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The effect of zinc limitation on the transcriptome of *Pseudomonas protegens* Pf-5

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Summary

Zinc is an important nutrient but can be lacking in some soil environments, influencing the physiology of soil-dwelling bacteria. Hence, we studied the global effect of zinc limitation on the transcriptome of the rhizosphere biocontrol strain *Pseudomonas protegens* Pf-5 (formerly *Pseudomonas fluorescens*). We observed that the expression of the putative zinc uptake regulator (Zur) gene was upregulated, and we mapped putative Zur binding sites in the Pf-5 genome using bioinformatic approaches. In line with the need to regulate intracellular zinc concentrations, an array of potential zinc transporter genes was found to be zinc-regulated. To adapt to low-zinc conditions, a gene cluster encoding non-zinc-requiring paralogues of zinc-dependent proteins was also significantly upregulated. Similarly, transcription of genes encoding non-zinc-requiring paralogues of ribosomal proteins L31 and L36 was increased by zinc limitation. A strong transcriptional downregulation of the putative copper chaperone gene (*copZ*) was also observed, suggesting interplay between zinc and copper homeostasis. Importantly, zinc also affected biocontrol attributes in Pf-5, most notably reducing the expression of the gene cluster responsible for biosynthesis of the antibiotic 2,4-diacetylphloroglucinol (DAPG) under zinc limitation. This study clearly defines changes to the molecular physiology of Pf-5 that enable it to survive under zinc limitation.

Introduction

Pseudomonas protegens Pf-5 (previously called *Pseudomonas fluorescens* Pf-5) (Ramette *et al.*, 2011) is a soil-inhabiting biocontrol bacterium that can suppress a wide variety of plant pathogenic bacteria, fungi and oomycetes (Howell and Stipanovic, 1979; 1980; Loper *et al.*, 2007). Pf-5 suppresses the growth of these pathogens primarily via the secretion of a range of secondary metabolites such as hydrogen cyanide, pyoluteorin, pyrrolnitrin, rhizoxin analogues and 2,4-diacetylphloroglucinol (DAPG) (Howell and Stipanovic, 1979; 1980; Nowak-Thompson *et al.*, 1994; Whistler *et al.*, 1998; Loper *et al.*, 2008). Genome sequencing further highlighted the biocontrol properties of Pf-5, revealing that a large proportion of its genetic information is dedicated to biocontrol functions, such as the production of secondary metabolites, a number of which were previously unknown (Paulsen *et al.*, 2005).

The concentrations of micronutrients vary considerably within the soil environments that may be inhabited by *P. protegens* Pf-5 and related biocontrol organisms. Nonetheless, micronutrients are essential to most organisms and are likely to have profound effects on the physiology of soil-dwelling microbes. Zinc is considered an essential micronutrient mainly due to its unique chemical properties. Because zinc does not undergo redox reactions and can act as a Lewis acid or an electrophile, it serves as a cofactor for many proteins, mediating distinct catalytic reactions in a large number of enzymes (Andreini *et al.*, 2006; Haas *et al.*, 2009). For example, it has been estimated that zinc may act as a cofactor within approximately 5% of the proteins found in bacteria (Berg and Shi, 1996; Andreini *et al.*, 2006), including ribosomal proteins, DNA and RNA polymerases (Blaby-Haas *et al.*, 2011) and DNA primases (Pan and Wigley, 2000), which serve in the essential housekeeping functions of DNA, RNA and protein synthesis. Additionally, zinc is a cofactor for a range of accessory proteins, such as some β-lactamases (Andreini *et al.*, 2006) that may be essential under particular stress conditions. In line with its importance in the cell, zinc ions have been shown to accumulate to the same levels as iron and calcium in some bacterial cells (Outten and O'Halloran, 2001).

Although it is important for bacterial cells to accumulate a sufficient quantity of zinc to fulfil the range of essential cellular reactions, zinc in excess can be toxic as it can compete with other metals for binding sites in proteins (Loisel *et al.*, 2008; Blaby-Haas *et al.*, 2011). Therefore, bacteria must employ measures to maintain a fine zinc

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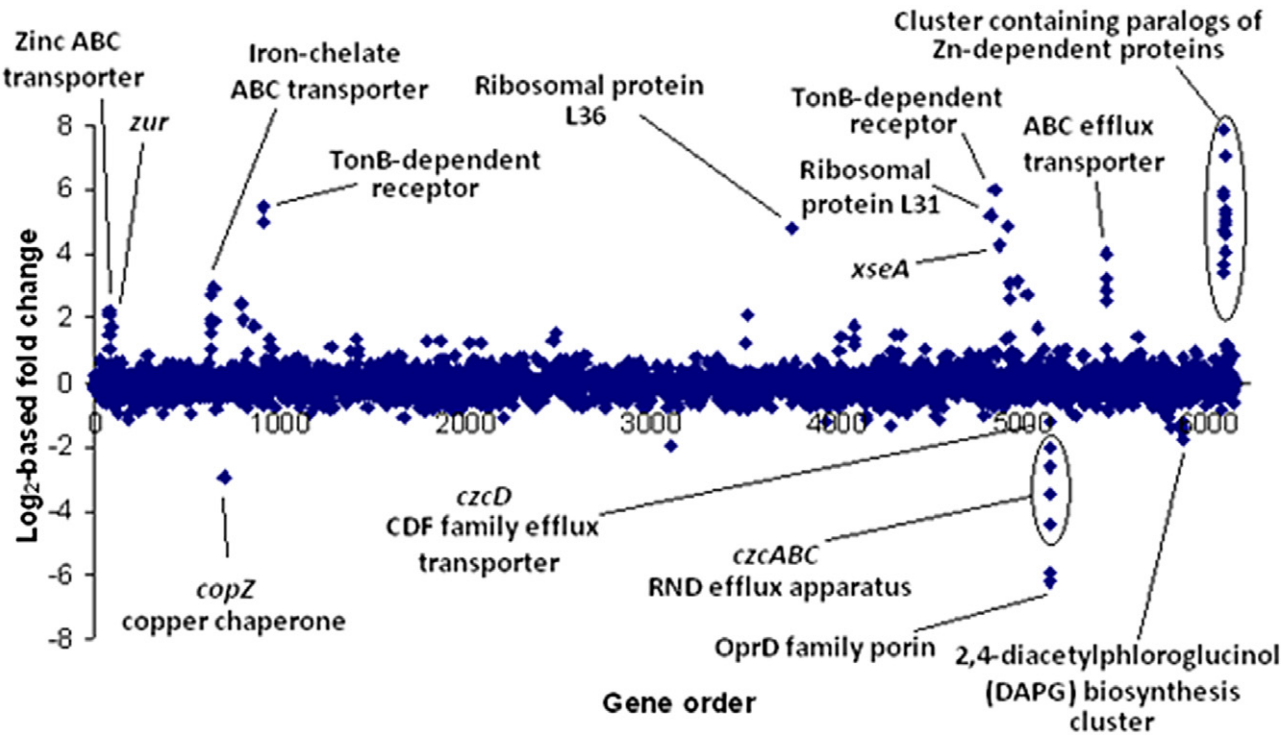

Fig. 1. The transcriptional profile of Pf-5 grown in zinc-limited versus zinc-replete culture medium. Each of the annotated genes in the Pf-5 genome is represented by a dot. The *x*-axis is the gene order with the origin of replication situated at both ends, while the *y*-axis is the $\log_2$-based fold change of transcript abundance for each gene in cultures of Pf-5 grown in a zinc-limited medium as compared with a zinc-replete medium.

balance. Zinc homeostasis in bacteria is frequently controlled by the zinc uptake regulator (Zur) (Hantke, 2005), a member of the ferric uptake regulator (Fur) family (Lee and Helmann, 2007) that is widely conserved across a range of bacterial genera, including the proteobacteria (Panina *et al*., 2003). Similar to the iron concentration-dependent regulatory mechanism of Fur, Zur functions as a repressor of target genes under zinc-replete conditions. When zinc is abundant, zinc-bound Zur proteins bind to the operator sequences upstream of target genes to prevent transcription. Under zinc-limited conditions the Zur equilibrium shifts to the unloaded form, causing Zur to dissociate from target gene operators allowing gene transcription to proceed (Blaby-Haas *et al*., 2011).

In addition to the normal cellular functioning of bacterial cells, zinc can be an important factor in the expression of biocontrol phenotypes of some bacteria. For example, zinc was found to increase biocontrol properties of *Pseudomonas* spp., including *P. protegens* strains (Défago and Haas, 1990; Ownley *et al*., 1991; 2003). This may be due to the zinc-induced production of biocontrol secondary metabolites such as phenazine 1-carboxylic acid (Slininger and Jackson, 1992), DAPG and pyoluteorin (Duffy and Défago, 1999) in these strains. Given that zinc deficiency in soil is widespread globally (Alloway, 2009), zinc concentrations might affect the capacity of biocontrol strains of *Pseudomonas* spp. to suppress plant pathogens. Consequently, we investigated the genome-wide impact of zinc deficiency in the model biocontrol strain, *P. protegens* Pf-5, using a transcriptomic approach.

Results and discussion

Overview of microarray study

Although zinc in excess can be toxic, previous studies showed that the growth of some strains of *P. fluorescens* and *P. protegens* were unaffected by $ZnSO_4$ at concentrations up to 0.8 mM (Duffy and Défago, 1999; 2000). Thus, M9 minimal medium supplemented with 0.8 mM $ZnSO_4$ was used as the zinc-replete condition versus non-amended M9 minimal medium. No notable differences were observed in cell growth and biomass of Pf-5 between the conditions. Preliminary evaluation with quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) found differences in transcription of probable zinc homeostasis genes between the conditions tested.

Microarray experiments determined that 73 genes were upregulated and 28 genes were downregulated by at least twofold under zinc-limited conditions (Fig. 1; Table S1). The microarray data were validated by qRT-PCR (Fig. 2; Table 1; Table S2). Genes were clustered according to 18

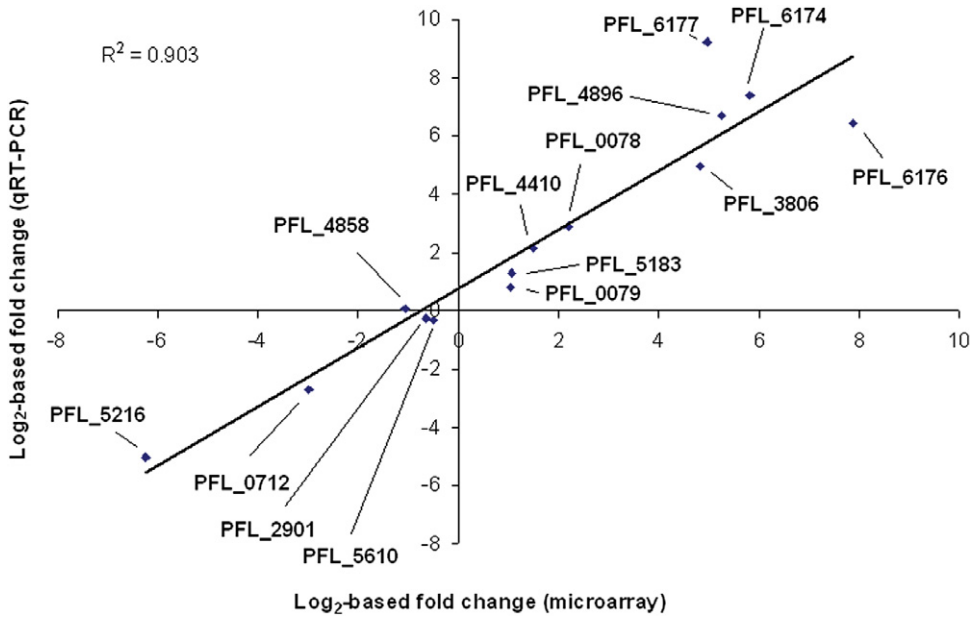

Fig. 2. qRT-PCR validation of the microarray data. The chart shows the correlations of log_2-based fold changes for 14 genes (Table S2), as derived from the microarray study and qRT-PCR. A strong correlation of coefficient (R^2) of 0.903 was observed.

functional role categories (Hassan *et al*., 2010). This analysis showed that eight of the downregulated genes were in the 'cellular processes' category, which may reflect adjustments made to the core physiology of Pf-5 to reduce the reliance on zinc-dependent enzymes or pathways (Fig. S1). Many of the genes in the 'hypothetical proteins' and 'unknown function' categories were upregulated, which indicates a potential role in zinc homeostasis for these currently uncharacterized genes. Importantly, genes in the 'transport and binding proteins' role category were differentially regulated in both directions, reflecting a need to modulate the intracellular zinc concentration.

Identification of a Zur-binding motif

In line with its predicted role as a major regulator of zinc homeostasis, the *zur* regulatory gene (PFL_0078) was significantly upregulated in cells grown under zinc-limited conditions (Table 1). Potential Zur binding sites in the Pf-5 genome were identified using a bioinformatic approach. Assuming Zur modulates expression of many of the genes affected by low-zinc conditions, we searched for conserved sequence motifs in the upstream regions of these genes using MEME (Bailey *et al*., 2006) and utilized MAST (Bailey and Gribskov, 1998) to search the remainder of the Pf-5 genome for the consensus Zur motif. This analysis revealed a putative consensus Zur binding site, consisting of a 17 bp palindrome, upstream of 16 genes (Fig. 3; Table 1; Table S3). This consensus Zur-binding motif was similar to the Zur-binding motif predicted upstream of PFL_6174 and PFL_6177 genes in Pf-5 by Haas and colleagues (2009), who used a different methodology to obtain their prediction. In their approach, they searched for a Zur-binding motif using the SignalX program (Gelfand *et al*., 2000) with a previously predicted Zur-binding motif for gammaproteobacteria (Panina *et al*., 2003). Also, the Zur-binding motif predicted in this study is similar to that of Pf-5 predicted in RegPrecise (Novichkov *et al*., 2010), a manually curated database of transcriptional regulation sites in bacterial genomes. In terms of genes with predicted upstream Zur-binding motifs, comparison between our study and that of the RegPrecise database revealed that many of the predictions are in concordance (Table 1; Table S3). In our study, most of the genes downstream of a putative Zur binding site showed increased levels of transcription under zinc-limited conditions (Table 1; Table S3). Nonetheless, the Zur motif was not found upstream of all genes modulated by zinc-limited conditions, suggesting that not all zinc-regulated genes were controlled directly by Zur.

Transporter genes regulated by zinc

In the current study, a large number of transporter genes were affected by zinc limitation, as determined using our role category analysis (Fig. S1; Table S1). The zinc-regulated transporters observed in Pf-5 corresponded well with zinc transport systems previously predicted bioinformatically using the TransportDB resource (Ren *et al*., 2004) by Haritha and colleagues (2008).

The array data showed that four gene clusters encoding ABC transporters were upregulated under zinc limitation.

Table 1. Transcriptional regulation of selected genes by zinc limitation.

Genes	Annotated functions	Microarray fold change (log_2)	qRT-PCR fold change (log_2)	Presence of putative Zur binding site upstream of gene, as predicted in
	Regulators			
PFL_0078	Transcriptional regulator, Zur	2.2	2.9	RegPrecise
PFL_5221	DNA-binding heavy metal response regulator, CzcR	–3.5		
	Transporters			
PFL_0076	High-affinity zinc ABC transporter, permease protein, ZnuB	2.1		
PFL_0077	High-affinity zinc ABC transporter, ATP-binding protein, ZnuC	1.5		
PFL_0079	High-affinity zinc ABC transporter, periplasmic zinc-binding protein, ZnuA	1.0	0.8	RegPrecise
PFL_0644	Iron-chelate ABC transporter, FeCT family, periplasmic iron-chelate-binding protein	1.9		
PFL_0645	Iron-chelate ABC transporter, FeCT family, ATP-binding protein	1.9		
PFL_0879	Outer membrane porin, OprD family	1.8		
PFL_4993	Outer membrane porin, OprD family	3.1		
PFL_5216	Outer membrane porin, OprD family	–6.2	–5.0	
PFL_5218	Cobalt/zinc/cadmium resistance protein, CzcA	–4.4		
PFL_5219	Cobalt/zinc/cadmium resistance protein, CzcB	–2.1		
PFL_5220	Cobalt/zinc/cadmium resistance protein, CzcC	–2.6		
PFL_5222	Cobalt/zinc/cadmium resistance protein CzcD	–1.2		
PFL_5529	ABC transporter, ATP-binding protein	2.9		
PFL_5530	ABC transporter, permease protein	3.3		
PFL_5892	Cadmium-translocating P-type ATPase, CadA_1	–1.5		
PFL_6179	Manganese(II)/zinc(II) ABC transporter, permease protein, MntB	7.1		
PFL_6180	Manganese(II)/zinc(II) ABC transporter, periplasmic manganese(II)/zinc(II)-binding protein, MntC	5.4		
PFL_6191	Cadmium-translocating P-type ATPase, CadA_2	1.1		
	TonB-dependent receptors			
PFL_0646	TonB-dependent outer membrane receptor	2.8		This study
PFL_0648	TonB-dependent outer membrane receptor, OprC	1.8		
PFL_0932	TonB-dependent outer membrane receptor	5.5		This study
PFL_4912	TonB-dependent outer membrane receptor	6.0		This study; RegPrecise
	Ribosomal proteins			
PFL_3806	Ribosomal protein L36, RpmJ	4.8	4.9	This study
PFL_4896	Ribosomal protein L31, RpmE_1	5.2	6.7	This study
	Genes in the cluster containing non-zinc-requiring paralogues of zinc-dependent proteins			
PFL_6171	Cobalamin synthesis protein/P47K family protein	3.7		
PFL_6172	Conserved hypothetical protein	4.7		
PFL_6173	CobW/P47K family protein	6.0		
PFL_6174	RNA polymerase-binding protein, DksA	5.8	7.4	This study; RegPrecise
PFL_6175	Conserved hypothetical protein	3.5		This study; RegPrecise
PFL_6176	*N*-acetylmuramoyl-L-alanine amidase, AmiC	7.9	6.4	This study; RegPrecise
PFL_6177	GTP cyclohydrolase I type 2	5.0	9.2	This study; RegPrecise
PFL_6181	Phosphoribosyl-AMP cyclohydrolase 2, HisI2	5.3		
PFL_6182	Carbonic anhydrase	4.6		
PFL_6183	Dihydroorotase	5.1		
PFL_6184	Threonyl-tRNA synthetase, ThrS_2	4.1		
	DNA repair			
PFL_4944	Exodeoxyribonuclease VII, large subunit, XseA	4.3		
	Copper homeostasis			
PFL_0712	Copper chaperone, CopZ	–3.0	–2.7	
	DAPG biosynthesis gene cluster			
PFL_5953	Transcriptional repressor, PhlF	–1.8		
PFL_5954	2,4-Diacetylphloroglucinol biosynthesis protein, PhlA	–1.5		
PFL_5955	2,4-Diacetylphloroglucinol biosynthesis protein, PhlC	–1.5		
PFL_5956	2,4-Diacetylphloroglucinol biosynthesis protein, PhlB	–1.2		

Two of these gene clusters were annotated as being putatively involved in zinc uptake, i.e. the PFL_0076-77, PFL_0079 cluster encoding *znuABC* and the PFL_6178-80 cluster encoding *mntABC* (Table 1). The *znuABC* genes are co-located with *zur*, whereas the *mntABC* cluster is located near genes encoding non-zinc-requiring paralogues of zinc-dependent genes. In many bacterial species, ZnuABC is the major uptake system for zinc (Patzer and Hantke, 1998; Ammendola *et al.*, 2007; Desrosiers *et al.*, 2010).

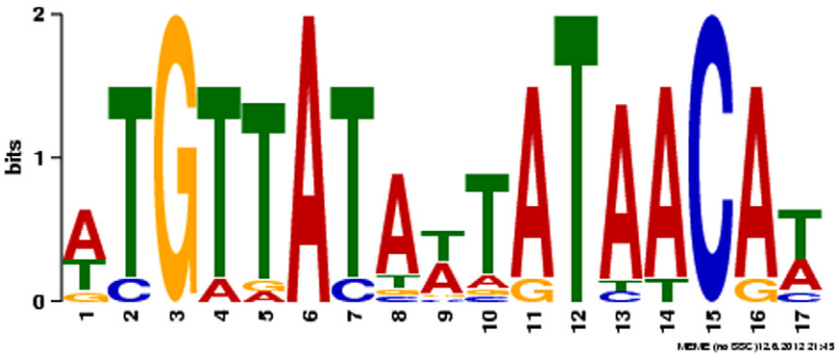

Fig. 3. The 17 bp palindrome consensus nucleotide sequences of the putative Zur binding sites identified in this study. The size of the nucleotide at each position indicates its relative prevalence in sequences used as training set for MEME algorithm (Bailey *et al.*, 2006).

Interestingly, genes in a third ABC transporter gene cluster (PFL_0643-45), which is annotated as encoding a FeCT family iron-chelate transport system, were upregulated under zinc limitation (Table 1). In a separate study examining the effects of iron limitation on the transcriptome of Pf-5, no change in regulation was observed for this cluster (Lim *et al.*, 2012). Based on these results, we speculate that this transport system might be involved specifically in zinc rather than iron homeostasis.

The PFL_5529-30 gene cluster is also likely to encode an ABC transporter and was upregulated under zinc limitation (Table 1). The genes encoding this ABC transport system are in a probable operon with genes encoding a conserved hypothetical protein (PFL_5528) and a putative lipoprotein (PFL_5531), which were also highly expressed (Table S1). Interestingly, the PFL_5528 polypeptide is histidine-rich, which is characteristic of zinc-binding proteins. Moreover, this protein is functionally predicted to be a possible zinc-binding protein by the SVMProt server (Lin *et al.*, 2006). The PSORTb 3.0 algorithm (Yu *et al.*, 2011) indicated that both PFL_5528 and PFL_5531 are likely to be non-cytoplasmic in nature but their exact cellular location is unknown. Support for this operon playing a role in zinc homeostasis comes from a Zur-binding motif found in its upstream region (Table S3). The possible function of this transporter operon in zinc homeostasis is intriguing.

In contrast to the ABC transport systems described above, zinc limitation resulted in the downregulation of genes encoding an RND superfamily efflux system (PFL_5218-20, *czcABC*), which is annotated as being involved in the efflux of metals, including zinc (Table 1). The orthologous *czcABC* system in *Alcaligenes eutrophus* is involved in the resistance to metals, such as zinc, cadmium and cobalt, through efflux (Nies, 1995). In *Pseudomonas aeruginosa*, the orthologous system is regulated by the CzcR–CzcS two-component system and is downregulated in response to zinc limitation (Hassan *et al.*, 1999; Perron *et al.*, 2004). In concordance, we also observed the downregulation of *czcR* (PFL_5221) in Pf-5 grown in zinc-limited medium.

The *czcD* (PFL_5222) gene, which is adjacent to the *czcR–czcS* cluster and encodes a protein classified within the cation diffusion facilitator (CDF) family of transport proteins, was also transcriptionally repressed under zinc limitation (Table 1). CDF family proteins function to transport metal ions from the host cell (Paulsen and Saier, 1997; Anton *et al.*, 2004). The CzcD transporter has been implicated in resistance to excess cobalt, zinc and cadmium in some bacterial genera (Anton *et al.*, 1999). As in our study, *czcD* orthologues in other species, such as *Staphylococcus aureus* (Kuroda *et al.*, 1999) and *Streptococcus pneumoniae* (Jacobsen *et al.*, 2011; Shafeeq *et al.*, 2011a), are also regulated in response to the zinc concentration.

In *P. protegens* Pf-5, the transcription of several genes (PFL_0879, PFL_4993 and PFL_5216) encoding outer membrane porins was affected by zinc concentration of the growth medium (Table 1). These porins belong to the OprD family, which facilitates the movement of a range of small molecules across the outer-membrane of Gram-negative bacterial cells (Trias and Nikaido, 1990; Huang and Hancock, 1993). Although two of the porin-encoding genes (PFL_0879 and PFL_4993) are upregulated under zinc-limiting conditions, PFL_5216, which is clustered with the *czcABC* and *czcD* genes described above, was downregulated. PFL_5216 is homologous to *opdT* (PA2505) of *P. aeruginosa*, which encodes a porin putatively involved in tyrosine uptake (Tamber *et al.*, 2006). Exposure to copper stress induces transcription of PA2505, suggesting that the OpdT porin could be regulated by heavy metals (Teitzel *et al.*, 2006). In addition, Conejo and colleagues (2003) showed that OpdT porin expression in *P. aeruginosa* PAO1 is positively correlated with the presence of zinc, similar to Pf-5 in this work. In *P. aeruginosa*, expression of OprD is regulated by heavy metals such as zinc (Conejo *et al.*, 2003; Perron *et al.*, 2004) and copper (Teitzel *et al.*, 2006; Caille *et al.*, 2007) (although transcription of the *oprD* orthologue, PFL_4773, in Pf-5 is not affected in this study), probably via heavy metal-responsive two-component system regulators, e.g. CzcRS and CopRS that are perceptive to zinc and copper respectively (Perron *et al.*, 2004; Caille *et al.*, 2007). Similarly, porins encoded by the

zinc-regulated genes identified in this study may play a role in heavy metal transport in Pf-5.

Two transporter genes of the P-type ATPase family, PFL_5892 and PFL_6191, were transcriptionally under- and overexpressed, respectively, under low-zinc conditions (Table 1). Transporters of the P-type ATPase family are involved in cation translocation across the membrane (Lutsenko and Kaplan, 1995). Bioinformatic analysis by Haritha and colleagues (2008) predicted that PFL_5892 and PFL_6191 transport zinc/cadmium and cadmium respectively. PFL_5892 is homologous to the *Escherichia coli* zinc P-type ATPase (*zntA*) (Rensing *et al*., 1997). Mutational inactivation of the PFL_5892 orthologue in *P. aeruginosa* (PA3690) rendered the mutant strain hypersensitive to zinc treatment (Teitzel *et al*., 2006). Based on these observations and the downregulation of PFL_5892 under zinc-limited conditions, it is likely that PFL_5892 has a role in transporting zinc out of the cell. As for PFL_6191, its overexpression might be a response mechanism to compensate for the reduced expression of the other cadmium metal efflux transporters (i.e. *czcABC*, *czcD* and possibly PFL_5892) in order to maintain a balanced cadmium level in the cells.

Zinc limitation increased transcription of genes encoding TonB-dependent receptors

Various extracellular compounds, such as ferric-siderophore complexes (Hartney *et al*., 2011), vitamin B12 (Chimento *et al*., 2003) and nickel (Schauer *et al*., 2007), are transported into cells through TonB-dependent receptors (TBDR), outer membrane proteins which are energized by the TonB–ExbB–ExbD apparatus for active uptake of substrates (Krewulak and Vogel, 2011). Recently, TBDRs that might be specific for zinc uptake were discovered in *Neisseria meningitidis* (ZnuD) (Stork *et al*., 2010) and in cyanobacterium *Anabaena* sp. strain PCC 7120 (Napolitano *et al*., 2012). In our study, a homologue of ZnuD (PFL_4912) (27% identity based on BLASTP) in Pf-5 was upregulated upon zinc limitation, suggesting that this receptor may facilitate zinc uptake in Pf-5 (Table 1).

In addition to the *znuD* orthologue, we observed the upregulation of several other TBDR genes under zinc limitation, i.e. PFL_0646, PFL_0648 and PFL_0932, which do not resemble ZnuD but may function in zinc uptake (Table 1). A possible reason for the participation of several TBDRs in zinc homeostasis could be to facilitate the uptake of many complexed forms of zinc that exist in soil (Karlsson and Skyllberg, 2007). For example, these receptors may act as an uptake channel for zinc-loaded siderophores, such as pyridine-2,6-bis(thiocarboxylic acid) (PDTC) (Leach *et al*., 2007). The transcription of PFL_0932 was also found to be upregulated under iron-limited conditions in Pf-5 (Lim *et al*., 2012), suggesting that it is regulated by multiple environmental factors. Interestingly, the gene adjacent to PFL_0932, PFL_0931, was also highly transcribed under low-zinc conditions. This gene encodes a hypothetical protein containing a CbiK superfamily domain, which is represented by the periplasmic component of cobalt ABC type transporters, probably indicating a function of metal transport within the periplasm.

PFL_0648 is a homologue of *oprC* (PA3790) of *P. aeruginosa*, which encodes a copper-binding protein that may function in copper utilization (Yoneyama and Nakae, 1996). Expression of *oprC* by *P. aeruginosa* is repressed under elevated copper conditions (Yoneyama and Nakae, 1996; Teitzel *et al*., 2006) but is not altered significantly under copper starvation (Frangipani *et al*., 2008). The influence of copper on expression of *oprC* by Pf-5 is yet to be determined, and there is precedence whereby homologues of TBDRs in different species of a genus may be differentially regulated. For example, the *znuD* orthologue of *Neisseria gonorrhoeae* is iron-regulated (Ducey *et al*., 2005; Jackson *et al*., 2010; Cornelissen and Hollander, 2011), whereas *znuD* of *N. meningitidis* is zinc-regulated, as mentioned above. Similarly, *oprC* might be regulated by different metal ions in *P. protegens* versus *P. aeruginosa*.

Effect of zinc limitation on transcriptional regulation of ribosomal proteins

Certain ribosomal protein genes, such as those encoding L31 and L36, are present in two copies (C+ and C– forms) in many bacterial genomes, including *P. aeruginosa* (Makarova *et al*., 2001) and *P. protegens* Pf-5 (Paulsen *et al*., 2005). The C+ form is able to bind a metal such as zinc, whereas the C– form lacks metal chelating capacity (Boysen and Hearn, 2001). Under zinc starvation, zinc can be released from the C+ form to fulfil cellular needs (Nanamiya *et al*., 2004; Akanuma *et al*., 2006). The functions of the C+ forms associated with the ribosome machinery can be replaced by the C– forms, which are de-repressed when the zinc concentration in the cell is low (Li *et al*., 2009). With this mechanism, the ribosomes can act as zinc reservoirs and zinc can be directed to cellular processes of higher priority during zinc starvation conditions (Gabriel and Helmann, 2009; Li *et al*., 2009). Of the two paralogues of ribosomal protein L31 in Pf-5 (PFL_0441 and PFL_4896), one is classed as C+ and the other C–. As observed in other bacteria (Graham *et al*., 2009), expression of the C– form (PFL_4896) increased under zinc-limited conditions (Table 1), while expression of the C+ form (PFL_0441) did not change (confirmed by qRT-PCR, data not shown). Likewise, the gene encoding the C– form of ribosomal protein L36 (PFL_3806) was upregulated when zinc was limited (Table 1). The gene encoding the C+ form of the ribosomal protein L36 in Pf-5

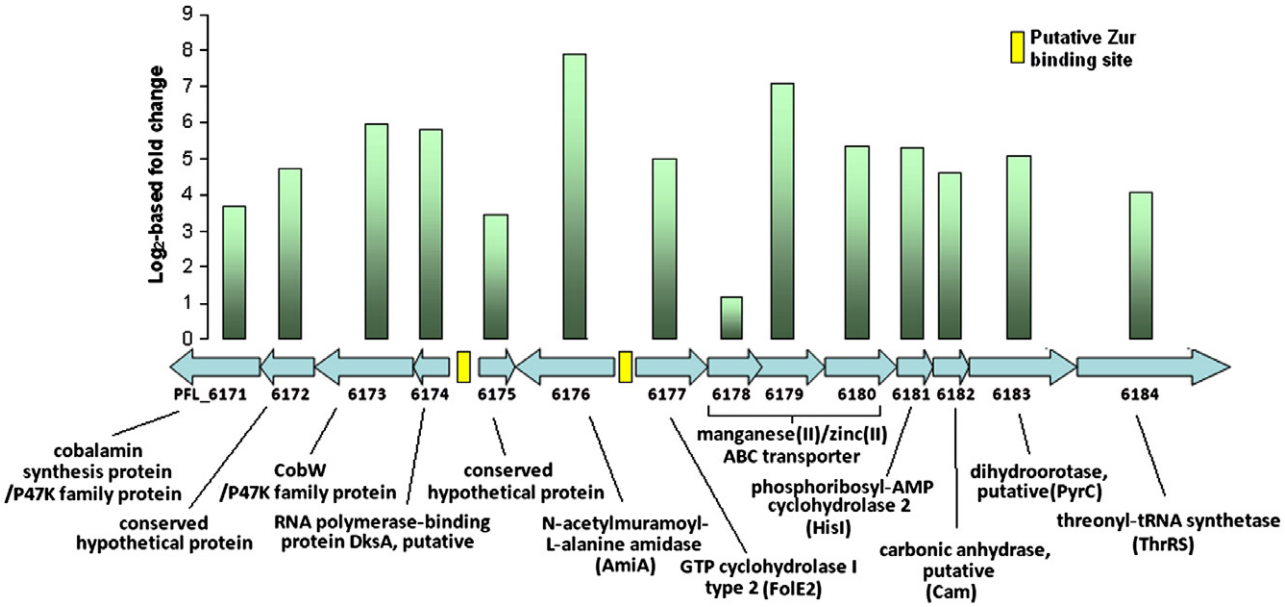

Fig. 4. The gene cluster (PFL_6171-84) encoding non-zinc-requiring paralogues of zinc-dependent proteins was significantly upregulated as derived from microarray data. The fold change for PFL_6178 exceeded two but was not registered as significant when analysed with SAM at FDR of less than 5%. Putative Zur binding sites, as determined by Haas and colleagues (2009) and in this study, are located between PFL_6174 and PFL_6175 as well as PFL_6176 and PFL_6177.

(at coordinates 6351985–6351869; Refseq accession NC_004129.6) was not annotated in the original version of the Pf-5 genome and therefore was not included in the microarray used in this study. However, we observed no change in transcriptional regulation of the gene when tested with qRT-PCR (data not shown).

Upregulation of a gene cluster containing non-zinc-requiring paralogues of zinc-dependent proteins

Recently, Haas and colleagues (2009) described a gene cluster (PFL_6171-84) having two putative Zur binding sites in the Pf-5 genome. In concordance with their prediction, we found that zinc limitation upregulated the transcription of genes in this cluster (Fig. 4; Table 1). Two upregulated genes belonging to the COG0523 family are found in this cluster in Pf-5 (PFL_6171 and PFL_6173). Members of the COG0523 family are involved in many functions, including participating in cobalamin biosynthesis (Crouzet *et al*., 1991), serving as a nitrile hydratase activator (Nojiri *et al*., 1999) and possibly acting as a metallochaperone (Lee and Helmann, 2007). Although the exact functions of the COG0523 family members found within this zinc-regulated gene cluster in Pf-5 are unknown, based on their location in a cluster that is likely involved in zinc homeostasis, Haas and colleagues (2009) postulated that these genes might serve as zinc-specific chaperones. Interestingly, some COG0523 members in other bacterial species such as *Bacillus subtilis* (Gabriel *et al*., 2008) and *Corynebacterium glutamicum* (Schröder *et al*., 2010) were also controlled by zinc availability.

Other upregulated genes within the PFL_6171-84 cluster code for non-zinc-requiring paralogues of zinc-dependent metabolic enzymes such as GTP cyclohydrolase I type 2 (PFL_6177, FolE2), Cam (γ-class carbonic anhydrase) (PFL_6182) and PyrC (dihydroorotase) (PFL_6183). These enzymes may use metals other than zinc as cofactors or they may function in an apo form to provide a back-up for zinc-dependent proteins under zinc starvation conditions (Haas *et al*., 2009). For example, in *Methanosarcina thermophila*, γ-class carbonic anhydrase could function with iron as a cofactor in addition to zinc (Tripp *et al*., 2004; Macauley *et al*., 2009). The *P. aeruginosa* homologue (PA5541) of the Pf-5 non-zinc-requiring PyrC (PFL_6183) was found to be functional (Brichta *et al*., 2004). Likewise, the zinc-dependent and non-zinc-requiring paralogues of FolE2 were found to be functionally redundant (Sankaran *et al*., 2009).

Of particular interest is the upregulation of the gene encoding a non-zinc-requiring paralogue (PFL_6174) of the DksA regulator found within the aforementioned cluster. DksA has a zinc-finger motif that is essential for its functionality (Paul *et al*., 2004; Perron *et al*., 2005). In *P. aeruginosa*, DksA functions as a regulator in response to nutrient starvation and quorum sensing that can be functionally replaced by the non-zinc-requiring paralogue

when there is insufficient zinc availability (Perron *et al*., 2005; Blaby-Haas *et al*., 2011).

Some of the genes in the PFL_6171-84 cluster encode enzymes retaining zinc-binding motifs, including AmiA (*N*-acetylmuramoyl-L-alanine amidase) (PFL_6176), HisI (phosphoribosyl-AMP cyclohydrolase) (PFL_6181) and ThrRS (threonyl-tRNA synthetase) (PFL_6184) (Haas *et al*., 2009). Haas and colleagues (2009) suggested that the purpose of upregulation of these zinc-requiring enzymes is to increase their product copy number under low-zinc conditions.

Increased transcription of DNA repair gene, xseA*, under low-zinc conditions*

Upregulation of *xseA* (PFL_4944) was observed under zinc-limited conditions (Table 1). XseA complexes with XseB to form exodeoxyribonuclease VII (Vales *et al*., 1982), one of the enzymes involved in methyl-directed mismatch DNA repair (Cooper *et al*., 1993; Burdett *et al*., 2001). Given that many DNA-repair proteins, such as UvrA (Pakotiprapha *et al*., 2008), Fpg (O'Connor *et al*., 1993) and Ada (Myers *et al*., 1995), require zinc as cofactors, the over-transcription of exodeoxyribonuclease VII during zinc limitation might compensate for the possible increased rate of DNA replication errors due to reduced activities of the zinc-dependent DNA repair enzymes. Experiments with exodeoxyribonuclease VII of *E. coli* (Chase and Richardson, 1974) and *Thermotoga maritima* (Larrea *et al*., 2008) have shown that it does not rely on zinc for functionality.

Zinc limitation reduced transcription of the putative copper chaperone CopZ

Interestingly, *copZ* (PFL_0712), which is located in a gene cluster related to copper metabolism (Zhang and Rainey, 2008; Nawapan *et al*., 2009; Corbett *et al*., 2011), was greatly downregulated under zinc limitation (confirmed by qRT-PCR) (Table 1). In *Enteroccoccus hirae*, CopZ functions as a metallochaperone that delivers copper to the CopY repressor, which replaces the bound zinc ions with copper ions under excess copper conditions (Strausak and Solioz, 1997; Cobine *et al*., 2002). A BLASTP search did not reveal a CopY homologue in the genome of Pf-5; therefore, the exact function of CopZ in Pf-5 remains unknown. However, CopZ has also been suggested to serve as a shuttle to transport copper to ATPase efflux pumps as well as to enzymes that require copper as a cofactor (Multhaup *et al*., 2001; Banci *et al*., 2003). For *P. fluorescens* strain SBW25, Zhang and Rainey (2008) suggested that CopZ is involved in delivering copper to copper-requiring enzymes, but noted that *copZ* (Pflu0660; *cueZ*) is regulated by other heavy metals, such as gold, silver and mercury, although not by zinc at a lower concentration than used here (i.e. up to 1 μM; Zhang and Rainey, 2008). Taken together, these observations suggest an interplay between zinc and copper homeostasis in *Pseudomonas* spp. Such interplay has been described in other bacteria (Kershaw *et al*., 2005; Jacobsen *et al*., 2011). For example, in *S. pneumoniae*, zinc starvation resulted in the upregulation of the *cop* (copper resistance) operon (Shafeeq *et al*., 2011b). In *P. aeruginosa*, the CopR regulator, which regulates the *cop* operon, was suggested to regulate the CzcR–CzcS two-component system that in turn controls the CzcABC efflux pump (Caille *et al*., 2007), as inferred from the existence of a probable CopR binding site upstream of the *czcR* gene (Chen *et al*., 2004). A similar mechanism may link zinc and copper homeostasis in *P. protegens* Pf-5.

Effect of zinc limitation on secondary metabolite production

Downregulation of several genes in the gene cluster involved in DAPG production was observed in the transcriptional profile of Pf-5 under zinc-limited conditions (Table 1). Using HPLC, we quantified levels of DAPG extracted from culture supernatants. On average, the amount of DAPG was slightly higher in zinc-replete cultures (0.45 ± 0.27 μg ml^{-1}) compared with non-amended cultures (0.34 ± 0.16 μg ml^{-1}). In accordance with our observation, Duffy and Défago (1999) have shown that production of DAPG by Pf-5 can be enhanced significantly by zinc amendment. However, two-tailed *t*-test statistical tests showed that the higher DAPG production in zinc-replete cultures in our study was not significant. This could be attributed to a myriad of factors that influence DAPG biosynthesis in *Pseudomonas* spp. (Duffy and Défago, 1999; Schnider-Keel *et al*., 2000; de Werra *et al*., 2009; 2011; Kidarsa *et al*., 2011). One of these is the repression of its biosynthesis by pyoluteorin (Schnider-Keel *et al*., 2000; Brodhagen *et al*., 2004), and we detected high concentrations of pyoluteorin (53.7–92.2 μg ml^{-1}) extracted from culture supernatants of Pf-5 grown in both zinc-replete and zinc-limited culture medium.

Conclusions

The effect of zinc limitation on the transcriptome of *P. protegens* Pf-5 was examined. Our observations showed an upregulation of the global regulatory gene, *zur*, involved in zinc homeostasis. Moreover, by examining the upstream regions of highly overexpressed genes, we identified putative binding sites for Zur. Of the genes shown to be significantly differentially expressed in response to zinc limitation, only 33 out of 73 (45%) were located downstream of putative Zur binding sites (including genes in predicted operons), suggesting that not all zinc-regulated

genes are directly controlled by Zur. Interestingly, a few genes encoding TBDRs were upregulated in response to low-zinc conditions. Upon zinc deficiency, non-zinc-requiring paralogous genes of zinc-dependent proteins such as ribosomal proteins L31 and L36, as well as a gene cluster containing non-zinc-requiring paralogues of zinc-dependent proteins, were induced, possibly to compensate for the loss of functions of zinc-requiring enzymes in the absence of zinc cofactors. The downregulation of *copZ* was also detected, which suggests an interplay between zinc and copper homeostasis in the bacterium. A lack of zinc could increase DNA replication errors due to the need for zinc cofactors in various DNA repair enzymes and, therefore, could be the reason for increased transcription of *xseA*, which encodes a component of the DNA repair enzyme exodeoxyribonuclease VII. Consistent with previous studies on the importance of zinc on DAPG production in Pf-5, we observed its effect at the transcriptional level. Overall, this study revealed a broad adaptive nature of Pf-5 towards zinc limitation.

Experimental procedures

Bacterial strains and growth conditions

Pseudomonas protegens strain Pf-5 was grown in M9 minimal medium (Sambrook *et al*., 1989) supplemented with 100 μM calcium chloride ($CaCl_2$), 2 mM magnesium sulfate ($MgSO_4$), 100 μM iron(III) chloride ($FeCl_3$) and 0.4% glucose. For growth under zinc-limited conditions, this medium was used without zinc supplementation, whereas zinc-replete conditions were achieved with the addition of 0.8 mM zinc sulfate ($ZnSO_4$) into the media. The bacteria were grown to OD_{600} = 1.1 aerobically at 25°C with shaking.

RNA extraction, cDNA synthesis and labelling

RNA was extracted from 20 ml of bacterial cultures using the RNAeasy kit (Qiagen) according to the manufacturer's protocol, with the addition of an initial Trizol (Invitrogen) extraction step. The RNA was treated with Turbo DNase (Ambion) after purification and cleaned by processing with the RNAeasy kit (Qiagen). The concentration and purity of RNA extracted were determined with a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesized from approximately 7 μg of RNA using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen) using random hexamer primers for each reaction. The synthesized cDNA was labelled with either Alexa Fluor 555 or Alexa Fluor 647 fluorescent dyes (Invitrogen). The RNA was hydrolysed and the reactions cleaned up using the QIAquick PCR purification kit (Qiagen). The cDNA concentration and labelling efficiency were determined using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies).

Microarray experiments

Labelled cDNA samples were hybridized to microarray slides spotted with 70-mer DNA oligonucleotides representing 6147 genes annotated in Pf-5 (Hassan *et al*., 2010). Experiments were performed using three biological replicates and three technical replicates including two 'dye flip' replicates. An Axon 4000B scanner with GenePix 4.0 software was used to detect hybridized spots. The resulting data were analysed with Spotfinder 3.2.1 (TIGR) and normalized using the LOWESS algorithm incorporated in the TIGR-MIDAS 2.20 package with block mode and smoothing parameter values of 0.33. Statistical analyses were conducted using the Statistical Analysis of Microarrays (SAM) 3.02 algorithm (Tusher *et al*., 2001) with a false discovery rate (FDR) of less than 5%. Genes displaying a fold change in expression of twofold or more between the zinc-limited and zinc-replete conditions were identified as significantly differentially expressed. The microarray data were deposited in the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with the Accession No. GSE32921.

qRT-PCR validation of microarray data

The microarray data were verified using qRT-PCR on 14 differentially expressed genes using primers designed with the help of Primer3 program (Rozen and Skaletsky, 2000) (Table S2). The *ppsA* pyruvate kinase gene (PFL_1869), which has an orthologue in *P. aeruginosa* (Khan *et al*., 2008), was used as an internal control in qRT-PCR experiments. cDNA for qRT-PCR experiments was reverse-transcribed from 2.5 μg of the same RNA samples utilized for microarray experiments using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). qRT-PCR was performed with GoTaq® qPCR Master Mix (Promega) in a Mastercycler ep Realplex^4 S (Eppendorf) and processed using Eppendorf Mastercycler ep Realplex 2.2 software. Each reaction was performed in triplicate. The $\Delta\Delta C_T$ algorithm was used to determine the fold change of the genes tested (Livak and Schmittgen, 2001).

Bioinformatic analyses

Putative Zur-binding motifs were identified in regions upstream of open reading frames using the Multiple Em for Motif Elicitation (MEME) version 4.8.1 tool (Bailey *et al*., 2006). The upstream intergenic regions of genes upregulated by at least fourfold under zinc limitation were used as an initial training set in this analysis. The scoring matrix obtained was subsequently used to search for Zur binding sites globally in the intergenic regions of Pf-5 using the Motif Alignment and Search Tool (MAST) version 4.8.1 (Bailey and Gribskov, 1998). Significant MAST hits were identified as having an *E*-value below 10, a motif *P*-value of less than 0.0001 and a palindromic sequence. The consensus sequence for the putative Zur binding site was displayed using Weblogo 3.0 (Crooks *et al*., 2004). Our predicted Zur binding sites were compared with those in the RegPrecise database version 1.7 (Novichkov *et al*., 2010). Information pertaining to probable operons in the Pf-5 genome was obtained from Pseudomonas Genome Database (Winsor *et al*., 2011). Possible functionality of a protein is predicted using SVMProt server (Lin *et al*., 2006) while the subcellular location is predicted by PSORTb 3.0 algorithm (Yu *et al*., 2011).

Chemical detection of secreted secondary metabolites

Concentrations of DAPG and pyoluteorin were quantified from 10 ml aliquots of supernatant from Pf-5 cultures grown in M9 minimal media containing the supplements mentioned previously. The supernatants were acidified to pH 1–2 with hydrochloric acid (HCl) prior to repeated extraction with 4 ml of ethyl acetate. The extract was dried under reduced pressure using a SpeedVac DNA 110 (Savant) and dissolved in 250 μl of methanol. High-performance liquid chromatography (HPLC) was used to detect the secreted secondary metabolites of interest using conditions described previously (Whistler *et al*., 1998; Hassan *et al*., 2010). HPLC analysis was performed on a Shimadzu HPLC instrument equipped with an SPD-M10A diode array detector using a Phenomenex Luna C18(2) column (4.6 × 250 mm, 5 μm). Triplicate cultures were extracted and analysed. Concentrations of the above compounds in extracts were calculated based on the standard curves made using purified compounds.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes significantly regulated by at least twofold by zinc limitation, as identified by SAM analysis of microarray data at a false discovery rate (FDR) of less than 5%.

Table S2. List of qRT-PCR primers utilized in this study.

Table S3. The putative Zur-binding motifs found using MAST version 4.8.1 (Bailey and Gribskov, 1998) with transcriptional fold changes of downstream genes, as determined from microarray data. The fold changes are reported in $\log_2$-based format.

Fig. S1. Role category analysis of microarray data. The numbers of upregulated and downregulated genes of Pf-5 grown in zinc-limited media versus zinc-replete media are respectively represented as blue and red bars. The genes are categorized according to 18 functional role categories (Hassan *et al.*, 2010) and some genes can be placed in more than one category and thus counted more than once.