubiquitin-like protein
HT09I02u_at		6.1	No homolog
EBro07_SQ001_A12_at		4.9	No homolog
Contig484_x_at		4.0	Probable cytochrome P450 monooxygenase
Contig10247_at		3.7	F-box containing protein TIR1
Contig20590_at		3.7	No homolog
HB21C01r_at		3.7	No homolog

The barley seed transcriptome was examined in Hoagland solution at 25°C after infecting for 12 h

Discussion

In this study, we identified some putative cation transport systems that come into play as novel virulence factors when the PA1207, PA5021, PA1820, and PA2647 mutants of *P. aeruginosa* interact with germinating barley seeds. These observations showed that the barley germination assay is a rapid way to identify virulence factors. We provide evidence that two of the four mutants (PA1207 and PA5021) encode K^+-transporting proteins. This suggests that K^+ transport may be crucial for virulence in *P. aeruginosa* as well as Na^+ transport (Kuroda et al. 2004) as was seen for the PA1820 mutation. The PA2647 protein is a homolog of the large subunit of the multigene-type Na^+ antiporter (PA1054), but we did not observe Na^+-dependent growth inhibition. The PA2647 protein is likely a part of a NADH/ubiquinone oxidoreductase complex (Nuo) cluster that mediates primarily H^+ translocation. Truncated NuoL has Na^+ activity in *E. coli*, but it may be minor (Steuber 2003).

The PA5021 protein is similar to the *E. coli* YcgO, which is a Na^+/H^+ antiporter (Verkhovskaya et al. 2001). The LB2003 cells grow poorly under K^+-limited conditions due to their inability to uptake K^+. The expression of the PA5021 gene repressed the growth of LB2003 further with low concentrations of K^+ (Fig. 5a). In contrast, LB2003 expressing the PA5021 gene gained tolerance to high concentrations of K^+ (Fig. 5b). Even though K^+ is the essential mineral for bacterial growth, excess K^+ inhibits the growth of LB2003 (Radchenko et al. 2006a, b). Taken together, these results show the PA5021 gene encodes a K^+ antiporter. However, the PA5021 protein did not show Na^+ antiporter activity in the KNabc mutant, although growth of the *P. aeruginosa* PA5021 mutant was inhibited by adding higher concentrations of NaCl and $NaNO_3$ in a minimal medium (Fig. 4c,f). This may be due to inactive PA5021 or only low Na^+/H^+ antiporter activity in the *E. coli* KNabc mutant. Most living organisms from bacteria to humans have developed functional redundancy in their gene structure. For example, *E. coli* possesses three Na^+/H^+ antiporters, NhaA, NhaB, and ChaA (Ivey et al. 1993; Padan and Schuldiner 1994). In spite of this redundancy, *E. coli* growth is repressed at high Na^+ conditions upon disruption of *nhaA* (Padan and Schuldiner 1994). These observations imply that NhaA plays a major role in Na^+ extrusion. Therefore, the PA5021 protein may play a major role in K^+ or Na^+ transport under high salinity. On the other hand, YcgO was identified as a Na^+/H^+ antiporter that is critical under hypoosmotic conditions but not during high salinity conditions (Verkhovskaya et al. 2001). Probably, the PA5021 protein regulates cellular K^+/Na^+ homeostasis in a manner different from YcgO. We suggest that the

Table 5 Genes repressed more than 3.7-fold in barley upon infection with wild-type *P. aeruginosa* PAO1 and with the PA5021 mutant (K^+ and Na^+ exporter)

Probe set name	Fold change		
	PAO1	PA5021	Description
Common repressed genes			
Contig3776_s_at	−59.7	−39.4	Putative lipid transfer protein
Contig758_at	−24.3	−6.5	Disulfide isomerase
Ebem10_SQ002_L14_s_at	−11.3	−14.9	No homolog
Contig3670_at	−8.0	−6.5	Ice recrystallization inhibition protein
Contig16010_at	−8.0	−6.5	Putative 1,4-beta-xylanase
Contig7937_s_at	−7.5	−3.7	High p*I* alpha-glucosidase
Ebro08_SQ003_I19_s_at	−7.0	−7.0	No homolog
Contig1326_s_at	−6.5	−4.9	Cold-regulated protein BLT14
Contig3185_s_at	−6.1	−7.5	No homolog
HW02O23u_s_at	−6.1	−7.0	No homolog
U19359_s_at	−5.7	−6.1	Cysteine proteinase EP-B 1 precursor
Contig568_s_at	−5.7	−4.3	Heat shock protein 90
Contig8703_at	−5.3	−6.1	Isocitrate lyase
Contig6859_at	−5.3	−5.7	Low temperature induced protein
Contig6593_s_at	−5.3	−4.6	Putative DNA replication licensing factor, mcm5
Contig4194_at	−4.9	−4.3	Patatin-like phospholipase
Contig1852_at	−4.6	−4.9	Probable peroxidase
Contig3391_at	−4.6	−3.7	Probable glucan 1,3-beta-glucosidase
Contig6515_at	−4.3	−5.7	Class III peroxidase
HVSMEf0019H18r2_s_at	−4.3	−4.3	Putative tonoplast membrane integral protein
Contig14651_at	−4.3	−4.0	Ascorbic acid oxidase
Contig4194_s_at	−4.3	−3.7	Patatin-like protein
Contig5067_at	−3.7	−5.3	Nicotianamine synthase 2
Contig5180_at	−3.7	−4.6	Putative transcription factor
AF069331_s_at	−3.7	−4.0	Low temperature induced protein
Contig7315_at	−3.7	−3.7	No homolog
Repressed genes upon wild-type PAO1 infection			
HW07D08u_s_at	−10.6		Cysteine synthase
HB25F09r_x_at	−7.0		Hypothetical protein
Ebro03_SQ001_C05_at	−4.6		Hypothetical protein
Contig10893_at	−4.3		Putative serine carboxypeptidase II
HVSMEc0016D02f_at	−4.3		Photosystem I P700 chlorophyll A apoprotein A1 (PsaA)
Contig71_s_at	−4.0		Heat shock protein hsp90
Contig7032_at	−4.0		Alpha-L-arabinofuranosidase/beta-d-xylosidase isoenzyme ARA-I
HVSMEl0010A17f_s_at	−4.0		No homolog
Contig682_s_at	−4.0		Serine carboxypeptidase III precursor (CP-MIII)
Contig21515_s_at	−4.0		Putative cytochrome p450
Contig6897_at	−3.7		Serine carboxypeptidase I precursor (Carboxypeptidase C; CP-MI)
Contig2504_at	−3.7		Putative IAA1 protein
HU11N21r_x_at	−3.7		No homolog
Contig17190_at	−3.7		Putative heat shock protein
Contig15987_at	−3.7		Putative dioxygenase, ethylene-forming enzyme
Repressed genes upon PA5021 infection			
Contig7079_at		−19.7	Putative peroxidase
Contig4115_s_at		−9.2	Putative nuclease
Rbasd23b02_s_at		−5.7	No homolog
Contig600_at		−4.6	Carboxypeptidase C
Contig15099_s_at		−4.6	Pathogenesis-related protein 4
HA16L09r_s_at		−4.3	No homolog
HVSMEn0024H20r2_s_at		−4.0	Amino acid permease AAP3
Contig5281_at		−3.7	Cysteine proteinase EP-B 2 precursor
Contig9373_at		−3.7	DNA replication licensing factor MCM3 homolog

Table 5 (continued)

Probe set name	Fold change		
	PAO1	PA5021	Description
Contig1861_at		−3.7	Peroxidase
HVSMEc0013C02f_at		−3.7	Putative NADH dehydrogenase 49-kDa protein
Contig20222_at		−3.7	Putative carboxypeptidase D

The barley seed transcriptome was examined in Hoagland solution at 25°C after infecting for 12 h.

PA5021 protein should be KnaA for the K^+ and Na^+ antiporter.

In contrast to PA5021, the expression of the PA1207 gene restored the growth of the LB2003 cells with low concentrations of K^+ (Fig. 5a) but repressed its growth with high concentrations of K^+ (Fig. 5b). These results show the PA1207 protein facilitates K^+ uptake in the LB2003 cells. We suggest that the PA1207 protein should be KchA for the K^+ channel.

Similar phenotypes were also found with LB2003 expressing *mvp* and *mjk1* encoding K^+ channels from *Methanococcus jannaschii*; the expression of either *mvp* or *mjk1* restored growth of the LB2003 cells under low K^+ conditions but repressed its growth under high K^+ conditions (Hellmer and Zeilinger 2003). Hence, the PA1207 protein may encode a K^+ channel in *P. aeruginosa*.

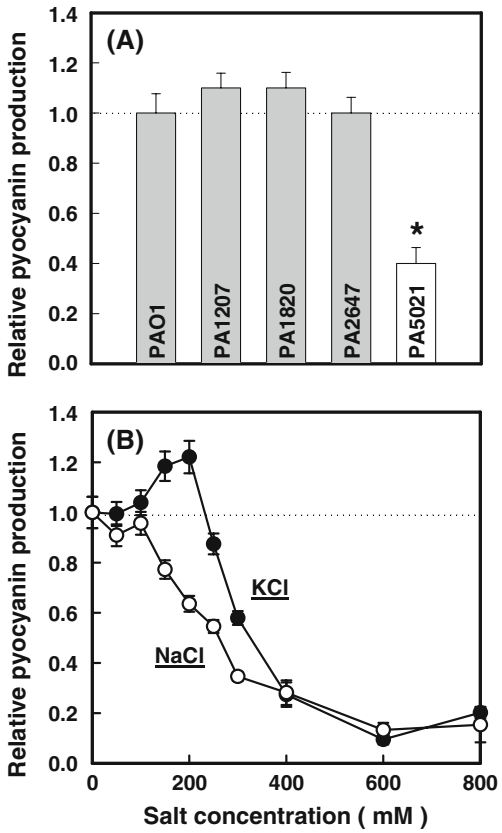

Fig. 6 Pyocyanin production by less virulent *P. aeruginosa* mutants (**a**) and the effect of high salinity on pyocyanin production (**b**). Pyocyanin content was determined after 24 h and normalized by growth. Data are the average of two independent experiments, and one standard deviation is shown. *Asterisks* indicate significant differences as estimated by Fisher's LSD test ($p<0.05$)

Both the PA5021 and PA1207 proteins are hydrophobic proteins, have putative transmembrane regions from amino acid positions 16 to 341 for the PA5021 protein and from 4 to 384 for the PA1207 protein, and have hydrophilic C-terminal regions. The C-terminal regions of the PA5021 protein (position 361–443, score of 1,754) and the PA1207 protein (position 408–522, score of 3,055) also have a similarity to the regulating the conductance of K^+ (RCK) domain that is well conserved in K^+-transporting proteins such as with the *B. subtilis* KtrAB ion transporter (Albright et al. 2006). This finding is consistent with the phenotypes of K^+ transport observed in *E. coli* LB2003 with PA5021 and PA1207 expression (Fig. 5a,b). We propose that the PA5021 protein participates in K^+ transport through its RCK domain, even though both K^+ and Na^+ inhibited growth phenotype of the PA5021 mutant (Fig. 4b,c).

The barley pathogenicity assay identified two other Na^+ transporters, the PA1820 and PA2647 proteins. The PA1820 gene encodes a Na^+/H^+ antiporter (*nhaB*), and NhaB was able to recover impaired growth of the KNabc cells under high NaCl conditions (Kuroda et al. 2004). Although the function of the PA2647 protein was not identified, the mutations for PA2637-PA2649 in the *nuo* gene cluster (except PA2640 and PA2647) were found to be determinants for anaerobic growth screening (Filiatrault et al. 2006). Similar to our results, most of the mutations in the *nuo* cluster caused less virulence to lettuce (Filiatrault et al. 2006), suggesting that the Nuo function is necessary for pathogenicity in *P. aeruginosa*.

Mutating the PA1054 gene, which encodes a Na^+/H^+ antiporter, did not affect its virulence for barley, although the PA1054 mutant was less virulent for mice (Kosono et al. 2005). This might be due to the difference in ionic environments between animals and plants. Na^+ plays important roles in animal tissue, but plants prefer to use K^+, instead of Na^+ (Zhu 2003).

The mechanism of how cation-transporting systems regulate virulence in *P. aeruginosa* is still unknown. We found that higher concentrations of KCl and NaCl inhibit

production of the virulence factor pyocyanin in PAO1 (Lau et al. 2004; Fig. 6b). This may be one of the reasons why pyocyanin production by the PA5021 mutant lacking K^+ and Na^+ antiporter activity was decreased (Fig. 6a): The PA5021 mutant may accumulate intracellular salt, which reduces pyocyanin synthesis. Our results show clearly that cells that lack pyocyanin production (*phzM* and *phzS*) have reduced virulence to barley, which corroborates the reduced virulence to animal cells (Rahme et al. 2000). Furthermore, it is well known that plant-hypersensitive reactions are triggered by pathogenic attacks (Klement and Goodman 1967) and that increased efflux of K^+ from the plant cells occurs (Atkinson et al. 1985). Because K^+ efflux reaches 35% of the total cellular K^+ in plant cells (Atkinson et al. 1985), the ionic environment in the phyllosphere is expected to be K^+ rich upon bacterial infection. Therefore, this K^+-rich environment may suppress bacterial virulence by reducing pyocyanin production and the increased extracellular salt may inhibit growth, as observed for the K^+-mediated severe inhibition in the growth of the PA5021 mutant (Fig. 4b). In support of this, bacterial cation-transporting systems participate in establishing symbiotic relationships between bacteria and plants. For example, a *R. meliloti pha* mutant lacking K^+ antiporter activity lost the ability to invade the alfalfa root nodule (Putnoky et al. 1988). Although direct protein data have not been obtained, Pha may encode a K^+ efflux system (Putnoky et al. 1998). Hence, K^+ transport may play an important role in both symbiotic and antagonistic relationships of bacteria and plants.

Understanding how the host responds against pathogenic attack is important, but little is known about the global response of gene expression in plant seeds upon pathogenic infection. Our whole-genome, comparative expression analysis identified that many genes were differentially regulated upon infection of barley by wild-type PAO1 and the less virulent PA5021 mutant; however, the expression of some common determinants in disease resistance were induced, such as the ethylene response factor (ERF) transcription factor (Berrocal-Lobo et al. 2002), the Cys2/His2 zinc-finger protein (Kim et al. 2004), and the lipid transfer protein (Molina and García-Olmedo 1997; Table 4). In addition to these genes, wild-type PAO1-infected barley induced more disease-related genes, as seen in the expression of the GAST protein, two other zinc finger proteins, the heat stress transcription factor, cinnamoyl CoA reductase, and glutathione *S*-transferase (Table 4). Typical water stress-related genes were also upregulated in PAO1-infected barley, such as the expression of late embryogenesis abundant protein, dehydrin 11, and dehydrin 3. Wilting is one of the well-known symptoms upon bacterial infection (Godiard et al. 2003), and in our previous work, we showed increased wilting of poplar trees by *P. aeruginosa* infection (Attila et al. 2008). For seeds, water breaks dormancy and promotes germination and further development. Hence, barley seeds might undergo water deficit upon PAO1 infection, although the mechanisms are still not known about how *P. aeruginosa* inhibits germination of plant seeds.

Instead of induction of water stress-related genes upon PAO1 infection, the genes for proteolysis were induced by infection of the PA5021 mutant (e.g., 26 proteasome regulatory subunit and ubiquitin). The ubiquitin/26S proteasome pathway is the mechanism for selective protein breakdown and regulates diverse functions in plants, including cell cycle, hormone signaling, and disease resistance (Vierstra 2003). SGT1/E3 ubiquitin protein ligase, one of the components of the ubiquitin/26S proteasome, plays a major role in plant disease resistance (Azevedo et al. 2002). Although the function of ubiquitin and the 26S proteasome regulatory subunit upon *P. aeruginosa* infection is still unknown, both proteins may participate in the disease response in barley. The LysM domain-containing protein was first found in the symbiotic interaction between pea and *Sinorhizobium meliloti* (Limpens et al. 2003). LysM is thought to be a receptor for Nod factor signals (Limpens et al. 2003), and LysM in barley may function in signaling pathways for interactions with the less virulent PA5021 mutant.

Clearly, our barley pathogenicity assay is effective, i.e., inexpensive, rapid, and convenient, and allows scrutiny of virulence factors in *P. aeruginosa*. In addition, we have used this assay to identify three new virulence factors (PA1207, PA2647, and PA5021), and it appears K^+ transporters are important for the bacterial response to K^+ efflux from damaged plant cells.

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