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Transcriptome and secretome analyses of the adaptive response of *Pseudomonas aeruginosa* to suboptimal growth temperature

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Summary. *Pseudomonas aeruginosa* is an opportunistic pathogen involved in several diseases, including cystic fibrosis and nosocomial infections. Although the behavior of this bacterium at 37°C has been intensively studied, little is known about its capacity to adapt and survive at suboptimal temperatures, such as those encountered in hospitals. In this work, transcriptomic and proteomic analyses were used to identify factors that allow *P. aeruginosa* to become established at room temperature (close to 25°C) and thus facilitate host infections. Since the virulence of this pathogen is multifactorial and dependent on the extracellular release of toxins and degradative enzymes targeted to the host by several secretory systems, the study focused on genes activated at 25°C, namely, those encoding either components of the secretory machinery or secreted proteins. These observations were enhanced by 2D-PAGE analyses, which showed that the production of effectors from type I and type II secretion systems (respectively, proteases AprA and PrpL) and of a hemolysin co-regulated protein (Hcp) related to the type VI secretion system was specifically stimulated when the growth temperature was lowered from 37 to 25°C. The results provide a fundamental basis for investigating the processes that allow *P. aeruginosa* to adapt to suboptimal growth temperatures and which thereby promote nosocomial infection. [**Int Microbiol** 2009; 12(1):7-12]

Key words: *Pseudomonas aeruginosa* · transcriptome · secretome · extracellular proteins · secretory systems · growth temperature

Introduction

Pseudomonas aeruginosa is a common environmental microorganism and an opportunistic pathogen involved in a wide range of severe and sometimes fatal diseases, including cystic fibrosis, nosocomial pneumonia in intubated and mechanically ventilated patients, and infections in surgery patients. Its high virulence makes *P. aeruginosa* one of the key agents of hospital-acquired infections [1]. Bacterial infections in humans are mostly caused by mesophilic bacteria, whose optimum growth temperature is around 37°C, i.e., normal human-body temperature. Several plant pathogenic bacteria express pathogenicity factors that are thermoregulated [15,16,18,22]. Similarly, temperature has been shown to affect the secretion of virulence factors by *Yersinia enterocolitica*. These Yop effectors are efficiently produced and secreted at 37°C but poorly at 27°C, although the latter temperature is close to the bacterium's optimal growth temperature [25]. In contrast, other virulence factors are expressed at low temperatures, including urease [2] and O-antigen outer core lipopolysaccharide [21]. Only a few studies have been carried out regarding the effect of growth temperature on the opportunistic pathogen *P. aeruginosa*. One such study showed that thermal adaptation involved changes in the phospholipid, lipopolysaccharide, and outer membrane protein composition of *P. aeruginosa* strain PAO1 [10]. It was

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also shown that the major *P. aeruginosa* porin (OprF) changes its channel size according to temperature, with a decrease in pore size at suboptimal temperatures [8]. This behavior might help to prevent the entry of toxic compounds into the bacterial cell and to retain essential metabolites. It has been suggested that the regulatory effect is counterbalanced by the overproduction at low temperature of extracellular degradative enzymes such as proteases, which would allow the cell to make use of nutrient sources [6]. Thus, it seems likely that secretion systems play a role in the survival of pathogenic bacteria growing at temperature below 37°C. Indeed, the virulence of gram-negative pathogens is multifactorial and depends either on their direct injection into host cells or on the extracellular release of toxins and degradative enzymes into the host's surrounding environment by a battery of secretory systems. The secretion machinery of gram-negative bacteria can be roughly subdivided into two large classes, one in which the substrates are secreted in a two-step process, including a periplasmic step, and another in which the secreted factors are directly addressed to the medium or the host in a one-step process. Sec- or Tat-dependent substrates bearing a signal peptide comprise the former pathway (type II secretion system or T2SS, T4SS in *Bordetella pertussis*, T5SS), and exoproteins devoid of signal sequence the latter pathway (T1SS, T3SS, T4SS, T6SS) [3,5,19]. *P. aeruginosa* is one of the rare bacterial species equipped with almost all secretion systems described so far, it lacking only T4SS. Results obtained with *Legionella pneumophila* indicated that T2SS is required for optimal bacterial growth at 25°C and that the system becomes increasingly more important as the temperature decreases [23]. Even if this observation is specific for *L. pneumophila*, the study of bacterial secretion of virulence factors and gene expression at the temperature typically encountered in hospitals could provide insight into the nature of biological factors that facilitate the establishment of pathogens at early steps of human colonization by bacteria. *P. aeruginosa* has a wide spectrum of growth temperatures, ranging from 4°C to >40°C. This characteristic suggests that the virulence factors of *P. aeruginosa* are differentially expressed and secreted according to the growth temperature.

Although the production of virulence factors by *P. aeruginosa* has been thoroughly studied at 37°C, it is not clear whether growth at lower temperatures affects the secretion of known virulence factors and/or promotes the production and secretion of factors that have not been detected under the bacterium's usual growth conditions. Identification of bacterial factors that promote a thermal adaptive response and thereby infection at local temperature would be a key step in understanding the persistence of pathogens in the environment and thus, the basis of nosocomial infection.

The goal of this study was to use global transcriptomic analysis to detect *P. aeruginosa* genes up-regulated at suboptimal growth temperature, and 2D-PAGE to identify secreted proteins present in higher amounts under these culture conditions.

Materials and methods

Bacterial strain and growth conditions. *P. aeruginosa* strain PAO1 (prototroph, *chl-2*, B. Holloway collection) was grown in tryptic soy broth (TSB) liquid medium (Difco) at either 37 or 25°C under agitation. Samples were withdrawn at intervals for optical density monitoring at 600 nm (OD_{600}) and cells from both cultures were harvested at the early stationary phase of growth.

***Pseudomonas aeruginosa* microarray production.** The complete PAO1 gene collection of LaBaer et al. [11] was used to PCR-amplify all genes of the library cloned into an entry vector of the Gateway system (Invitrogen). Microarray production was carried out essentially as previously described by Bordi et al. (submitted) with the following modifications uploaded at ArrayExpress [http://www.ebi.ac.uk/arrayexpress] under the accession A-MEXP-1258.

Microarray cDNA sample preparation. Overnight bacterial cultures were diluted in TSB to an OD_{600} of 0.1. Separate cultures of *P. aeruginosa* PA01 wild-type strain were grown at 37 or 25°C to an OD_{600} of 4.4 (37°C) or 4 (25°C), corresponding to the early stationary phase of growth. Cells from 500 µl of each culture were centrifuged at 4°C after which cellular RNA was extracted using the SV Total RNA Isolation System Kit (Promega). The procedure followed the manufacturer's instructions, with minor modifications, including DNAse digestion, which was carried out twice in order to minimize DNA contamination. RNA was quantified by measuring the optical density at 260 nm, and its integrity controlled by agarose gel electrophoresis. Twenty µg of each RNA extract was reverse-transcribed in labeled cDNA using the ChipShot Direct Labeling System (Promega), followed by purification on a ChipShot Membrane Clean-Up System (Promega) according to the manufacturer's instructions. cDNA preparations from the wild-type strain grown at 25°C were labeled with cyanine 3 and those from cells grown at 37°C with cyanine 5. After purification, the two cDNA preparations were concentrated with a speed-vacuum concentrator and mixed in 50 µl of DigEasy buffer (Roche Diagnostics). The labeled cDNA was denatured at 95°C for 5 min and then hybridized on a microarray for 16 h at 42°C in an oven. The arrays were washed twice with 2× SSC, 0.1% SDS, 5 mM DTT buffer at 60°C followed by one wash with 0.5× SSC buffer and wash with 0.1× SSC buffer. They were then dried and rapidly scanned with a ScanArray 5000 (Perkin Elmer) in a transcriptome platform (IBSM-CNRS-Marseille, France) at 550 and 650 nm, respectively, for cyanine 3 and cyanine 5.

Microarray analysis. Data from scanned TIF images were quantified with the Genepix Pro 6.0 program (Molecular Devices) and subsequent data were analyzed using Acuity software (Molecular Devices). All data were normalized by performing a Lowess regression and then filtered to remove genes weakly expressed at both temperatures (signal noise ratio < 2). Two criteria (>2-fold change and Student's *t* test $P \leq 0.05$) were used to determine significant changes. Each microarray experiment was carried out in triplicate from three independent bacterial cultures. The array design was loaded into ArrayExpress under the accession number A-MEXP-1258.

Transcriptome analysis. Global gene expression profiling for strain PAO1 was analyzed using specific *P. aeruginosa* microarrays generated in our laboratory (ArrayExpress accession: A-MEXP-1258). To this end, strain

PAO1 was grown in TSB medium at 37°C (control conditions) and 25°C (assay conditions) (Fig. 1A) after which the cells were processed for cDNA preparation from RNA extracts. The genes responding reproducibly and with a difference >2-fold following the change in growth temperature were selected after statistical analysis. The expression of >153 genes was found to be altered by the growth temperature, with 68 (44.4 %) activated at low temperature. To examine the functional distribution of these genes, they were classified according to the categories described in the *Pseudomonas* genome database [http://v2.pseudomonas.com].

Protein analysis. Bacterial cells were harvested by centrifugation (5000 ×*g*, 10 min, 4°C) and the supernatant, containing the extracellular proteins, was filtered through 0.22-µm Acrodiscs (PALL). The filtrate was mixed with 3 µg of carrier tRNA (type X, Sigma)/ml for 15 min and the proteins precipitated in ice for at least 30 min with 10% trichloroacetic acid (TCA) (final concentration, w/v). The precipitated material was washed twice with 90% acetone (v/v) to remove TCA, and the proteins analyzed by two-dimensional gel electrophoresis (2D-PAGE) as described in Michel et al. [13]. Proteins were stained with imperial blue (Pierce) according to the manufacturer's instructions. Acrylamide gel slices containing the protein spots were cut out with a sterile scalpel and trypsin-digested, followed by MALDI-TOF mass spectrometry analysis. Results were drawn from three independent experiments, each carried out in duplicate. Only reproducible changes were taken into consideration.

Results and Discussion

Transcriptome analysis. The genes identified in our transcriptional analysis were distributed in 20 categories, the last consisting of unknown/unclassified genes, which comprised the major class evaluated in this study (Fig. 1B). Note that, among the *P. aeruginosa* genes activated at suboptimal growth temperature, those involved in cell metabolism (amino acid biosynthesis, carbon compound catabolism, energy, fatty acid and phospholipid metabolism), protein secretion (protein secretion/export apparatus and secreted factors classes), cell wall/LPS/capsule, motility/attachment, protein synthesis (translation, post-translational modification, degradation), and transcriptional regulation were particularly affected by the decrease in temperature (Table 1-SI, and Fig. 1B). These changes most probably correlated with the bacterium's adaptation to this new temperature environment. Given the importance of secreted factors in the virulence of *P. aeruginosa*, as a first approach we focused on genes encoding proteins of the secretion apparatuses and on those encoding secreted proteins.

Few of the genes encoding secreted factors were found to be activated at the suboptimal growth temperature. However, genes encoding the proteases PrpL (PA4175 or protease IV) and PA3535 (an autotransporter probably catalyzing serine protease activity) were up-regulated under these culture conditions (9- and 2-fold, respectively) (Table 1-SI). Another gene, encoding the hemolysin co-regulated protein HcpB (PA5267), was found to be very strongly up-regulated (19-fold). Interestingly, this gene is present in triplicate at different loci in the *P. aeruginosa* genome (PA0263, PA1512, PA5267). The three genes encode three identical proteins (100% identity), HcpC, HcpA, and HcpB, which are located outside the three typical T6SS *hsi* clusters, *hsi-I*, *hsi-II*, and *hsi-III* [5,14]. Hcp proteins are generally thought to be substrates of the recently described T6SS [14], involved in *P. aeruginosa* pathogenicity; they are secreted in a Sec- or Tat-independent manner [5]. As reported in that study, several *vgrG* genes located outside the T6SS clusters are found in association with *hcp* genes. Indeed, another low-temperature-activated gene, *vgrG* (PA0262, 3.4-fold increase), is situated on the *P. aeruginosa* chromosome at a locus close to *hcpC* (PA0263). Although PA5267 and PA0262 belong, respectively, to the Hcp and VgrG families, their genes are not located within any loci assigned to the *P. aeruginosa* T6SS systems but are present as orphan elements on the chromosome. VgrG proteins form homotrimeric or heterotrimeric complexes that could assemble into a putative phage-like tail. This structure might constitute a needle-like device used to penetrate either the bacterial cell envelope or host cell membrane [5,17].

In addition, a gene belonging to HSI-I T6SS, *hsiB1* (PA0083), and the *secB* gene involved in the Sec export system were shown to be up-regulated (respectively, 2- and 2.6-fold) as the growth temperature decreased. These results suggested that both Sec-dependent (such as T2SS) and Sec-independent (such as T6SS) secretion systems are specifically affected by slight changes in temperature (Table 1-SI). *P. aeruginosa* T5SS is represented by the autotransporter class PA3535 (AT or T5aSS) and by the two-partner secretion class (TPS or T5bSS) [3,7]. The gene PA4625, found to be up-regulated at 25°C (2.8-fold), encodes the TpsA component (passenger) of the TPS constituted in association with the TpsB transporter element PA4624. The role of this TPS system has not yet been described.

2D-PAGE analysis of extracellular proteins produced at the suboptimal growth temperature. To validate the microarray data, we focused on one of the mechanisms actively involved in the pathogenicity of *P. aeruginosa*, i.e., the secretion of extracellular proteins. We also sought to determine a correlation between transcription analysis and proteins present in increasing amounts in the secretome of cells grown at the suboptimal temperature. Establishment of such a correlation could expand our understanding of how altered protein secretion helps cells to adapt to new environmental conditions. Strain PAO1 was grown either at 37°C (control conditions) or at 25°C (suboptimal temperature) (Fig. 2A) and extracytoplasmic proteins isolated from both cultures at the same growth phase (OD_{600} = 5 and

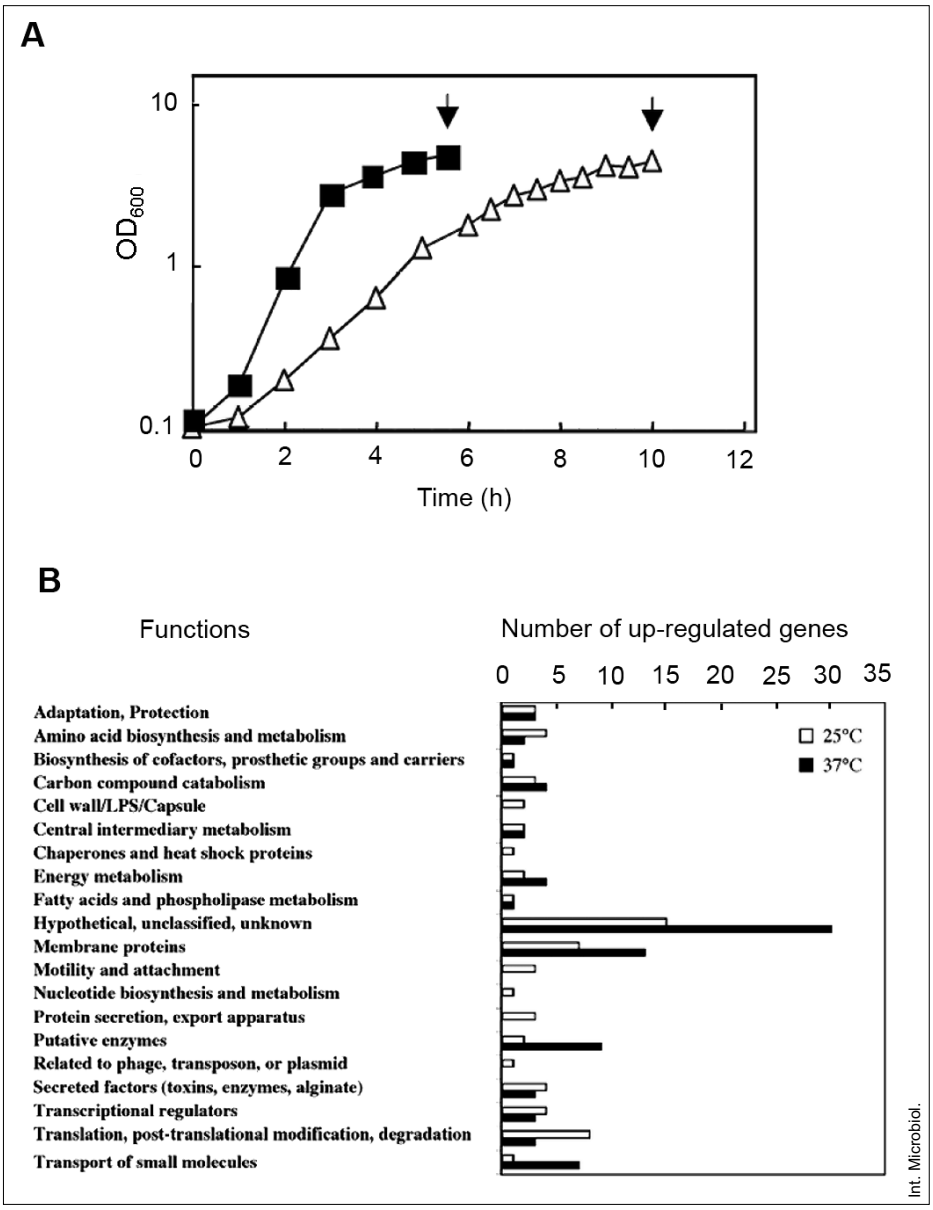

Fig. 1. Transcriptional analysis of *Pseudomonas aeruginosa* PAO1 grown at different temperatures: (**A**) Growth at 37°C (closed squares) and 25°C (open triangles) was monitored by measuring the optical density at 600 nm (OD_{600}). Arrows indicate the time of sampling. (**B**) Microarray studies of thermoregulated gene expression in *P. aeruginosa*. Genes up-regulated at 37 or 25°C were classified in 20 different functional categories and their distribution expressed in number of genes affected by temperature.

4.6, respectively) were analyzed by 2D-PAGE, as described in Michel et al. [13]. There were a few slight variations from one experiment to another between the protein profiles of the secretome produced at 37 or 25°C. However, only protein spots whose increase could be reproduced at 25°C were considered. At least three protein spots were repeatedly increased at 25°C compared to 37°C (Fig. 2B, arrows). Mass spectrometry analysis of these proteins identified spot 1 as the alkaline protease AprA (PA 1249), a T1SS substrate, spot 2 as the T2SS-dependent protease PrpL (PA 4175), and spot 3 as Hcp (hemolysin co-regulated protein). As described above, there are at least five Hcp proteins in *P. aeruginosa* PAO1, including three whose predicted protein sequences are 100% identical. Although spot 3 is representative of one of these three Hcp, mass spectrometry was unable to determine which of the three genes encoded the isolated product. Nevertheless, the results obtained by transcriptomic studies (Table 1-SI) suggested that spot 3 is HcpB (PA5267). In a previous study, the Hcp1 protein of the Hsi-I system was detected in the sputum of cystic fibrosis (CF) patients colonized with *P. aeruginosa*, and Hcp1 antibodies were found in CF patient sera, suggesting that T6SS is involved in pathogenicity [14]. However, the role of each component of the HSI systems is still poorly understood. Similarly, the roles of the orphan Hcp and VgrG proteins in cell adaptation or pathogenicity remain to be established.

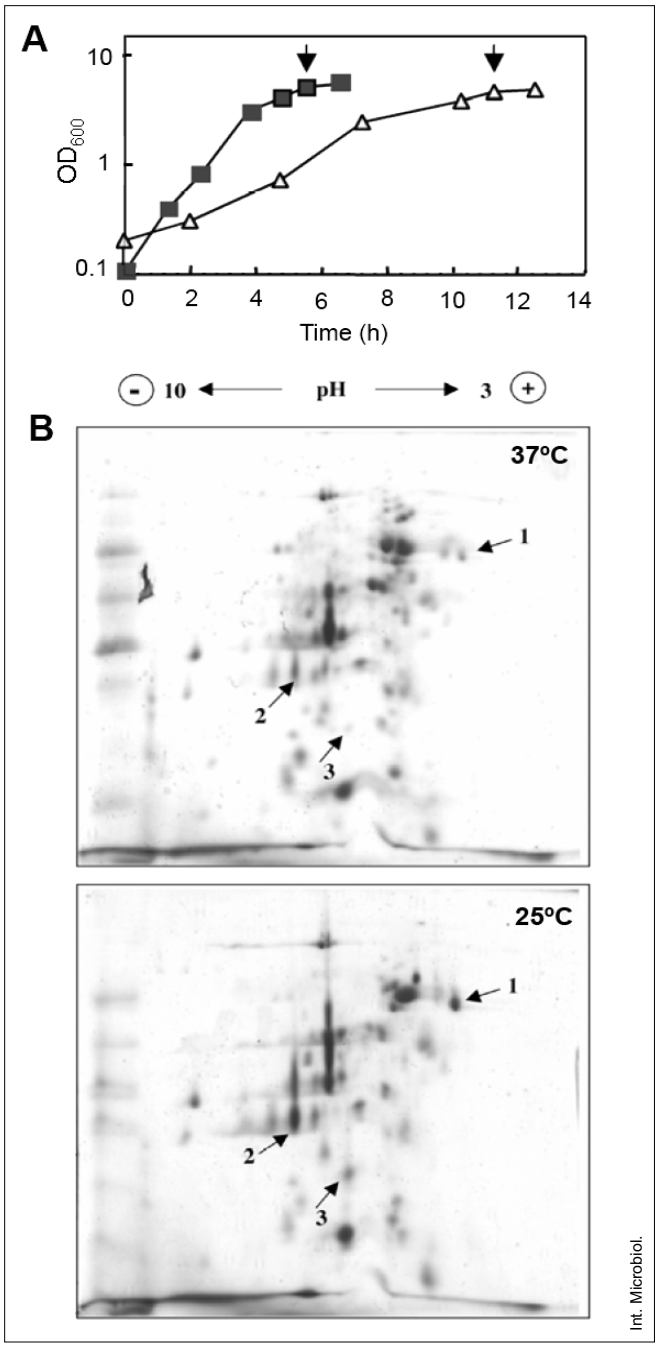

Fig. 2. Comparative analyses of the secretomes of *Pseudomonas aeruginosa* PAO1 grown at 37 and 25°C. (**A**) Growth at 37°C (black squares) and 25°C (white triangles). Arrows indicate the time of sampling. (**B**) Influence of the growth temperature on the secretome pattern. Extracellular proteins were analyzed by 2D-PAGE as described in Materials and methods. Only the proteins repeatedly found to be increased at 25°C compared to 37°C in the three experiments were taken into consideration (indicated by arrows).

The increased production of PrpL and HcpB at 25°C corroborates the results obtained by transcriptional analysis and suggests that these proteins are involved in a process probably promoting thermoadaptation and cell survival. However, although 2D-PAGE showed that the alkaline protease AprA (PA1249) was present in higher amounts at 25 than at 37°C (Fig. 2B), gene expression was not activated at low temperature (Table 1-SI). These results indicated that either the activity of the proteases involved in AprA degradation was lower at 25 than at 37°C, or that these proteases were produced in decreased amounts at the suboptimal growth temperature, or that AprA was more efficiently secreted by T1SS at the lowest temperature. Consistent with the last hypothesis, we observed that *secB* was up-regulated at the suboptimal temperature (Table 1-SI). This gene encodes a bifunctional protein, SecB, involved in two separate pathways: protein export via the Sec machinery and the secretion of HasA, a T1SS substrate from *Serratia marcescens* [20]. Therefore, it cannot be ruled out that, as for HasA, AprA could be more efficiently secreted in the presence of higher amounts of SecB and thus recovered in greater amounts at 25 than at 37°C. The role of AprA in the pathogenicity of *P. aeruginosa* has not been clearly defined, but as a fibrin protease it may participate in acute lung injury [9]. The presence of higher amounts of PrpL in the surrounding medium of *P. aeruginosa* grown at suboptimal temperature could impact the virulence of this bacterium. Indeed, *P. aeruginosa* is known to cause devastating infection of the human eye and the most common infection of the cornea among contact lens users. It was determined that virulence is due to PrpL (previously named protease IV) [4,24]. This protein was also shown to contribute to the ability of *P. aeruginosa* to persist in a rat model of chronic pulmonary infection [26] and to the inhibition of lung surfactant host defense and biophysical functions [12].

In summary, this study shows that protein secretion by *P. aeruginosa* is affected by the growth temperature and that this thermoadaptation is likely to be specific for typical exoproteins secreted by the T1SS (AprA), T2SS (PrpL), or T6SS-related (HcpB) pathways. The modulation of protein secretion in *P. aeruginosa* adapted to low temperature could give the cells a competitive advantage over other environmental bacteria, allowing them to become established at local temperature and to infect hosts, especially in hospitals. This study, which also identified a number of genes up-regulated at 25 compared to 37°C, provides a fundamental basis for investigating the processes that allow *P. aeruginosa* to adapt to suboptimal growth temperatures and which thereby promote nosocomial infection.

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Table 1-SI. Genes up-regulated in *Pseudomonas aeruginosa* growing at 25°C compared to 37°C

Name /function Gene number (name)	Gene product description	*P*-value	Fold change
Adaptation, Protection			
PA0177	Purine-binding chemotaxis protein	0.0205	3.10
PA2385 (*pvdQ*)	3-oxo-C12-homoserine lactone acylase	0.0024	2.24
Amino acid biosynthesis and metabolism			
PA1818	Orn/Arg/Lys decarboxylase LdcC	0.0016	2.27
PA0402 (*pyrB*)	Aspartate carbamoyltransferase	0.0268	2.83
PA3987 (*leuS*)	Leucyl-tRNA synthetase	0.0111	2.24
Carbon compound catabolism			
PA0210 (*mdcC*)	Malonate decarboxylase delta subunit	0.0084	2.87
PA2517 (*xylY*)	Toluate 1,2-dioxygenase beta subunit	0.0071	3.18
PA4901 (*mdlC*)	Benzoylformate decarboxylase	0.0146	2.08
Cell wall/LPS/Capsule			
PA1082 (*flgG*)	Flagellar basal body	0.0442	2.91
PA3548 (*algI*)	alginate-O-acetyl transferase	0.0064	2.58
Central intermediary metabolism			
PA0710 (*gloA2*)	Lactoylglutathione lyase	0.0155	2.25
PA2821	Probable glutathione S-transferase	0.0067	2.27
Chaperones & heat shock proteins			
PA5195	Heat shock protein	0.0240	2.71
Energy metabolism			
PA5558 (*atpF*)	ATP synthase B chain	0.0184	2.99
PA4587 (*ccpR*)	Cytochrome c551 peroxidase precursor	0.0326	2.25
Fatty acid and phospholipid metabolism			
PA3487 (*pldA*)	Phospholipase D	0.0012	5.75
Hypothetical, unclassified, unknown			
PA0049		0.0186	11.81
PA0587		0.0039	2.06
PA1592		0.0510	3.07
PA1734		0.0119	3.34
PA1817		0.0070	2.59
PA1895		0.0299	2.03

PA1907		0.0214	2.34
PA1939		0.0225	2.27
PA2208		0.0067	3.31
PA2282		0.0019	2.88
PA2746		0.0005	3.18
PA2763		0.0267	2.55
PA3208		0.0009	23.02
PA3613		0.0358	2.34
PA3695		0.0012	3.31
PA4352		0.0441	2.63
PA4537		0.0077	3.40
PA4578		0.0173	2.49
PA5120		0.0090	2.60
PA5228		0.0123	2.39
Membrane proteins involved in transport of small molecules			
PA2391 (opmQ)	Outer membrane TolC homologue	0.0324	2.82
PA5097	Amino acid permease	0.0097	3.66
PA5099	Nucleotide transport	0.0004	3.02
PA5479 (gltP)	Proton-glutamate symporter	0.0012	2.67
Motility/Attachment			
PA1460 (motC)	Flagellar assembly, chemotaxis	0.0085	2.85
PA4555 (pilY2)	Type IV pilus assembly	0.0056	3.18
PA5043 (pilN)	Type IV pilus assembly	0.0032	4.89
Nucleotide biosynthesis and metabolism			
PA3163 (cmk)	Cytidylate kinase	0.0335	2.32
Protein secretion/Export apparatus			
PA0083	T6SS HSI-I component	0.0433	2.07
PA0262	T6SS VgrG homologue	0.0204	3.42
PA5128 (secB)	Export system	0.0298	2.62
PA4625	hypothetical adhesin	0.0235	2.78
Putative enzymes			
PA1881	Oxidoreductase	0.0022	2.28
PA3535	Probable serine protease	0.0028	2.01
PA4512 (lpxO1)	Lipopolysaccharide biosynthetic protein	0.0012	2.29
Related to phage			
PA0644	Pyocin F, phage-related	0.0195	3.15
Secreted Factors (toxins, enzymes, alginate)			

PA4175 (prpL)	Protease IV	0.0116	8.93
PA5267 (hcpB)	Secreted protein	0.0036	19.37
Transcriptional regulator			
PA0765 (mucC)	Positive regulator (alginate biosynthesis)	0.0147	2.54
PA4315 (mvaT)	Transcriptional regulator	0.0195	2.06
PA4577	Post-transcriptional control regulator	0.0095	3.53
PA4596	Transcriptional regulator, NfxB homologue	0.0121	8.63
Translation, post-translational modification, degradation			
PA4246 (rpsE)	30S ribosomal protein S5	0.0031	2.47
PA4252 (rplX)	50S ribosomal protein L24	0.0026	4.06
PA4256 (rplP)	50S ribosomal protein L16	0.0241	3.34
PA4263 (rplC)	50S ribosomal protein L3	0.0018	3.20
PA4678 (rimI)	Peptide n-acetyltransferase	0.0147	4.20
PA4891 (ureE)	Urease accessory protein	0.0096	3.90
PA1793 (ppiB)	Peptidyl-prolyl *cis-trans* isomerase B	0.0178	4.23
PA4572 (fklB)	Peptidyl-prolyl cis-trans isomerase	0.0013	2.61
Transport of small molecules			
PA4687 (hitA)	Ferric iron-binding periplasmic protein	0.0368	2.23