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Identification of meat spoilage gene biomarkers in *Pseudomonas putida* using gene profiling

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ABSTRACT

While current food science research mainly focuses on microbial changes in food products that lead to foodborne illnesses, meat spoilage remains as an unsolved problem for the meat industry. This can result in important economic losses, food waste and loss of consumer confidence in the meat market. Gram-negative bacteria involved in meat spoilage are aerobes or facultative anaerobes. These represent the group with the greatest meat spoilage potential, where Pseudomonas tend to dominate the microbial consortium under refrigeration and aerobic conditions. Identifying stress response genes under different environmental conditions can help researchers gain an understanding of how Pseudomonas adapts to current packaging and storage conditions. We examined the gene expression profile of *P. putida* KT2440, which plays an important role in the spoilage of meat products. Gene expression profiles were evaluated to select the most differentially expressed genes at different temperatures (30°C and 10°C) and decreasing glucose concentrations, in order to identify key genes actively involved with the spoilage process. A total of 739 and 1269 were found to be differentially expressed at 30°C and 10°C respectively; of which 430 and 568 genes were overexpressed, and 309 and 701 genes were repressed at 30°C and 10°C respectively.

***Keywords*:**

Meat spoilage; Pseudonomas putida; gene expression; profiling; biomarkers

1. Introduction

Meat represents one of the most important consumed food commodities worldwide. Food availability data indicate that overall meat consumption in developed countries is increasing, with the USA standing as the leading country in total meat consumption (Daniel *et al.,* 2011). The United States Census (The-United-States-Census-Bureau, 2012) estimates that world consumption of beef (including veal), pork and broiler (chicken) in 2010 was 56,544, 102,953 and 75,127 thousands of metric tons, respectively. This suggests that not only does the meat industry have to meet this increasing demand but it also has to adapt to seemingly different market demands. Consumers expect food products of upgraded functional and nutritional properties in conjunction with low processing interventions and fewer additives, and yet that they possess a long shelf life and guaranteed product safety (Nychas *et al.,* 2008). These changes in demand, as well as the introduction of new food products that broaden food choices in conjunction with the adoption of new technologies by the industry, have a direct influence on food loss rates in the overall meat supply chain (Buzby *et al.,* 2009).

Meat is described as spoiled when it is considered unacceptable by consumers due to physical and chemical changes that alter its sensory characteristics. As spoiled food is not necessarily unsafe, spoilage has not received the same attention as microbial changes that lead to foodborne illnesses. However, spoilage leads to important economic losses, food waste and loss of consumer confidence in the meat market. Therefore understanding the underlying mechanisms involved in meat spoilage is crucial for minimising its economic, social and environmental impacts. Although several attempts have been made over the past few years to associate specific metabolites with microbial spoilage in meat, none of them have been completely successful due to a poor understanding of the phenomena (Nychas *et al.*,

2007). Therefore, novel technologies are arising in order to overcome this lack of information.

Predominance of a certain group of microorganisms on meat depends on the characteristics of the meat product, the processing that meat may undergo and environmental storage conditions. The intrinsic characteristics of meat are favourable for the growth of most microorganisms. Gram-negative bacteria involved in meat spoilage are aerobes or facultative anaerobes, and represent the group with the greatest meat spoilage potential. In chilled meat stored aerobically, members of the genera *Pseudomonas, Acinetobacter, Psychrobacter* and *Moraxella* display the fastest growth rates (García-López *et al.,* 1998). However, even though small numbers of *Acinetobacter, Psychrobacter* and *Moraxella* appear in meat, members of these genera fail to effectively compete against *Pseudomonas* (Stanbridge & Davies, 1998).

Although *Acinetobacter* could compete for amino acids and lactic acid with *Pseudomonas*, its low oxygen affinity favours the dominance of *Pseudomonas* (Baumann, 1968). Therefore, *Pseudomonas* tends to dominate the microbial consortium under refrigeration and aerobic conditions.

The genus *Pseudomonas* comprises a group of gram-negative rods, motile and non-spore-forming which are mostly aerobic (Liao, 2006). Its classification has suffered modifications since the assortment made by Palleroni in 1973, in which the genus was subdivided into five rRNA similarity groups according to DNA-DNA hybridization studies. Despite these taxonomic changes, the most relevant species involved in meat spoilage are located in group I, including *P. aeruginosa, P. fragi, P. lundinensis, P. fluorescens, P. putida, P. chlororaphis, P. cichorii, P. viridiflava* and *P. syringae.* Of these species, *P. fragi* is considered the main component of the microbial association, with an incidence between 56.7% and 79.0% on spoiled meat, followed

by *P. lundinensis*, which is the second most common species responsible for meat spoilage. Due to the importance and spoilage potential of this genus, several studies have been conducted in broth and in model systems such as meat extracts and gel cassettes in order to monitor the chemical changes associated with metabolic attributes of *Pseudomonas* spp. (Nychas *et al.,* 2007).

The genomics, transcriptomics and metabolomics of spoilage compounds must be understood to effectively develop technologies that can prevent meat spoilage. Therefore, identifying the genes involved in the metabolic pathways that lead to spoilage compound production is crucial. Moreover, identifying stress response genes under different environmental conditions can help researchers increase their knowledge of how *Pseudomonas* adapts to current packaging and storage conditions. This could lead to the potential of finding optimum solutions to decrease its role in meat spoilage.

In the field of genomics, microarrays have already been implemented for the study of meat spoilage via the construction of a mixed species microarray for identifying spoilage bacilli in a wide variety of food products (Caspers *et al.,* 2011). This study aimed to detect *Bacillus* bacteria in a rapid and cost-effective approach since spoilage of food products is often caused by thermo-stable spores from the Bacillaceae family. Another example is the use of microarrays for the detection of bacterial species that play a significant role in beer spoilage (Weber *et al.,* 2008). In this case, the use of microarrays allowed the distinction between bacteria with the potential to grow in beer and unviable bacteria.

This article presents a novel approach to identify spoilage biomarker genes by identifying genes whose expression was significantly different between different glucose concentrations and temperature conditions to obtain a better understanding

of the regulation of spoilage in meat products at 30°C and 10°C at a decreasing glucose concentration.

2. Materials and Methods

A full account of the experimental procedures can be found in the accompanying supplementary materials.

2.1 Growth experiments

P. putida KT2440 cells were grown in Luria Bertani broth (LB) at 10°C and 30°C in the presence of glucose. During the experiment growth kinetics was determined and the concentration of glucose was monitored. In brief, samples were taken at different time points to assess gene expression profiles at different glucose concentrations (Table 1). Time points were selected in such a way as to provide similar glucose concentrations in the experiments performed at different temperatures. The glucose concentration was determined at the different growth stages using the D-Glucose/D-Fructose determination kit (R-Biopharm, Germany). At these time points, cells were immediately cooled, collected by centrifugation and subsequently stored at -80°C for RNA isolation.

2.2 RNA isolation and cDNA synthesis

Total RNA was prepared based on the Progenika protocol and the article of (Yuste *et al.,* 2006). For cDNA synthesis 20 µg total RNA in a maximum volume of 18 µl was mixed with 3 µl random hexamer primers (3 µg/µl, Life Technologies, UK), incubated at 70°C for 5 minutes and subsequently placed on ice for at least 1 minute. Purification of the cDNA was carried out using S.N.A.P. columns (Invitrogen, UK) according to the manufacturer's instructions. Full details are available in S1.2 and S1.3 respectively of the supplementary materials.

2.3 Fluorescent labelling

Cy3™ and Cy5™ dyes (Amersham Pharmacia, UK) were each dissolved in 5 µl dimethylsulfoxide (included in the "SuperScript Indirect cDNA Labeling" system (Invitrogen, UK)). To 5 µl cDNA in coupling buffer either 2.5 µl Cy3-dye or 2.5 µl Cy5-dye was added and 2.5 µl Sigma water. Full details are available in S1.4 of the supplementary materials.

2.4 Microarray hybridization and washing

For the transcriptomics experiments the *P. pudita* whole genome microarray of Progenika Biopharma S.A. (Spain) was used. This microarray (*P. putida* KT2440 Genome Microarray) represents 5,539 *P. putida* open reading frames (ORFs). The hybridization and washing are described in S1.5 of the supplementary materials.

2.5 Experimental analysis

P. putida KT2440 cells were collected during growth experiments by centrifugation and immediately stored at -80ºC for RNA isolation. mRNA was extracted from the samples, purified and reverse transcribed into cDNA. Control samples were prepared by pooling the extracted RNA from all the samples of a given experiment. Controls were labelled with Cy5 while samples were dyed with Cy3. After labelling, samples and controls were applied in equal proportions (1:1 mix of Cy3 labelled sample and Cy5 labelled pooled RNA) onto the microarray slides to enable the hybridization reaction between samples and probes. Once hybridization had taken place, microarray slides were washed and scanned. The resulting image was the starting point for the data analysis. The commercial microarray platform used was the *P. putida* Genome Oligonucleotide Array from Progenika Biopharma, SA (Spain), which consists of single stranded oligonucleotides printed in a repeating spot pattern onto γ-aminosylane treated microscope slides. This array was generated from a *P. pudita* oligonucleotide collection, which represents 5,539 *P. pudita* open reading frames (ORFs), with each ORF represented by a 50-mer length oligonucleotide. As a result,

10 microarrays were performed, with each microarray representing a different temperature with a different glucose concentration point for each experiment.

2.6 Data analysis

Pixel intensities originating from the scanners were converted into numeric probe-level datasets. ArrayVision software (by Biocompare, USA) was used to determine which pixels corresponded to the fluorescence emitted by the hybridized samples (foreground) or to the glass slide (background). The background correction, data normalisation and statistical analysis were performed using R in conjunction with Bioconductor and the Limma package. R is an open source environment and programming language for statistical computing and visualisation (http://www.r-project.org). Bioconductor (http://www.bioconductor.org) is a collection of R packages that provide tools specialized in the analysis of genomic data. The Limma package (Smyth, 2005) in Bioconductor offers a wide collection of pre-processing and statistical analysis methods for two-channel arrays. Therefore, this package was used for analysis.

The analysis of the microarrays was performed using a “two-channel normalisation approach”. This approach was selected as log-ratios were considered to be more stable than absolute intensities across slides.

A time course analysis based on the Limma approach (Smyth, 2005) was performed on the data within each set, considering the points of decreasing glucose concentration at 30ºC and 10ºC. The analysis was conducted by fitting a linear model to the expression data of all the arrays in one set for each gene, using the function “lmFit” from the Limma package. For this purpose, a design matrix was created specifying the different RNA sources that had been hybridized to the arrays and the common references used in each set (e.g. “Pool1”). The two replicates for each gene were also specified when fitting the linear model by the function

“duplicateCorrelation”. Subsequently, a contrast matrix was determined, allowing the coefficients outlined in the design matrix to become contrasts of interest. Each contrast corresponds to a comparison of interest between the RNA sources. The contrast and fitted matrix were used by “contrasts.fit” function in Limma for computing fold changes and t-statistics for the contrasts of interest. Moderated t-statistics and log-odds of differential expression were computed in each contrast for each gene using a simple empirical Bayes model. This model was used to decrease the standard errors into a common value and increase the degrees of freedom of individual variances (Smyth, 2004). The function used was “eBayes”, which allowed genes to be ranked in order of evidence of differential expression by computing a moderated F-statistic. The F-statistics tested if any of the contrasts were non-zero for a gene, this is, if a gene was differentially expressed on any contrast.

The results of the contrasts were corrected for multiple testing using the Benjamini and Hochberg´s (BH) method to control false discovery rate (Benjamini & Hochberg, 1995). The significance p-value was set at 5%. This function provides average log intensities, log fold changes, moderated t-statistics, t-statistics p-values, adjusted p-values after multiple testing, F-statistics and F-statistics p-values. Once genes were classified as differentially or non-differentially expressed for the contrasts of interest, this information was used for selecting spoilage biomarkers and for monitoring genes which had already been identified to play a role in spoilage.

The Limma “write.fit” function was used to select biomarkers, which had either incremental or decreasing differential expression when glucose concentration was decreased. A list of potential biomarkers was computed for each temperature (10ºC and 30ºC).

3. Results

Two gene expression microarray studies were performed using *P. putida* KT2440. Cells were grown in the presence of glucose at different temperatures of 10ºC and 30ºC, with the highest temperature being representative of spoilage. Growth curves and glucose consumption were determined for *P. putida* KT2440 in LB broth (Figure 1). Scatterplots were used to compare raw intensities between the red channel (X-axis) and green channel (Y-axis) for raw and normalised data (Figure 2); the results followed the expected pattern of intensities distributed symmetrically to a straight line of slope 1 and an intercept of 0, with positive values for the spots with greater red than green intensities (R>G) and negative values for spots with higher green intensities (R<G). Data were treated for background correction using the method "normexp" with an offset of 50. This method was preferred to the traditional subtraction method after a visual assessment using scatterplots for the corrected values and to avoid negative values, which would lead to missing log-ratios (Figure 2c and 2d).

MA-plots (where 'M' captures the ratio between the red and green intensities, and 'A' captures the average of the red and green intensities) identified "print tip loess" and "aquantile" methods as the best transformations for within and between normalisation. As shown in Figure 3a, the experiment presented considerable variation between both arrays and channels. After normalisation of the M-values using the print-tip-loess method, the distribution of the green and red channels for each array became essentially similar, although variation between arrays was still notable (Figure 3b). After the application of "aquantile" normalisation to the A-values, the distributions became similar across channels and arrays (Figure 3c). Differentially expressed genes were identified for each contrast of interest. An average of 4,114 genes showed significant differential expression in at least one of the contrasts. Genes that could be used as potential biomarkers for spoilage were selected at each

temperature (30ºC and 10ºC). The criterion set for biomarker selection was either an increase or a decrease in gene expression for the initial glucose consumption period and for the interphase between intermediate glucose concentrations to glucose depletion (i.e. a systematic decrease or increase in gene expression when glucose concentration was reduced). For this purpose, genes were classified as overexpressed, repressed or not differentially expressed by means of the F-statistic. The number of biomarkers identified in each treatment is summarised in Table 2. Figure 4 shows two heatmap clusters of the top 100 most differentially expressed genes under both 30ºC (Figure 4a) and 10ºC (Figure 4b) across different glucose concentrations; where genes are clustered according to similar behaviour in expression levels. Spoilage biomarkers that expressed consistently (either continuously increasing or decreasing their expression) at different temperatures and decreasing glucose concentrations were then selected for further analysis.

The top 100 most differentially expressed genes shown in Figure 4 were then shortlisted further into two lists of 20 potential biomarkers for each temperature (30ºC and 10ºC), where a further analysis was conducted using STRING (http://string-db.org/) and InterPro (www.ebi.ac.uk/interpro/) to identify the corresponding proteins and their functional domains (Table 3 and 4). This list could be applied to monitor the degree of glucose consumption in the meat muscle both at 30ºC and 10ºC, giving an indication of the degree of spoilage associated with this decrease in glucose concentration.

4. Discussion

The expression pattern revealed differences in the level of expression between the two temperatures (10ºC and 30ºC). The fact that these differences were higher at initial glucose concentrations, and considerably lower when approximating glucose depletion, suggest that glucose concentration plays a more important role than temperature in determining bacterial gene expression (Nychas *et al.,* 1988). At

optimal glucose concentrations the effects of temperature were noticeable. However, when glucose was close to depletion, the effect of temperature was less relevant and the lack of glucose became the limiting factor in determining gene expression. The importance of the role of low molecular compounds, and especially of glucose, in meat spoilage has been highlighted (Nychas *et al.,* 1988; Nychas *et al.,* 2008).

It has thus been stipulated that the concentration of glucose, lactic acid and certain amino acids affect the type and rate of spoilage (Lambropoulou *et al.,* 1996). The criterion used for biomarker selection for ideal spoilage indicators also satisfied the specific characteristics previously defined (Nychas *et al.,* 2007; Nychas *et al.,* 2008). This suggests that optimum biomarkers should: (i) be absent or at low levels in unspoiled fresh muscle tissue; (ii) increase with storage time; (iii) be produced by the dominating microbes during spoilage; and (iv) have a good correlation with sensory testing (Nychas *et al.,* 2007; Nychas *et al.,* 2007; Nychas *et al.,* 2008).

As the preferred energy source for *Pseudomonas* is D-glucose, which has been found to be a precursor of many off-odour compounds during storage (Nychas *et al.,* 1988; *Ercolini et al.,* 2010; Casaburi *et al.,* 2014), our study contemplated the identification of potential biomarkers which were significantly differentially expressed (either up or down regulated) for continuous glucose decreasing concentrations. The decrease therefore in glucose concentration through time was considered to be representative of glucose consumption in meat muscle that supports the growth of *Pseudomonas* during storage. This criterion aimed to identify genes activated or repressed during the initial glucose consumption phase and the stage equivalent to glucose depletion. This last phase was considered particularly interesting as it represented the stage when spoilage is known to occur, in which the available glucose does not meet microbial glucose demand, and bacteria must switch to amino acids as the new energy source (García-López *et al.,* 1998; Nychas *et al.,* 2007).

Monitoring the expression patterns of spoilage genes aims to increase the understanding of spoilage regulation. This is necessary for developing accurate storage and packaging technologies. Interestingly, spoilage genes showed more differential expression activity after a decrease in glucose concentration at 10ºC than at 30ºC. These results were surprising; as abuse temperatures like 30ºC constitute more optimal conditions for spoilage. However, a deeper examination of the expression patterns when reaching glucose depletion revealed that at 30ºC, 35.0% of the differential expression corresponded to up-regulated genes, whereas at 10ºC only 21.7% of the genes were overexpressed. These findings are in agreement with higher temperatures being more favourable for spoilage, as a higher percentage of spoilage genes were found to be up-regulated at 30ºC than at 10ºC. Therefore, the overall higher differential expression found at 10ºC corresponds to genes being more repressed than at 30ºC.

The obtained results also support that it is precisely at the final stage of glucose consumption where spoilage is more pronounced (García-López *et al.,* 1998). This is because the total number of activated genes at the stage of glucose depletion was considerably higher than the number of repressed genes at 30ºC. However, at 10ºC this pattern was not followed, as the number of activated and repressed genes was similar. This could be the result of the effect of low temperature, which would be delaying the spoilage process. Furthermore, genes involved in the production of malodorous end-products appeared to be repressed under low temperatures, whereas genes participating in ammonia production showed a more varied pattern.

In order to gain more supporting evidence about this pattern in expression profiling, an in-depth functional annotation analysis was performed on the top differentially expressed genes at both 30ºC and 10ºC temperatures; through the annotation of these genes using Gene Ontology (GO) terms, protein signature databases (InterProScan), followed by mapping of the retrieved enzyme GO terms to their

corresponding KEGG pathways. At 10ºC, a total of 66 hits of the top DE genes GO terms are related to the metabolic and cellular process. Closer examination of the genes group that shows continuous increase of expression [Figure 3.a (B)] revealed that a large proportion of this group are involved in metabolic or catabolic activity. For instance, PP3139, Glycoside hydrolase, is involved in synthesis and breakage of glycosidic bonds; while PP0035 is a family protein involved in the polysaccharide biosynthetic process. At 30ºC, a different pattern was observed, where 39 hits are related to metabolic and cellular process. The gene group showing a steady increase in expression [Figure 3.a(B)] are mostly related to main cellular function, such as PP1131 catalyses the transposition of transposable elements of transposons, and PP1028 involved in the transcription regulation.

Another interesting finding was that several of these spoilage genes had the appropriate pattern to become potential spoilage biomarkers. For example, at 30ºC, “ubiA” (4-hydroxybenzoate octaprenyltransferase) catalyses the decarboxylation of 3-octaprenyl-4-hydroxy benzoate to 2-octaprenylphenol, and “PP4030” (Enoyl-CoA hydratase) is involved in the degradation of even *cis*-unsaturated fatty acids, therefore they can be used as indicators for the production of malodourous compounds.

It is also noted that most of the differentially expressed genes at 30°C are associated with fundamental cellular activities, such as transcription regulation, further studies of such proteins using the “String” database revealed direct association with the spoilage process. For example, “PP5337” is found to be directly linked with “aspA” (aspartate ammonia-lyase) and “purE (phosphoribosylaminoimidazole regulator), indicating that it can also be associated with the malodours production (Figure 5a). Similarly, at 10°C, “aruF” (arginine N-succinyltransferase, alpha subunit) is found to be linked to a series of genes involved with the production of ammonia such as “astb” (succinylarginine dihydrolase), which catalyzes the hydrolysis of N_2-succinylarginine

into N_2-succinylornithine, ammonia and CO_2 (Figure 5b). soxA (Sarcosine oxidase) is responsible for the demethylation of sarcosine to yield glycine, and has shown a constant increase in expression levels over the course of the experiment (Figure 5c). On the other hand, these two lists include a series of hypothetical proteins. Further studies of these unknown genes are required to identify the suitability of using these genes as spoilage biomarkers.

5. Conclusions

The aim of this study was to analyse the data from microarray experiments performed with *P. putida* KT2440 to obtain a better understanding of the regulation of spoilage in meat products at 10°C and 30°C at a decreasing glucose concentration. As a result, a list of the *P. pudita* genes differentially expressed between the two temperatures was generated. The fact that there is a lack of a sound definition for spoilage makes the task of identifying spoilage indicators difficult (Nychas *et al.,* 2008). This is because spoilage is mainly due to the growth of bacteria in the food substrate. As a result, genes which showed differential expression were found to be participating in a wide range of metabolic pathways, some related directly to the production of metabolites that have been correlated with spoilage (Casaburi *et al.,* 2014), whilst most of them were involved in reactions from the basal metabolism that maintain the growth of the bacteria.

To our knowledge we are the first to reveal the role of specific genes from bacteria that contribute significantly to meat spoilage. The influence of parameters such as the glucose concentration and the temperature storage of meat were also evident.

As a future recommendation, the results from this study should be validated by other high throughout techniques such as *de novo* RNA-Seq analysis for *P. putida* species, as well as other specific strains that also have been found to be associated with the ecological phenomenon of spoilage. This will allow us to gain a greater depth of

understanding of the genome, especially for the genes with currently unknown function.

The type and the rate of spoilage can only be controlled by a thorough understanding of the metabolic pathways that lead to spoilage. This can only be achieved by carrying out further research. Therefore additional experiments in the line of this study could help increase the knowledge on spoilage of food products.

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Table 1. Experimental set up for growth experiments.

		Time points for similar glucose concentration (h)				
Set No.	**Temperature (ºC)**	**1**	**2**	**3**	**4**	**5**
1	30	8	9	11	13	14
2	10	50	66	68	71	75

Time points represent (1) initial glucose concentration, (2) and (3) intermediate glucose concentrations, (4) and (5) glucose depletion.

Table 2. Total number of genes selected as potential biomarkers in each treatment for being constantly overexpressed or repressed at decreasing glucose concentrations.

		Number of potential biomarkers	
Set	**Temperature**	**Overexpression**	**Repression**
1	30°C	430	309
2	10°C	568	701

Table 3: The top 20 most differentially expressed genes at 30ºC showing the predicted protein information.

Gene ID	Protein information	Protein function
PP4444	transposase family protein	Essential for efficient DNA transposition (InterPro: IPR021896)
PP3088	Hypothetical protein	Unknown
PP4956	Acetyl transferase	Responsible for transferring organic acids, typically fatty acids, onto hydroxyl groups of membrane-embedded targets. (InterPro: IPR024194)
aer-1	aerotaxis receptor Aer-1	Responsible for mediating chemotaxis to diverse signals, responding to changes in the concentration of attractants and repellents in the environment by altering swimming behaviour [PMID: 16359703] (InterPro: IPR004090).
fliK	Flagellar hook-length control protein F	This is the C terminal domain of FliK. FliK controls the length of the flagella hook by directly measuring the hook length as a molecular ruler (InterPro: IPR021136)
PP4030	enoyl-CoA hydratase	Enoyl-CoA hydratase/isomerase family protein. In Arabidopsis, enoyl-CoA hydratase 2 is involved in the degradation of even cis-unsaturated fatty acids (InterPro: IPR027090)
PP0580	acyl dehydratase MaoC	The C terminus of the MaoC protein is found to share similarity with a wide variety of enzymes. This domain is found in parts of two enzymes that have been assigned dehydratase activity (InterPro: IPR002539)
PP4314	Hypothetical protein	Unknown
PP0102	Hypothetical protein	Unknown
ubiA	4-hydroxybenzoate octaprenyltransferase	Catalyses the decarboxylation of 3-octaprenyl-4-hydroxy benzoate to 2-octaprenylphenol
PP1729	Hypothetical protein	Unknown
PP5165	NLPA lipoprotein	D-methionine binding lipoprotein MetQ is the main member of this group. This protein is a component of a D-methionine permease, a binding protein-dependent, ATP-driven transport system (InterPro: IPR004872)
cheW	purine-binding chemotaxis protein CheW	Interacts with the methyl accepting chemotaxis proteins (MCPs) and relays signals to CheY, which affects flagella rotation.
PP0098	Hypothetical protein	Unknown
PP2210	LysR family transcriptional regulator	InterPro:IPR017724. These are transcriptional regulators of the LysR family which contain a helix-turn-helix (HTH) domain (IPR000847) and a periplasmic substrate-binding protein-like domain (IPR005119).
PP3756	TetR family transcriptional regulator	Members of this family are transcriptional regulators belonging to the TetR-family (InterPro: IPR023851)
PP4924	serine protease	InterPRo: IPR022241. This domain family is found in eukaryotes, and is approximately 210 amino acids in length. The family is found in association with PF01694. Rhomboid is a seven-transmembrane spanning protein that resides in the Golgi and acts as a serine protease to cleave Spitz.
PP1328	cell division protein MraZ	These proteins may be DNA-binding transcription factors (InterPro: IPR003444)
PP4605	AraC family transcriptional regulator	Transcription regulator, AraC, predicted (InterPro: IPR016981)

Table 4. The top 20 most differentially expressed genes at 10ºC showing the predicted protein information.

Gene ID	Protein Information	Protein Function
PP3541	Mg^{2+} transporter	Protein belongs to the family MgtC/SapB/SrpB transporter (InterPro: IPR003416). The MgtC protein is found in an operon with the Mg^{2+} transporter protein MgtB (InterPro: IPR003416).
PP0023	hypothetical protein	Unknown
PP1680	alpha-ribazole-5'-phosphate phosphatase	Catalyses reactions involving the transfer of phospho groups between the three carbon atoms of phosphoglycerate [PMID: 2847721, PMID: 2831102, PMID: 10958932]. (InterPro IPR013078).
PP2534	LysR family transcriptional regulator	These are transcriptional regulators of the LysR family which contain a helix-turn-helix (HTH) domain (IPR000847) and a periplasmic substrate-binding protein-like domain (IPR005119) (InterPro: IPR017724).
PP3563	hypothetical protein	Unknown
PP3861	phage FluMu protein gp46	The characteristics of the protein distribution suggest prophage matches in addition to the phage matches (Interpro: IPR018755).
PP2188	tRNA--hydroxylase	This family consists of several bacterial tRNA-(MSIO[6]A)-hydroxylase (MiaE) proteins (InternPro: IPR010386).
PP0050	hypothetical protein	
argS	arginyl-tRNA synthetase	Catalyses the attachment of an amino acid to its cognate transfer RNA molecule in a highly specific two-step reaction (InterPro: IPR015945)
PP2427	hypothetical protein	Unknown
PP3088	hypothetical protein	Unknown
PP1728	hypothetical protein	Unknown
PP3027	hypothetical protein	Unknown
PP0640	hypothetical protein	Unknown
PP4903	ribosome-associated GTPase	NA
PP2235	hypothetical protein	Unknown
PP2867	pyridine nucleotide-disulfide oxidoreductase	The pyridine nucleotide-disulphide reductases (PNDR) use the isoalloxazine ring of FAD to shuttle reducing equivalents from NAD(P)H to a Cys residue that is usually a part of a redox-active disulphide bridge. (InterPro: IPR000103)
PP4345	GntR family transcriptional regulator	Involved with the regulation of transcription. Many bacterial transcription regulation proteins bind DNA through a helix-turn-helix (HTH) motif, which can be classified into subfamilies on the basis of sequence similarities. The HTH GntR family has many members distributed among diverse bacterial groups that regulate various biological processes (InterPro: IPR000524)
PP4444	transposasetransposase family protein	Essential for efficient DNA transposition (interPro: IPR021896)
PP4030	enoyl-CoA hydratase	This entry represents enoyl-CoA hydratase 2 (EC:4.2.1.119). It contains a MaoC-like domain. In Arabidopsis, enoyl-CoA hydratase 2 is involved in the degradation of even cis-unsaturated fatty acids (InterPro: IPR027090)

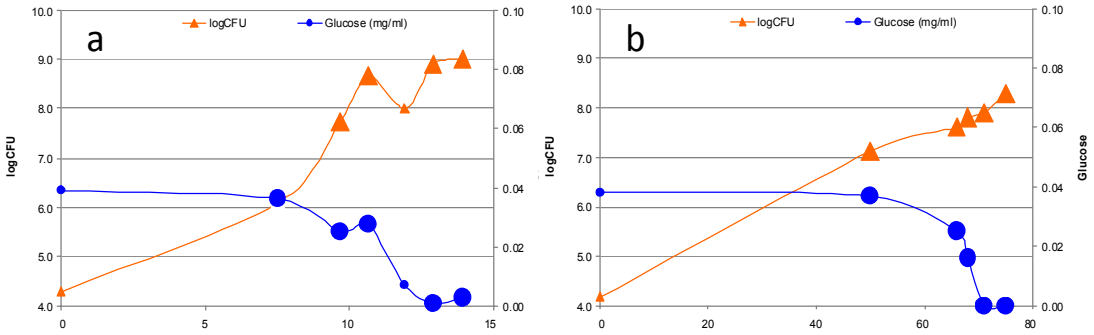


Figure 1. Growth of *P. putida* strain KT2440 (▲) and glucose consumption (●) in LB broth at (a) 30°C and (b) 10°C

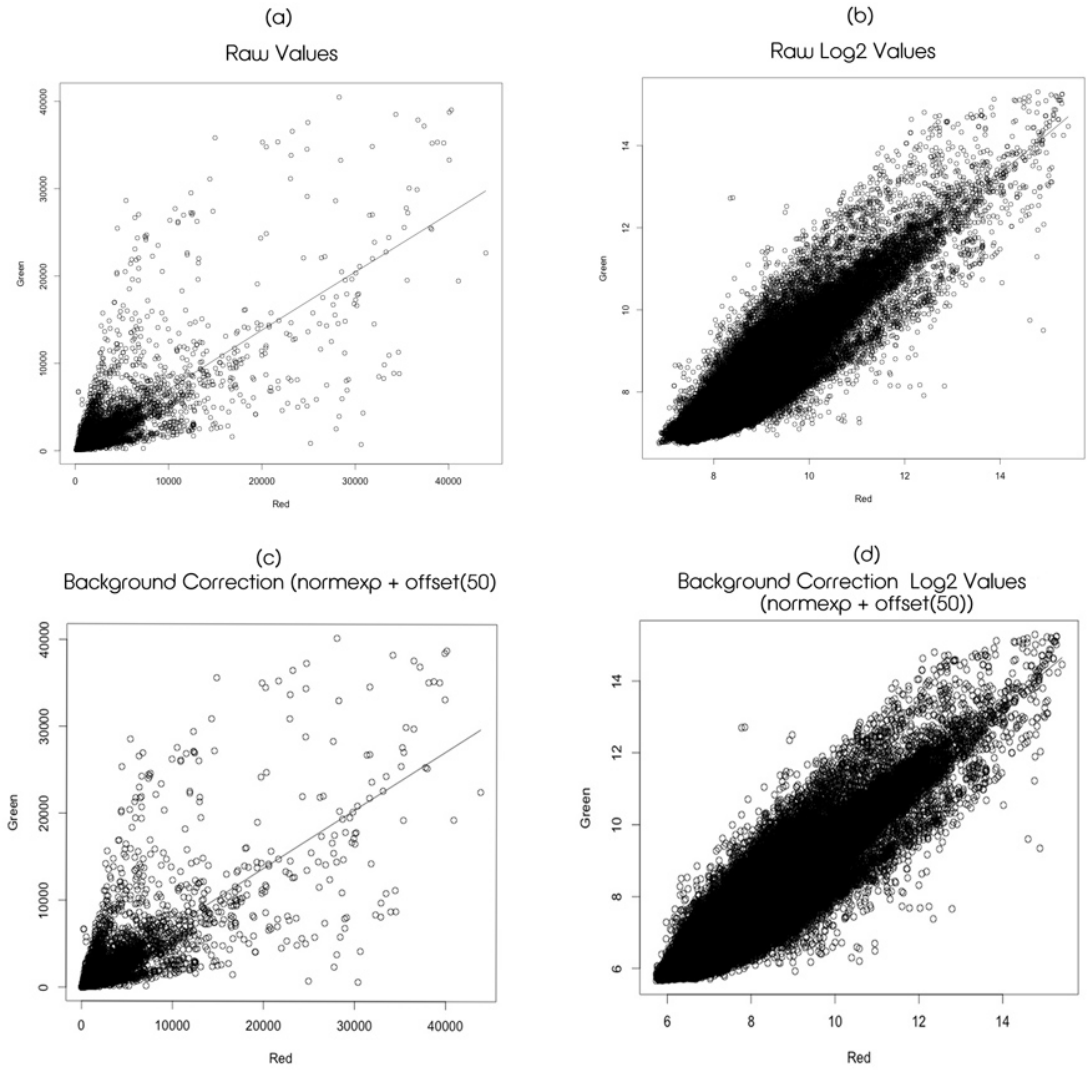


Figure 2. Relation between red (control – X-axis) and green (sample – Y-axis) channel intensities. Scatterplot (a) shows the distribution of raw intensities, with the x-axis representing the red intensity, and the y-axis representing the green intensity. Scatterplot (b) shows the effect of transforming the raw intensities of set 1 into log2 intensities. Scatterplot (c) shows the effect of background correction (normexp + offset 50) for raw and (d) log2 values.

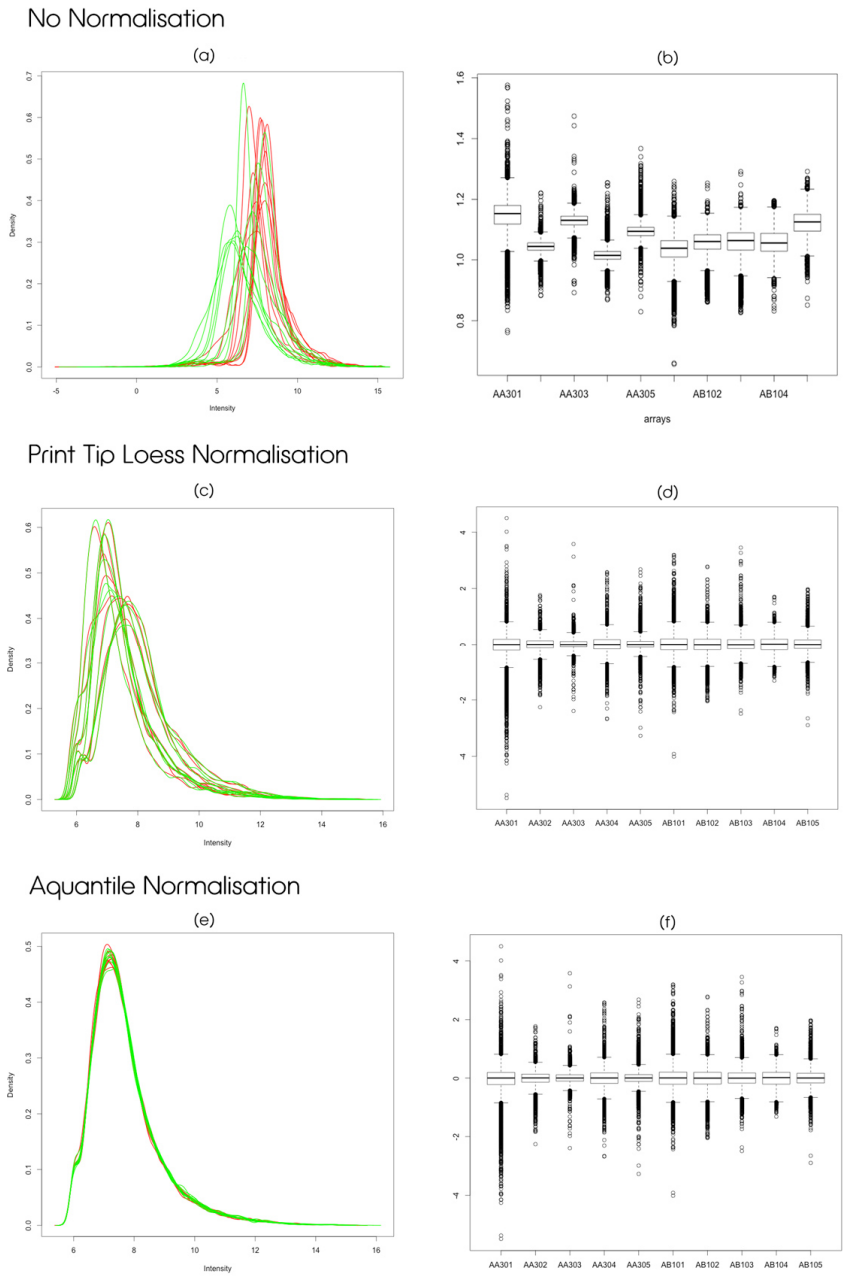


Figure 3. Density plots for each step of the normalisation process: (a) red (R) and green (G) intensities without normalisation correction, (c) R and G channels after print-tip-loess normalisation, and (e) distribution of R and G intensities after aquantile normalisation; (b) distribution of M-values before normalisation; (d) after within array print tip loess normalisation; and (f) after aquantile within array normalisation.

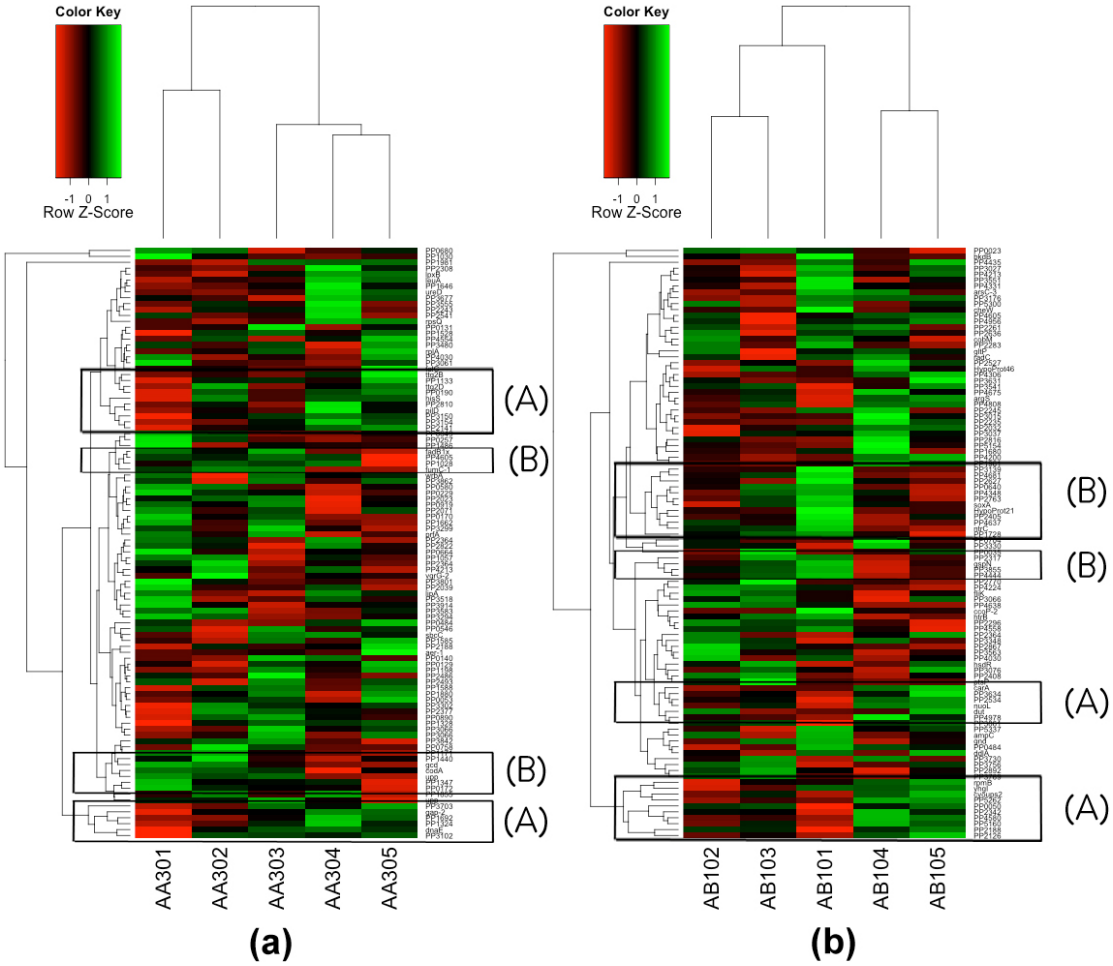


Figure 4. Heatmap clusters showing the top 100 most differentially expressed genes under 30°C (a) and 10°C (b). Group (A) shows genes that are continuously decreasing in their expressions, while Group (B) shows genes that are continuously increasing in their expression.

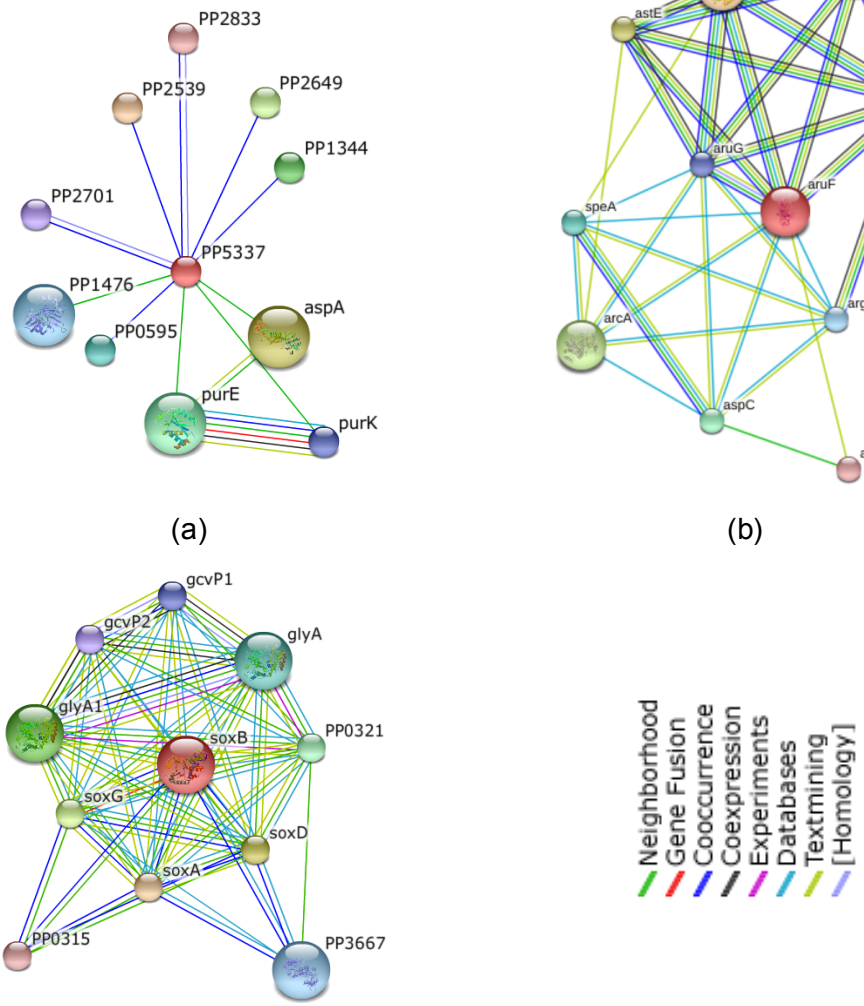


Figure 5. Summary network showing protein-protein interactions as identified by the STRING database (http://string-db.org/). (a) PP5337 (LysR family transcriptional regulator), (b) aruF (arginine N-succinyltransferase, alpha subunit) and (c) soxA (Sarcosine oxidase).



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