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DNA microarray analysis of the cyanotroph *Pseudomonas pseudoalcaligenes* CECT5344 in response to nitrogen starvation, cyanide and a jewelry wastewater

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ABSTRACT

Pseudomonas pseudoalcaligenes CECT5344 is an alkaliphilic bacterium that can use cyanide as nitrogen source for growth, becoming a suitable candidate to be applied in biological treatment of cyanide-containing wastewaters. The assessment of the whole genome sequence of the strain CECT5344 has allowed the generation of DNA microarrays to analyze the response to different nitrogen sources. The mRNA of *P. pseudoalcaligenes* CECT5344 cells grown under nitrogen limiting conditions showed considerable changes when compared against the transcripts from cells grown with ammonium; up-regulated genes were, among others, the *glnK* gene encoding the nitrogen regulatory protein PII, the two-component *ntrBC* system involved in global nitrogen regulation, and the ammonium transporter-encoding *amtB* gene. The protein coding transcripts of *P. pseudoalcaligenes* CECT5344 cells grown with sodium cyanide or an industrial jewelry wastewater that contains high concentration of cyanide and metals like iron, copper and zinc, were also compared against the transcripts of cells grown with ammonium as nitrogen source. This analysis revealed the induction by cyanide and the cyanide-rich wastewater of four nitrilase-encoding genes, including the *nitC* gene that is essential for cyanide assimilation, the cyanase *cynS* gene involved in cyanate assimilation, the *cioAB* genes required for the cyanide-insensitive respiration, and the *ahpC* gene coding for an alkyl-hydroperoxide reductase that could be related with iron homeostasis and oxidative stress. The *nitC* and *cynS* genes were also induced in cells grown under nitrogen starvation conditions.

In cells grown with the jewelry wastewater, a malate quinone:oxidoreductase *mqoB* gene and several genes coding for metal extrusion systems were specifically induced.

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1. Introduction

Large amounts of wastewaters with cyanide are produced by industrial activities like mining and metal processing, electroplating, goal coking and nitrile polymers synthesis (Kumar et al., 2011). Mining uses cyanide for extracting gold, silver and other metals from ores, processes that are considered the largest producers of cyanide-containing wastewaters. These liquid residues also contain heavy metals and metalloids that increase their toxicity. In these industrial wastes cyanide can be found not only as free ion (CN^-) but also as metal–cyanide complexes, since cyanide strongly binds to metals. For this reason, cyanide is toxic for most living organisms, inhibiting essential metalloenzymes like the cytochrome *c* oxidase, thus blocking aerobic respiration (Jünemann, 1997; Quesada et al., 2007).

The jewelry industry also produces large amounts of a liquid waste that contains high concentrations of cyanide and metals. Thus, the residue generated by the jewelry industry of Córdoba (Spain) displays an extremely alkaline pH (>13) and contains about 40 g l^{-1} cyanide (*ca.* 1.5 M) and several metals like copper, iron and zinc. Metals are required at very low concentrations by microorganisms, but metal accumulation at high concentrations cause cell toxicity, which is mainly related with their capacity to interact with cellular thiol components such as glutathione (Nies, 2003). Different types of metal efflux systems have been found in bacte-

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ria. *Cupriavidus metallidurans* CH34, a strain able to growth under high metal concentrations, contains about 40 systems involved in metal detoxification, including P-type ATPases for zinc and copper extrusion, RND (resistance-nodulation-cell division) proteins for zinc, copper, cadmium, cobalt, nickel and silver extrusion, and CDF (cation diffusion facilitator) systems for cobalt, cadmium, nickel and iron extrusion (Nies, 2003).

In addition to the physico-chemical treatments applied to remove cyanide from industrial residues, biological technologies for detoxification of free cyanide and some metal–cyanide complexes have been described (Akcil and Mudder, 2003; Dash et al., 2009).

Pseudomonas pseudoalcaligenes CECT5344 is an autochthonous bacterium that was isolated from the Guadalquivir River (Córdoba, Spain) by enrichment cultivation with cyanide (Luque-Almagro et al., 2005a,b). This bacterial strain uses free (ion) cyanide, metal–cyanide complexes like nitroferricyanide (nitroprusside), and cyano-derivatives like cyanate or several organic cyanides (nitriles) as the sole nitrogen source (Luque-Almagro et al., 2005a,b; Estepa et al., 2012). This bacterium is able to grow in batch reactor with sodium cyanide as the sole nitrogen source under alkaline conditions, avoiding cyanhydric acid volatilization (Huertas et al., 2010). The ability of the strain CECT5344 to grow with the metals- and cyanide-containing jewelry wastewater was also demonstrated (Luque-Almagro et al., 2007). All these characteristics make this bacterial strain a suitable candidate to be applied in bioremediation of industrial cyanide-containing residues.

Cyanotrophic microorganisms display different cyanide degradation/detoxification pathways, including hydrolytic, oxidative and substitution/addition reactions (Gupta et al., 2010; Luque-Almagro et al., 2011a). The cyanide degradation pathway of *P. pseudoalcaligenes* CECT5344 includes a cytoplasmic malate:quinone oxidoreductase that converts L-malate into oxaloacetate, which reacts chemically with cyanide to produce a cyanohydrin (nitrile) that is transformed into its corresponding carboxylic acid and ammonium by the nitrilase *NitC* (Luque-Almagro et al., 2011c; Estepa et al., 2012).

Recently, the whole genome sequence of *P. pseudoalcaligenes* CECT5344 has been elucidated (Luque-Almagro et al., 2013; Wibberg et al., 2014), which is useful to perform a global transcriptomic analysis. Over the past decades, studies on cyanotrophic microorganisms have been focused on specific genes and/or proteins required for cyanide degradation. This study describes the first global analysis of a cyanide-assimilating bacterial strain, *P. pseudoalcaligenes* CECT5344, in response to nitrogen starvation, sodium cyanide and cyanide- and metal-containing jewelry wastewater by transcriptional profiling using DNA microarrays.

2. Materials and methods

2.1. Growth of P. pseudoalcaligenes CECT5344

P. pseudoalcaligenes cells were grown in minimal medium M9 (Sambrook and Russel, 2001) containing 50 mM sodium acetate as carbon source and 2 mM ammonium chloride as nitrogen source. The pH of the media was adjusted to 9.5. The strain CECT5344 was cultured in 100 ml flasks filled with 25 ml of media, at 30 °C and continuous agitation at 220 rpm on a shaker. After 24 h, when ammonium was exhausted, three different nitrogen sources were added to different cultures: sodium cyanide, cyanide-containing jewelry wastewater or ammonium chloride (each at 2 mM concentration). 10 ml-aliquots were harvested by centrifugation at 4 °C and 4000 × *g* for 5 min when cultures reached the mid-exponential growth phase and the remaining nitrogen source in the media was about 30–50% (Fig. S1, Supplementary material). An additional culture was set up with a low amount of ammonium chloride (0.5 mM) and cells were harvested under the above described conditions 15 min after ammonium was consumed (nitrogen starvation condition). Pellets were kept at −80 °C until use and four independent biological replicates for each nitrogen source were carried out. The Dps mutant strain of *P. pseudoalcaligenes* CECT5344 was also grown in 100 ml flasks filled with 25 ml of media with ammonium, sodium cyanide or jewelry wastewater (2 mM concentration). When indicated, 2 mM $FeCl_3$ or 2 mM $CuCl_2$ were added to the media. The wild-type was cultured in the presence of nalidixic acid 10 (μg/ml) and the Dps mutant strain was cultured with both nalidixic acid 10 (μg/ml) and kanamycin (25 μg/ml).

2.2. Total RNA isolation

The frozen aliquots of *P. pseudoalcaligenes* CECT5344 cell material were suspended in 500 μl lysozyme-containing buffer and immediately disrupted using the RNAeasy Midi Kit (Qiagen) following instructions of the manufactures. DNase incubation was carried out in the column with RNase-free DNase set (Qiagen) and, when required, an additional post-column treatment was applied with DNase I (Ambion). Quality and quantity of the total RNAs were checked with Bioanalyzer (Agilent) and ND1000 spectrophotometer (Nanodrop 1000, Agilent Technologies-Wilmington, DE, USA). All samples showed an integrity number (RIN) higher than 7.

2.3. DNA microarray design and analysis

Oligonucleotides (60 bp) were designed for 4434 open reading frames of *P. pseudoalcaligenes* CECT5344 (Wibberg et al., 2014) with the software eArray (Agilent Technologies). Seven probes were designed for each gene.

RNA quality was assessed using a Tape Station (Agilent Technologies). RNA concentration and dye incorporation was measured using a UV–vis spectrophotometer (Nanodrop 1000, Agilent Technologies). Total RNA from each sample was reverse transcribed, labeled and hybridized to custom microarray, according to the protocol Two-Color Microarray-Based Prokaryote Analysis 1.4, Fair Play III Labeling, from Agilent Technologies. Briefly, reverse transcription was performed with AffinityScript^{TM} HC RT (Agilent Technologies), cDNA was labeled with a chemical coupling method, and hybridized following protocol of the manufacturers. Dye swaps (Cy3 and Cy5) were performed from each sample. Microarray chips were washed and immediately scanned by using a DNA Microarray Scanner (Model G2505C, Agilent Technologies).

Estimation of the RNAm from different samples was carried out previously they were sent to Bioarrays, S.L (Alicante, Spain) for microarrays construction and consisted of q-PCR analysis of all RNAm samples by using the three housekeeping genes, the α-subunit of the DNA polymerase III (BN5_2819), the ϵ-subunit of the DNA polymerase III (BN5_2215) and a 16S rRNA methyltransferase (BN5_0873). All RNA samples showed similar expression levels for all these genes. Normalization of the two color arrays was carried out by intensity normalization with the MA plot.

Gene expression analysis was carried out by using the Feature Extraction Software (v. 10.7) available from Agilent, using the default variables. Outlier features on the arrays were flagged by using the same software package. Data analysis was performed with a Bioconductor package, under R environment. Data preprocessing and differential expression analysis was performed with the Limma package (htpp:/www.bioconductor.org/), and the latest gene annotations available were used. Raw feature intensities were background-corrected with Norme xp background correction algorithm. Within-array normalization was carried out by using Aquantile normalization and spatial and intensity-dependent *loess* method. Gene expression was reported as log_2 of

the fold change, considering ≥2.1 fold with positive values (up-regulated) or negative values (down-regulated) and statistically significant (p-value<0.01). The microarray data can be accessed at the Gene Expression Omnibus (GEO) database (accession number GSE69930).

The functional analysis was performed by using GOStats (Bioconductor), which identifies significantly enriched GO terms among a list of genes by calculating the hypergeometric probability that a given GO term is represented more microarray features than would be expected by chance. Only GOStats results with a p-value <0.01 were considered.

2.4. Validation of DNA microarray data

P. pseudoalcaligenes CECT5344 cultures and RNA isolations were performed as previously described for DNA arrays generation. The concentration and purity of the RNA samples were measured in a ND1000 spectrophotometer (Nanodrop 1000, Agilent Technologies-Wilmington, DE, USA). Synthesis of total cDNA was achieved in 20 μl final volume, containing: 500 ng RNA, 0.7 mM dNTPs, 200 U SuperScript II Reverse Transcriptase (Invitrogen) and 3.75 mM random hexamers (Applied Biosystems). Samples were initially heated at 65 °C for 5 min and then incubated at 42 °C for 50 min, followed by incubation at 70 °C for 15 min. To carry out PCR reactions, 2 μl of each cDNA were initially heated at 98 °C for 2 min, followed by 30 cycles of amplification: 98 °C, 30 s; 60 °C, 30 s and 69 °C, 1 min. Polymerase extension reactions were completed by an additional incubation at 69 °C for 10 min. For real-time assays, the cDNA was purified using Favorprep Gel/PCR purification kit (Favorgen) and the concentration was measured using a ND1000 spectrophotometer. The iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) was used in a 25 μl reaction (final volume), containing 2 μl diluted cDNA (12.5, 2.5 and 0.5 ng) and 0.2 mM of each primer (Table S1, Supplementary material), and 12.5 μl iQ SYBR Green Supermix (Bio-Rad). Target cDNAs and reference samples were amplified three times in separate PCR reactions. Samples were initially denatured by heating at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C, 30 s; test annealing temperature, 60 °C, 30 s; elongation and signal acquisition, 72 °C, 30 s). For relative quantification of the fluorescence values, a calibration curve was made using dilution series from 50 to 0.0005 ng of *P. pseudoalcaligenes* CECT5344 genomic DNA sample. Represented data were normalized by using 16S rRNA methyltransferase (BN5_0873), 23S rRNA methyltranferase (BN5_2541), DNA polymerase III α-subunit (BN5_2819) and DNA polymerase III ϵ-subunit (BN5_2215) as housekeeping genes (Table S1, Supplementary material). Error bars represent standard deviation calculated from the results of three independent experiments.

2.5. Generation of a Dps mutant of P. pseudoalcaligenes CECT5344

A mutant strain of *P. pseudoalcaligenes* CECT5344 was generated by insertion of a kanamycin cassette in the *dps* gene (BN5_3091) that codes for a ferritin-like protein in this bacterium. Genomic DNA was isolated as indicated by the protocol of manufacturers (Wizard Genomic Purification Kit, Promega). The *dps* gene was amplified from genomic DNA by PCR using the oligonucleotides: Dps_F: 5′- AGCGGATCCTCTGTACCTGAAGACCCATAACTTC-3′ (*Bam*HI site is underlined) and Dps_R: 5′-AGCAAGCTTCGTTTGTTAAGTAAACGGCATTAC-3′ (*Hin*dIII site is underlined) to yield a 450 bp DNA fragment. The PCR program consisted of an initial denaturing step by heating at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C, 30 s; test annealing temperature, 65 °C, 30 s; elongation and signal acquisition, 72 °C, 30 s). The PCR fragment was cloned into pBluescrip-SK vector with the restriction sites *Bam*HI and *Hin*dIII. The kanamycin (Km) cassette was inserted to disrupt the *dps* gene by using a *Sph*I restriction site located at 200 bp from the 5-end of the *dps* gene. The *dps*::Km fragment was cloned into the mobilizable vector pK18*mob* previously digested with the restriction enzymes *Bam*HI and *Hin*dIII. This mobilizable construct was transferred to the wild-type strain by conjugation and transconjugants were selected by homologous recombination in media with nalidixic acid and kanamycin.

3. Results

The alkaliphilic bacterium *P. pseudoalcaligenes* CECT5344 uses free cyanide (CN^-) and different metal–cyanide complexes as the sole nitrogen source for growth (Luque-Almagro et al., 2005a,b), and the whole genome of this bacterium has been sequenced and annotated (Luque-Almagro et al., 2013 Wibberg et al., 2014). To obtain insights into the effect of different nitrogen sources, including cyanide and a cyanide-rich industrial residue, DNA microarrays from *P. pseudoalcaligenes* CECT5344 cells grown with different nitrogen sources have been constructed. DNA microarrays from cells grown with 2 mM sodium cyanide (NaCN) or cyanurated wastewater (CN-WW) from the jewelry industry (2 mM cyanide concentration) were compared against microarrays from cultures with 2 mM ammonium (NH_4Cl) as nitrogen source. A condition of nitrogen starvation (−N) was also compared against DNA microarrays from cultures with ammonium.

Four independent biological replicates for each nitrogen source/condition have been used to carry out the microarrays construction by Agilent Technologies, as previously and successfully used in other bacterial transcriptomic studies (Esclapez et al., 2015). The linkage of the four independent biological replicates for each nitrogen source/condition was statistically analyzed by different methods, including the Ward's linkage method (Fig. S2, Supplementary material). As expected, samples from the same nitrogen source were closer within them than to the samples from other conditions. In addition, cultures with cyanide (sodium cyanide or cyanide-containing industrial wastewater) were closer between them than to those from different nitrogen sources. Samples from ammonium, the preferential nitrogen source for many organisms, were more distant to samples from cyanide-containing cultures.

The whole genome of the strain CECT5344 includes 4434 protein coding sequences (Wibberg et al., 2014). Among them, a total of 1783 genes (40%) showed expression changes in the culture conditions used in this study, with 862 up-regulated genes and 921 down-regulated genes (Fig. 1). Genes were considered differentially expressed when they fulfilled the following filter parameters: expression ratio $\log_2$ fold change ≥2.1 with positive value for up-regulated genes or negative value for down-regulated genes, and an adjusted p-value≤0.01.

3.1. P. pseudoalcaligenes CECT5344 response to nitrogen starvation conditions

The limitation of nitrogen leaded considerable changes in the DNA arrays of *P. pseudoalcaligenes* CECT5344 when compared against microarrays from ammonium grown-cells. A total of 692 genes (15.6%) were found up-regulated in nitrogen-starved cells whereas 753 genes (17%) were down-regulated. Among up-regulated genes, 399 genes were specifically induced by nitrogen limitation and 293 genes were also induced by cyanide and/or the jewelry residue. Likewise, 352 genes were specifically down-regulated in nitrogen starved cells and 401 genes were also repressed by the presence of cyanide (Fig. 1). Induced genes by nitrogen starvation can be grouped in different functional (over-

Table 1
P. pseudoalcaligenes CECT5344 most relevant genes specifically affected by nitrogen starvation compared against ammonium.

Gene ID[a]	Annotation (function/gene name)	log_2FC	*p*-Value
BN5_0139	ABC-type amino acid transport/signal transduction systems, periplasmic component (*glnH*)	2.225	2.0E − 11
BN5_0140	ABC-type amino acid transport system, permease component (*glnP1*)	2.564	2.1E − 11
BN5_0141	ABC-type amino acid transport system, permease component (*glnP3*)	1.379	2.0E − 09
BN5_1009	ABC-type polar amino acid transport system, ATPase component (*aapP*)	1.162	4.2E − 08
BN5_0142	ABC-type polar amino acid transport system, ATPase component (*glnQ*)	2.101	9.1E − 10
BN5_3231	ABC-type branched-chain amino acid transport systems, periplasmic component	2.649	2.5E − 11
BN5_3113	Cyanate permease (*cynX*)	1.117	1.1E − 06
BN5_0499	Membrane transporter of cations and cationic drugs	1.582	9.5E − 08
BN5_2691	Permease of the drug/metabolite transporter (DMT) superfamily	1.277	3.0E − 07
BN5_2416	Fe^{2+}-dicitrate sensor, membrane component (*fecR*)	1.244	5.7E − 05
BN5_3557	ABC-type multidrug transport system, ATPase component	1.333	3.2E − 08
BN5_3747	Mn^{2+} and Fe^{2+} transporter of the NRAMP family	1.292	2.5E − 06
BN5_4476	Mercuric ion transport (*merT5*)	1.983	2.1E − 10
BN5_1137	Ammonia permease (*amt*)	1.984	3.4E − 10
BN5_0438	Transcriptional regulator containing PAS and DNA-binding domain (*cynF*)	1.419	7.2E − 07
BN5_0178	Nitrogen regulatory protein PII (*glnK*)	3.488	8.6E − 13
BN5_3961	NtrB signal transducer, responds to the nitrogen level and modulates NtrC activity (*ntrB*)	2.728	7.8E − 11
BN5_3962	Nitrogen response regulator containing DNA-binding domain (*ntrC3*)	2.596	4.9E − 13
BN5_4425	Transcriptional regulator. Nodulation protein D1 (*mexT5*)	1.332	1.6E − 06
BN5_0781	Transcriptional regulators (MocR family)	1.621	5.2E − 09
BN5_2516	Glutamate synthase	1.713	1.5E − 09
BN5_2542	Aconitase A (*acnA*)	1.081	2.6E − 06
BN5_1500	Carbon storage regulator, Global regulator protein family (*csrA*)	1.418	9.3E − 06
BN5_3204	Aliphatic amidase with a restricted substrate specificity, hydrolyzes formamide (*amiF*)	2.373	8.3E − 13
BN5_3668	Bacterioferritin (*bfr*)	1.507	1.2E − 09
BN5_0360	Glutathione *S*-transferase	1.464	1.5E − 07
BN5_1595	Glutathione peroxidase	1.319	2.5E − 08
BN5_0763	CRISPR-associated helicase Cas3 involved in defense mechanisms	1.251	3.7E − 08
BN5_0150	Cytochrome *c*, mono- and diheme variants (*cccA*)	1.115	4.2E − 08
BN5_1586	Cobalamin biosynthesis protein (*cobD/cbiB*)	1.591	2.3E − 08
BN5_4374	Cobalamin biosynthesis protein (*cobK*)	1.218	4.3E − 08
BN5_3669	Catalase-peroxidase I (*katG*)	1.563	4.0E − 10
BN5_1467	Molybdenum cofactor biosynthesis enzyme (*moaA1*)	1.177	3.3E − 09
BN5_2778	Uncharacterized protein involved in formation of periplasmic nitrate reductase (*napD*)	1.258	9.1E − 06
BN5_0412	Poly(3-hydroxyalkanoate) synthetase (*phaC2*)	2.358	5.6E − 10
BN5_0410	Poly(hydroxyalcanoate) granule associated protein (*phaF1*)	3.355	7.1E − 13
BN5_4096	Poly(hydroxyalcanoate) granule associated protein (*phaP*)	1.874	2.3E − 09
BN5_0413	Predicted hydrolase or acyltransferase (alpha/beta hydrolase superfamily) (*phaZ*)	1.554	4.3E − 09
BN5_3102	SAM-dependent methyltransferase (*ubiG3*)	1.465	6.5E − 09
BN5_0581	Urea amidohydrolase (urease) gamma subunit (*ureA*)	1.814	1.9E − 10
BN5_0578	Urea amidohydrolase (urease) alpha subunit (*ureC*)	1.443	9.5E − 10
BN5_0554	Putative gene involved in urea metabolism (*ureE*)	2.946	6.9E − 13
BN5_0551	Hydrogenase/urease accessory gene (*ureJ*)	2.328	2.1E − 11
BN5_4078	ABC-type polar amino acid transport system, ATPase component	−2.215	7.8E − 13
BN5_4080	ABC-type amino acid transport system, permease component	−1.646	2.5E − 07
BN5_4081	ABC-type amino acid transport system, permease component	−1.667	5.8E − 07
BN5_0814	Cation/multidrug efflux pump (*ttgB*)	−1.298	2.5E − 06
BN5_2733	Predicted exporter of the RND superfamily	−1.858	6.8E − 08
BN5_2616	ABC-type transport system involved in cytochrome *c* biogenesis (*ccmC*)	−1.078	7.1E − 07
BN5_4325	ABC-type metal ion transport system, permease component (*metI*)	−1.189	2.8E − 05
BN5_4326	ABC-type metal ion transport system, periplasmic component (*metQ*)	−3.823	1.8E − 10
BN5_0341	Predicted transcriptional regulator (*arsR3*)	−1.396	4.6E − 06
BN5_2792	Phosphoribosylaminoimidazole (AIR) synthetase (*purM*)	−1.544	1.8E − 05
BN5_1068	Formate-dependent phosphoribosylglycinamide formyltransferase GAR (*purT*)	−1.420	9.5E − 09
BN5_4231	NAD(P)H-nitrite reductase (*rubB*)	−2.014	1.7E − 09
BN5_1676	Phosphoserine aminotransferase (*serC1*)	−1.538	2.5E − 10
BN5_2026	Sulfite reductase, beta subunit (*sir*)	−2.507	6.2E − 12
BN5_3036	Peroxiredoxin (*ahpC1*)	−1.664	1.3E − 08
BN5_3035	Alkyl hydroperoxide reductase, large subunit (*ahpF*)	−1.085	2.8E − 07
BN5_2615	Putative gene involved in cytochrome *c* biogenesis (*ccmD*)	−1.256	2.4E − 07
BN5_2614	Cytochrome *c*-type biogenesis protein (*ccmE*)	−1.557	6.7E − 11
BN5_2613	Cytochrome *c* biogenesis factor (*ccmF*)	−1.643	3.3E − 11
BN5_2610	Cytochrome *c* biogenesis factor (*ccmH*)	−2.109	2.9E − 11
BN5_1342	Cbb3-type cytochrome oxidase, subunit 1 (*ccoN1*)	−1.259	9.6E − 07
BN5_4400	Precorrin-4 methylase (*cobM*)	−1.153	2.8E − 04
BN5_1592	Cobalamin-5-phosphate synthase (*cobS*)	−1.109	7.9 E − 05
BN5_0743	Putative Mg^{2+} and Co^{2+} transporter (*corC*)	−1.183	5.2 E − 08
BN5_0325	5,10-methylenetetrahydrofolate reductase (*metF1*)	−2.022	2.5E − 10
BN5_2035	Methionine synthase I, cobalamin-binding domain (*metH*)	−1.207	5.1E − 07

[a] Gene IDs refer to accession number HG916826 (Wibberg et al., 2014).

represented GO) categories according to GOStats analysis (Figs. S3 and S4, Supplementary material), including genes that code for DNA polymerase activity, motor activity, nucleic acid binding, nitrogen cycle and regulation of metabolic processes. The up-regulated genes exclusively induced in nitrogen-starved cells were related to transport of amino acids, cyanate or ammonia, amino

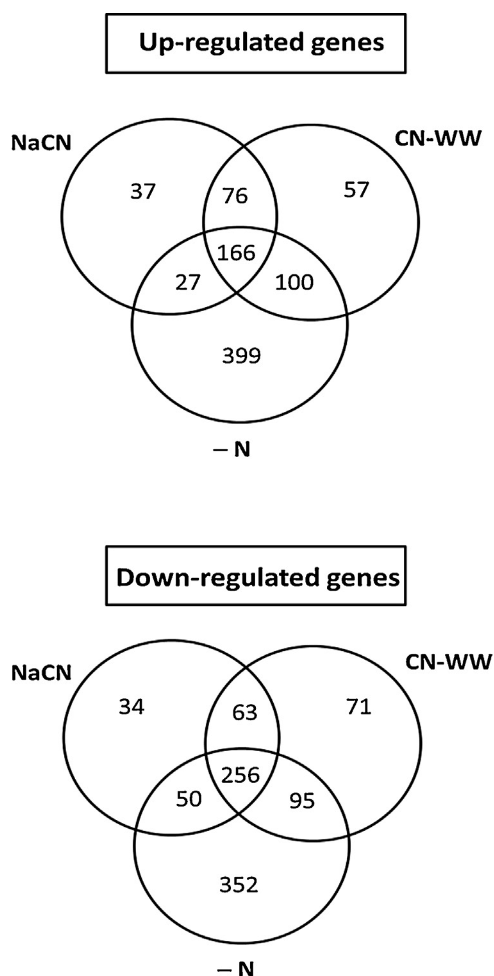

Fig. 1. Venn diagram with the number of up- and down-regulated genes in the transcriptomes of *P. pseudoalcaligenes* CECT5344 from sodium cyanide (NaCN), cyanide-containing jewelry wastewater (CN-WW) and nitrogen-limiting conditions compared against ammonium. An expression ratio of $\log_2$ fold $\geq$2.1 with positive values (up-regulated genes) and negative values (down-regulated genes) and an adjusted *p*-value of $\leq$0.01 were considered.

acid metabolism, regulation of nitrogen metabolism processes like global nitrogen control or cyanate and urea metabolism, enzymes and proteins involved in polyhydroxyalkanoate metabolism, and genes related to defense mechanisms, iron acquisition, metal transport and oxidative stress, such as glutathione peroxidase, CRISPR-associated helicase, catalase, bacterioferritin and the FecR Fe^{2+}-dicitrate sensor (Table 1).

Many genes were found down-regulated in the nitrogen starvation response. Most of the specifically down-regulated genes were involved in amino acids and metal transport, methionine metabolism, cytochrome *c* biogenesis and sulfite/nitrite reduction (Table 1). Expression of genes encoding ribosomal proteins, tRNA aminoacylation proteins for protein translation and translation factors was also decreased (Tables S2, S6–S8, Supplementary material).

3.2. P. pseudoalcaligenes *CECT5344 response to sodium cyanide and a cyanide-containing industrial wastewater*

The presence of cyanide provoked relevant changes in the transcripts of the strain CECT5344. Two different sources of cyanide were considered as nitrogen source for growth, and consequently, two different DNA microarrays were obtained and compared against microarrays from ammonium cultures: sodium cyanide (NaCN) that dissociates in aqueous solution to free ion cyanide, and cyanide-containing wastewater from the jewelry industry (CN-WW), which contains both free ion cyanide and stable cyano–metal complexes. Only 37 or 34 genes (0.8% genes in the whole genome) were specifically induced or repressed, respectively, in response to sodium cyanide (Fig. 1). Some genes exclusively up-regulated in sodium cyanide code for acyl-CoA transferases, aminotransferases and deamidases, a heme/copper-type cytochrome/quinol oxidase subunit, and the Nit3 nitrilase/cyanide hydratase (Table S3, Supplementary material). These cyanide-induced genes can be grouped in different functional categories by GOStats analysis (Figs. S5 and S6, Supplementary material) and include genes involved in metal- and iron–sulphur cluster binding, pyrydoxal phosphate cofactor binding, enzymes displaying oxidoreductase activity (L-serine family metabolic process) and hydroxymethyl-formyl and related transferases. Among genes specifically repressed by cyanide were highlighted those related to Fe-S oxidoreductases, metal-dependent hydrolases and cytochrome *c* biogenesis (Table S3, Supplementary material). The GOStats functional categories according to GOStats analysis for down-regulated genes in sodium cyanide are related to ATPase activity, C–C and C–N ligase activity, transport of ions and RNA binding.

Transcription of 128 genes (2.9% of total genes in the whole genome) was specifically affected in response to the cyanide-containing jewelry wastewater, with 57 (1.3%) induced and 71 (1.6%) repressed genes (Fig. 1). The up-regulated genes in the jewelry residue are shown in Table S4 (Supplementary material), and including genes for metal transporters, such as Fe^{3+}-siderophore ABC-type transporter, P-type ATPases for Cu^{+} or Cd^{2+} extrusion and multidrug efflux pumps from the RND family, regulatory genes involved in metal detoxification systems, and genes encoding arsenate reductase, D-methionine ABC-type transporter and malate:quinone oxidoreductase B (*mqoB*). According to the GOStats analysis, genes induced by the jewelry residue can be grouped in functional categories similar to those described for genes affected by sodium cyanide (Figs. S7 and S8, Supplementary material), which were relates to iron–sulphur and copper cluster binding, pyrydoxal phosphate binding, inorganic anion transport, and oxidoreductases (L-serine family metabolic process-related enzymes). The GOStats functional categories for the down-regulated genes by the jewelry residue were related to ATPase activity, racemase and ligase activities, acyl-transferase activities, transport of ions and RNA and NAD binding. Some genes specifically repressed by the jewelry liquid residue were the carbon–nitrogen hydrolase (nitrilase family) *aguB* gene, the DNA-binding ferritin-like *dps* gene, the iron and zinc uptake regulator (*zur*) gene, and genes encoding citrate synthase, acyl-CoA dehydrogenases and uncharacterized flavoenzymes (Table S4, Supplementary material). A Dps mutant strain of *P. pseudoalcaligenes* CECT5344 has been generated by insertion of a kanamycin antibiotic resistance cassette. Growth of Dps mutant strain in the presence of a high concentration of $CuCl_2$ (2 mM), with ammonium as nitrogen source, was much lower than growth of the wild-type strain. However, wild-type and Dps mutant strains showed similar growth in the presence of 2 mM $FeCl_3$ and ammonium. In cyanide-containing media, the Dps mutant of *P. pseudoalcaligenes* CECT5344 strain also showed a similar growth to the wild-type strain (not shown).

Expression of 139 genes was affected in cells grown with sodium cyanide or with cyanide-containing jewelry wastewater when compared against ammonium (Table S5). Induced genes (a total of 76) were, among others, two nitrilase-encoding genes (*nit2* and *nit4*), sulfur metabolism genes like those coding for sulfite reductase and for sulfate and sulfonates transporters, the *cioB* gene coding for a terminal *cbb*$_3$-type oxidase and the *ccoN1* and *cyoA* oxidase genes, several amino acid metabolism genes that are clustered together the *cioAB* genes and encode putative

aminotransferases for serine, histidine and arginine, genes encoding methionine synthases vitamin B12-dependent or -independent, the alkyl-hydroperoxide reductase *ahpC* gene that is adjacent to the sulfur metabolism genes, the Hmp flavohaemglobin *fpr* gene, the *isc* genes for Fe-S cluster assembly system, and the malic enzyme gene. The down-regulated genes presented different functions, and included those coding for RNA polymerase and CRISPR/Cas-associated proteins for defense mechanisms (Table S5, Supplementary material).

A total of 77 genes (1.7%) were found affected in common in the *P. pseudoalcaligenes* CECT5344 DNA microarrays from cells grown with sodium cyanide or under nitrogen-limiting conditions when both compared against ammonium. Transcripts with 27 genes (0.6%) were up-regulated whereas 50 genes (1.1%) were down-regulated (Fig. 1). The up-regulated genes (Table S6, Supplementary material) included, among others, the heme/copper-type cytochrome/quinol oxidase (*cox3*) and the aconitase A (*acnC*) genes. Several down-regulated genes code for succinate dehydrogenase subunits, efflux-type ATPase and RND efflux pump (*mexC*), glutamine synthetase (*glnA1*) and a rhodanase-related sulfurtransferase.

The DNA microarrays of *P. pseudoalcaligenes* CECT5344 in response to the jewelry wastewater and nitrogen-limiting conditions, both compared against ammonium, shared a total of 195 affected genes (4.4%), with 100 induced (2.3%) and 95 (2.1%) repressed genes (Fig. 1; Table S7, Supplementary material). Relevant induced genes were involved in nitrate/nitrite assimilation, including the two-component positive regulatory system that responds to nitrate and/or nitrite (*nasT* and *nasS* genes), the nitrate and nitrite transporters, and the catalytic nitrate and nitrite reductases, some regulatory genes like the metal regulator gene *cueR* and the polyhydrohyalkanoate structural and regulatory gene *phaI,* metal transporters like a Ni-transporter and a Cu- transporting ATPase (*actP* gene) and a Zn–Co–Cd efflux pump, and ABC-type transporters for amino acids, sugar and other compounds. Down-regulated genes code for aconitase B *acnB* gene, acetyl-CoA dehydrogenases, aminotransferases, biotin cofactor biosynthesis, sulfate transporters and glutamate synthase (*gltB1*).

DNA microarrays of *P. pseudoalcaligenes* CECT5344 in response to sodium cyanide, jewelry wastewater and nitrogen-limiting conditions, all three compared against ammonium, shared a total of 422 affected genes (9.5%), with 166 up-regulated (3.7%) and 256 (5.8%) down-regulated genes (Fig. 1; Table S8, Supplementary material). Some repressed genes code for aspartate kinase, ATPase subunits, cytochrome *c* oxidases, citrate synthase and oxaloacetate decarboxylase (Table 2). Relevant genes induced in all three experimental conditions tested were the nitrilase *nitC* gene that is essential for cyanide assimilation in the strain CECT5344 and other genes of unknown function that constitute the *nit1C* transcriptional unit, genes encoding GntR–MocR and pyridoxal phosphate (PLP)-dependent aminotransferases, biotin transport and synthesis genes, several genes that are usually up-regulated under nitrogen-limiting conditions like those coding for glutamine synthetase, glutamate synthase and ammonium transporter, genes for the ISC system for Fe-S cluster assembly, and genes coding for the ABC-type cyanate transporter (*cynABD*) and the cyanase (*cynS*) involved in cyanate assimilation.

3.3. qRT-PCR validation of microarray data

Microarray data were corroborated by quantitative RT-PCR gene expression of *P. pseudoalcaligenes* CECT53344 selected genes, like the nitrilase-encoding genes (*nit2*/*nitC*, *nit3*, *nit4* and *aguB*) and the *cioA* and *cioB* genes coding for the cyanide-insensitive terminal oxidase (Fig. 2). The genome of *P. pseudoalcaligenes* CECT5344 contains four nitrilase genes, *nit1* (BN5_0736), *nit2*/*nitC* (BN5_1632), *nit3* (BN5_3251) and *nit4* (BN5_1912), although only the *nitC* gene is essential for cyanide assimilation (Estepa et al., 2012). These four nitrilase-encoding genes have been found to be differentially expressed in the nitrogen conditions used in this study. In addition to these four nitrilase genes, an additional gene, *aguB*, which belongs to the nitrilase/C–N hydrolase superfamily was also affected in the DNA microarrays. Under nitrogen liming conditions, expression of all nitrilase genes was very low, except the *nitC* gene that was expressed at a high level. In cells grown with cyanide-containing media (sodium cyanide or jewelry residue), all nitrilase genes increased their expression, except *aguB* gene that was repressed. Repression of *aguB* gene was higher in cells grown with the jewelry residue than with sodium cyanide. These results confirmed the microarray data since the *nit3* gene was induced exclusively by sodium cyanide, the *nit4* gene was induced by both sodium cyanide and cyanide-containing jewelry wastewater, the *nitC* gene was induced by sodium cyanide and cyanide-containing wastewater and also under nitrogen starvation, and the *aguB* gene was repressed by the jewelry residue. Likewise, the microarrays analysis revealed that the *cioAB* genes were induced in the presence of cyanide (sodium cyanide and jewelry residue), displaying concordance with their expression profiles found by qRT-PCR analysis.

4. Discussion

DNA microarrays have been constructed from cells grown with sodium cyanide or cyanide-containing wastewater from the jewelry industry and compared against DNA microarrays from cultures with ammonium chloride as nitrogen source. Additionally, microarrays from cultures under nitrogen starvation have been also carried out. Microarrays analysis has been previously applied to study the response to nitrogen starvation in several bacterial strains like the Gram positive *Corynebacterium glutamicum* (Silberbach et al., 2005), the Gram negative *Pseudomonas putida* (Hervás et al., 2008), and the haloarchaeon *Haloferax mediterranei* (Esclapez et al., 2015). Although the response to a very low concentration of cyanide (1 mM) has been studied in *Nitrosomonas europaea*, this strain lacks of a cyanide assimilatory/detoxification pathway (Park and Ely, 2009). Therefore, there are no data available about transcriptomic studies of the response to cyanide in a cyanotrophic microorganism.

DNA microarrays revealed that large number of genes were up-regulated in cells grown under nitrogen starvation when compared against DNA microarrays from ammonium grown cells (Fig. 1). It has been previously described that under nitrogen-limiting conditions *P. putida* induces NtrC-dependent genes, including amino acid transport and urea assimilation genes, the PII-encoding *glnK* gene and the ammonium transporter *amtB* gene (Hervás et al., 2008). Under nitrogen deprivation, carbon storage is potentiated as suggested by the induction of polyhydroxyalkanoates synthase and phasin genes (Hervás et al., 2008). These genes were also induced in *P. pseudoalcaligenes* CECT5344 cells subjected to nitrogen starvation (Table 1). Interestingly, the carbon storage regulator *csrA* gene was also induced by nitrogen starvation in the strain CECT5344. In *Escherichia coli*, it has been described that the *csrA* gene acts as a repressor by mRNA binding, avoiding translation (Gutiérrez et al., 2005). This could be a mechanism to prevent an excess of catabolic flow through TCA cycle under nitrogen limiting condition for growth. GOStats analysis of *P. pseudoalcaligenes* CECT5344 microarrays revealed a reduced expression of genes encoding some ribosomal proteins, translation factors and tRNA aminoacylation proteins (Tables S2, S6–S8, Supplementary material), indicating that there is a general decrease of protein synthesis under nitrogen limiting conditions.

Table 2
P. pseudoalcaligenes CECT5344 most relevant genes affected by sodium cyanide, jewelry wastewater and nitrogen starvation compared against ammonium.

Gene ID[a]	Annotation (function/gene name)	CN^{-}-NH_4^+ log_2FC	WW-NH_4^+ log_2FC	N-NH_4^+ log_2FC	CN-NH_4^+ *p*-value	WW-NH_4^+ *p*-value	N-NH_4^+ *p*-value
BN5_1902	Cytochrome *bd* ubiquinol oxidase (*cioA3*)	8.128	7.702	1.151	3.1E − 12	3.0E − 12	2.9E − 3
BN5_1634	GCN5-related *N*-acetyltransferase	7.653	9.954	7.164	1.8E − 12	1.2E − 13	4.5E − 12
BN5_1632	NitC, Nitrilase/cyanide hidratase (*nit2*/*nitC*)	6.482	9.394	6.292	9.9E − 11	8.0E − 13	1.3E − 10
BN5_0442	Cyanate lyase, cyanate hydratase (*cynS*)	6.243	9.156	4.112	2.7E − 07	1.1E − 09	8.4E − 06
BN5_1899	PLP-dependent, GntR-regulator (*mocR*)	6.041	5.912	1.574	4.5E − 13	3.2E − 13	1.6E − 06
BN5_1353	Cytochrome *c* oxidase *cbb3* type (*ccoG*)	5.804	5.879	1.618	2.2E − 14	1.4E − 14	1.0E − 08
BN5_0439	ABC-type cyanate transporter (*cynA*)	5.695	10.330	5.867	9.6E − 08	2.9E − 11	4.2E − 08
BN5_1892	LysR, substrate-binding	5.311	5.226	1.520	1.0E − 12	6.8E − 13	9.4E − 07
BN5_0441	ABC-type cyanate transporter (*cynD*)	5.152	9.356	5.351	2.6E − 08	8.3E − 12	6.6E − 09
BN5_2689	Pyruvate ferredoxin/flavodoxin (*iorA1*)	4.580	4.226	1.666	2.8E − 10	4.3E − 10	1.4E − 05
BN5_2413	PLP-dependent aminotransferase	4.553	4.546	4.007	1.3E − 07	1.3E − 08	1.7E − 07
BN5_0440	ABC-type cyanate transporter (*cynB*)	4.202	8.812	4.185	3.1E − 06	1.9E − 10	9.2E − 07
BN5_0329	Glutamate synthase-GOGAT (*gltD1*)	2.907	3.087	5.773	2.7 E − 4	1.5 E − 4	8.5E − 08
BN5_1638	Isocitrate dehydrogenase (*aceK*)	2.523	3.782	1.789	2.7E − 06	1.2E − 08	7.8E − 06
BN5_2309	Glutamine synthetase-GS (*glnA5*)	2.383	2.512	3.199	1.6E − 07	5.3E − 08	1.6E − 10
BN5_2268	Biotine synthase	2.195	1.037	1.063	1.0E − 08	2.5E − 05	1.3E − 05
BN5_2535	Bacterial regulatory protein, MarR	2.160	2.427	3.093	7.6E − 10	9.4E − 11	1.1E − 11
BN5_2414	Bacterial regulatory protein, MarR	2.068	2.456	1.661	5.2E − 08	3.3E − 09	1.6E − 07
BN5_1587	PLP-dependent aminotransferase (*cobC*)	2.068	3.852	1.357	5.6E − 08	1.2E − 11	2.1E − 06
BN5_1338	LysR, GntR transcriptional regulators (*gstR*)	1.868	1.620	2.122	9.5E − 07	2.3E − 06	6.7E − 08
BN5_3729	FeS cluster assembly	1.762	2.440	1.579	1.3E − 06	7.6E − 09	7.2E − 07
BN5_3269	ISC system trasnscription regulator (*iscR*)	1.714	2.718	1.667	7.5E − 06	2.0E − 08	3.1E − 06
BN5_1807	Biotin/lipoyl, RND family efflux transporter	1.494	1.003	1.244	2.2E − 4	2.2E − 05	1.5E − 06
BN5_2308	PLP-dependent aminotransferase	1.410	1.705	1.488	3.5E − 07	1.9E − 08	5.9E − 08
BN5_0594	Bacterial regulatory proteins, gntR family	1.202	1.708	3.357	1.7E − 4	3.2E − 06	4.8E − 11
BN5_1373	Pyruvate kinase	1.197	1.441	2.208	2.5E − 05	2.1E − 06	1.0E − 08
BN5_2756	Asparagine synthetase (*asnB*)	1.146	1.197	1.963	2.4E − 05	8.1E − 06	2.2E − 08
BN5_0180	Ammonia permease (*amtB*)	1.118	2.213	4.699	6.8E − 5	2.9E − 8	4.4E − 12
BN5_2493	Bacterial regulatory protein, MarR	1.092	2.145	1.411	5.2E − 4	4.6E − 07	2.8E − 05
BN5_1499	Aspartate kinase	−1.078	−1.053	−2.352	2.6E − 4	2.1E − 4	3.2E − 08
BN5_2445	Cytochrome *c* oxidase *cbb3*-type (*ccoN3*)	−1.111	−1.444	−1.045	2.7E − 4	1.3E − 06	2.1E − 3
BN5_2705	ArsA, arsenite-activated ATPase (*arsA*)	−1.200	−2.052	−1.069	1.5E − 05	1.8E − 08	1.6E − 05
BN5_0188	PLP-dependent decarboxylase (*lysA*)	−1.270	−1.086	−1.168	9.8E − 06	3.0E − 05	7.5E − 06
BN5_4502	ATPase F0/V0 complex, sunibut C (*atpE*)	−1.635	−1.568	−3.1809	7.5E − 05	9.3E − 05	2.1E − 08
BN5_1328	Efflux transporter RND-HAE1 (*mexD*)	−1.730	−1.013	−2.097	1.0E − 05	1.1E − 3	3.8E − 07
BN5_2182	Citrate synthase I (*gltA*)	−1.774	−1.462	−3.282	1.2E − 05	4.1E − 05	4.3E − 09
BN5_4499	ATPase, F1 complex, alpha subunit (*atpA*)	−1.807	−2.160	−3.914	1.2E − 4	1.2E − 05	9.8E − 09
BN5_4497	ATPase, F1 complex, beta subunit (*atpD*)	−1.831	−2.277	−4.243	5.8E − 06	2.6E − 07	1.7E − 10
BN5_2444	Cytochrome *c* oxidase *cbb3*-type (*ccoP*)	−1.972	−3.515	−1.939	9.1E − 05	1.2E − 07	4.4E − 05
BN5_4498	ATPase, F1 complex, gamma subunit (*atpG*)	−1.995	−2.488	−4.306	4.2E − 05	2.4E − 06	2.6E − 09
BN5_4500	ATP synthase F1, delta subunit (*atpH*)	−2.114	−2.031	−3.572	4.1E − 06	4.3E − 06	3.7E − 09
BN5_4501	ATPase, F0 complex, subunit B (*atpF*)	−2.227	−1.942	−3.819	2.6E − 06	5.5E − 06	2.0E − 09
BN5_4309	Oxaloacetate decarboxylase (*oadA*)	−3.419	−3.827	−3.302	1.4E − 11	2.8E − 12	2.9E − 11

[a] Gene IDs refer to accession number HG916826 (Wibberg et al., 2014).

Among those genes that increased their expression exclusively in sodium cyanide it was found the nitrilase/cyanide hydratase *nit3* gene (Table S3, Supplementary material). Oxaloacetate has been described to be essential in the cyanide degradation pathway of *P. pseudoalcaligenes* CECT5344, since is specifically produced by a malate:quinone oxidoreductase in response to cyanide. Both compounds chemically react to generate the oxaloacetate–cyanohydrin, which is further used by the nitrilase NitC that converts the cyanohydrin into ammonium for its incorporation to carbon skeletons by the GS/GOGAT cycle (Luque-Almagro et al., 2011c; Estepa et al., 2012). Although the nitrilase NitC, but not the nitrilase Nit3, it is essential for cyanide assimilation in the strain CECT5344 (Estepa et al., 2012), a residual nitrilase activity that could be attributed to the nitrilase Nit3 activity remains in a NitC defective mutant strain of *P. pseudoalcaligenes* CECT5344. However, the NitC mutant strain is unable to grow with cyanide as the sole nitrogen source (Estepa et al., 2012). Another gene that increased its expression exclusively in sodium cyanide (Table S3, Supplementary material) encodes a 2-methylcitrate synthase, an acyl-CoA transferase that uses oxaloacetate and propionyl-CoA as substrates to yield 2-methylcitrate (Gerike et al., 1998). As indicated, oxaloacetate is produced as the first intermediate of the cyanide assimilation/degradation pathway in the strain CECT5344 (Estepa et al., 2012), and probably other enzymes that use this ketoacid as substrate are also induced. On the other hand, a large number of genes encoding metalloenzymes were repressed by cyanide (Table S3, Supplementary material) and this could be a collateral effect to the enzyme inhibition caused by cyanide, which displays a high affinity for the metallic centers. Several genes encoding metal transporters have been found induced in cells grown with the jewelry wastewater (Table S4, Supplementary material). The Fe^{3+}-siderophore transporters are ABC-type or TonB-dependent systems for iron acquisition, allowing cell survival in the presence of cyanide (Crosa and Walsh, 2002). The jewelry wastewaster contains free and metal−bound cyanide, metals like iron, copper and zinc, and also small traces of nitrite and cyanate (concentrations in the mM range). Metal extruders are induced as detoxification mechanisms in the presence of high concentrations of metals. Bacterial metal efflux pumps can be classified in three different types: the chemiosmotic gradient (H^+ or K^+)-dependent cation diffusion facilitator (CDF) family, the efflux P1-type ATPases, and the RND (resistance-nodulation-cell division) efflux pumps that are composed of an outer membrane protein, a periplasmic component with a small membrane hydrophobic region and an integral membrane component (Nies, 2003). Genes encoding several types of metal extruders have been found induced in cells grown with the jewelry wastewater, including P-type ATPases for Cu^+ or Cd^{2+} extrusion, multidrug efflux pumps of the

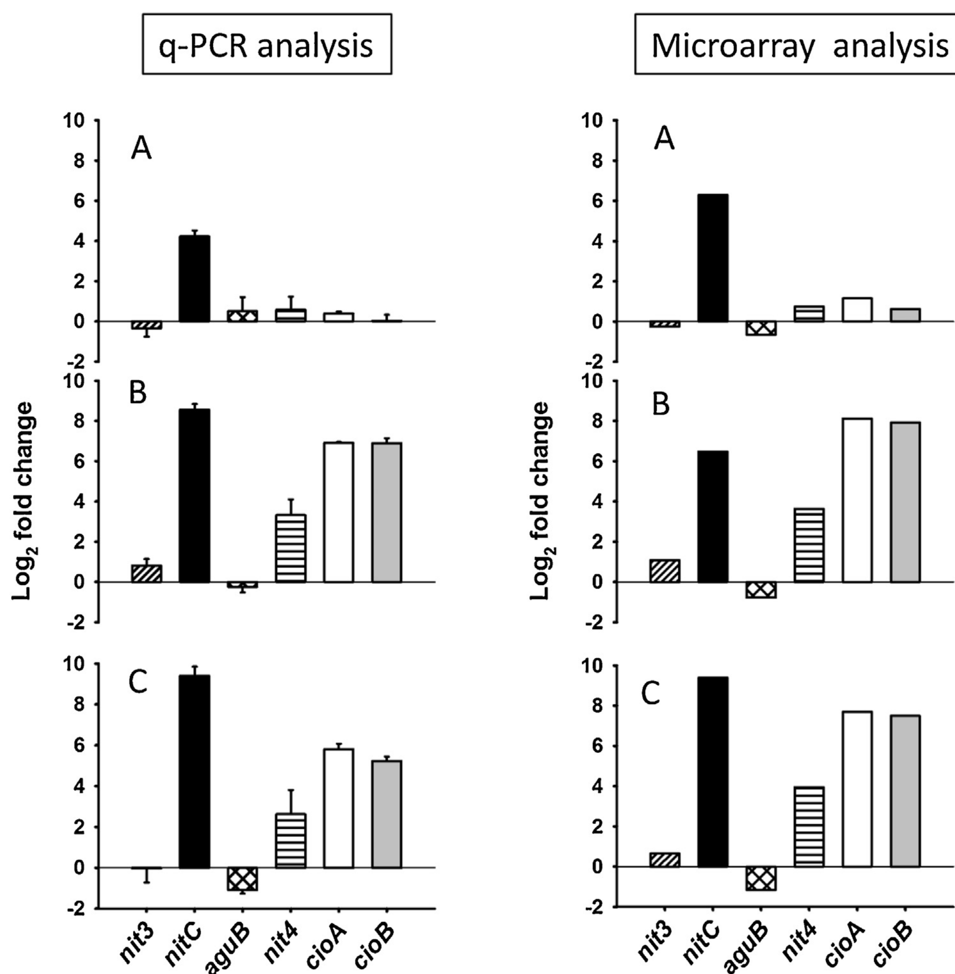

Fig. 2. Validation by qPCR of the microarray data. q-PCR analysis (left panels) and microarrays data (right panels). The selected genes *nitC/nit2* (BN5_1632), *nit3* (BN5_3251), *nit4* (BN5_1912) and *aguB* (BN5_0258) encode proteins that belong to the supernitrilase family (Podar et al., 2005), and the *cioA* (BN5_1902) and *cioB* (BN5_1903) genes code for the cyanide-insensitive terminal oxidase. (A) Gene expression in cells grown under nitrogen starvation. (B) Gene expression in cells grown with sodium cyanide. (C) Gene expression in cells grown with the cyanide-containing wastewater jewelry residue. Gene expression in all three nitrogen conditions was referred to that obtained in ammonium. q-PCR data represent an average of triplicate (±standard deviation).

RND family and regulatory genes involved in metal detoxification systems. Additionally, a gene that codes for a malate: quinone oxidoreductase B (*mqoB*) was induced by the jewelry residue. As mentioned above, a malate:quinone oxidoreductase is involved in the first step of cyanide degradation in *P. pseudoalcaligenes* CECT5344 by converting L-malate into oxaloactetate, and it is also a key enzyme in carbon metabolism since this bacterial strain lacks malate dehydrogenase (Luque-Almagro et al., 2011c). Analysis of the whole genome sequence of the strain CECT53344 reveals the existence of two-genes encoding malate:quinone oxidoreductases, *mqoA* (BN5_0860) and *mqoB* (BN5_1358), which share 52% identity and 71% similarity. The TargetP program (tp://www.cbs.dtu.dk/services/TargetP/) has predicted unequivocally a subcellular location of *MqoA* in the cytoplasm but an unclear subcellular location for the *MqoB* enzyme, which could be located either in the cytoplasm or in the periplasm. The *mqoB* gene was induced by the jewelry wastewater whereas the *mqoA* gene was expressed in cells grown with all different nitrogen sources tested in this work. Therefore, the *P. pseudoalcaligenes* CECT5344 *MqoA* is an essential key both in central carbon metabolism and in the cyanide assimilation/detoxification pathway, but function of the *mqoB* gene, which is specifically induced by the cyanide-containing jewelry wastewater, remains unknown. Although a gene encoding an arsenate reductase was also induced by the jewelry residue, arsenic derivatives could not be detected in this industrial waste. However, it has been described that other metals act as inducers of the arsenate reductase (Park and Ely, 2008). Specifically repressed by the jewelry liquid residue were the *zur* gene that codes for an iron and zinc uptake regulator and the DNA-binding ferritin-like *dps* gene (Table S4, Supplementary material). It has been previously demonstrated that the ferric-uptake regulator family protein Zur represses genes involved in zinc uptake in *Corynebacterium diphtheria* under zinc-rich conditions (Smith et al., 2009). The ferritin-like Dps proteins may participate in oxidative damage protection by iron-binding, avoiding free radicals and reactive oxygen species formation by the Fenton reaction (Bellapadrona et al., 2010). Recently, it has been postulated a role for a Dps protein in copper homeostasis in *E. coli*. Curiously, in this bacterium the intracellular levels of copper decrease when the Dps protein is overexpressed (Thieme and Grass, 2010). To investigate the role of the Dps ferrin-like protein in the cyanide assimilation/degradation process of the strain CECT5344, a Dps mutant strain of *P. pseudoalcaligenes* CECT5344 has been generated. Growth of the Dps mutant was affected only in the presence of a high concentration of copper with ammonium as nitrogen source, indicating a role for Dps protein as copper chelator. However, growth of the Dps mutant was not affected in cyanide-containing media or in the presence of high concentration of iron,

suggesting that iron homeostasis by the Dps protein during cyanide assimilation/detoxification does not occurs.

The nitrilase *nit2*/*nitC* and *nit4* genes, the latest coding for a putative 4-hydroxyalanine nitrilase, and a malic enzyme-encoding gene were induced in cells grown with both sodium cyanide and cyanide-containing jewelry wastewater (Table S5). The oxaloacetate cyanohydrin (a nitrile) that is produced during cyanide assimilation in *P. pseudoalcaligenes* CECT5344 (Estepa et al., 2012) might act as inducer of other nitrilases like Nit4 that also use nitriles as substrates. Thus, the nitrilase Nit4 could contribute to the residual activity found in the NitC mutant of *P. pseudoalcaligenes* CECT5344, but it is not essential for cyanide assimilation in the strain CECT5344 (Estepa et al., 2012). Malic enzyme catalyzes oxidative decarboxylation of malate to pyruvate and this 3-oxoacid also reacts with free cyanide to produce a cyanohydrin that can be assimilated through the nitrilase NitC (Estepa et al., 2012). This process may contribute to decrease the concentration of free cyanide, which is much more toxic and reactive than its derivative organic forms (cyanohydrins). The *cioB* gene that codes for the terminal cbb_3-type oxidase required for cyanide insensitive respiration has been also found induced by both sodium cyanide and the jewelry wastewater (Table S5). Mutational analysis of the *cioB* and *cioA* genes coding for the two subunits of the terminal oxidase revealed that these genes are essential to survive on cyanide (Quesada et al., 2007). Several genes located downstream *cioAB* genes that code for PLP-dependent aminotransferases were up-regulated in media with cyanide. Some of these genes have been demonstrated to be cotranscribed with the *cioA* genes, but their role on cyanide metabolism remains unknown (Quesada et al., 2007). The nitrilase *nit4* gene is also located downstream the *cioAB* genes in the strain CECT5344 and might be also co-transcribed with the *cioAB* genes. The *isc* genes for Fe-S cluster assembly, the a lkyl-hydroperoxide reductase *ahpC* gene and an *fpr* gene were also up-regulated by cyanide (Table S5). Alkyl-hydroperoxide reductase has been postulated to be involved in protection against hydrogen peroxide. In pathogenic *Staphylococci*, a link between AhpC and Hmp proteins has been established since they participate in global response to oxidative stress sharing common global regulators (Gaupp et al., 2012). Under oxidative stress conditions the Fe-S clusters of many proteins are very susceptible to oxidative inactivation; hence, in aerobic bacteria have evolved Fe-S cluster repair mechanisms like *isc* genes (Gaupp et al., 2012). Rhodanases are involved in detoxification of cyanide in some microorganisms since these enzymes catalyze the transfer of sulfur to cyanide producing thiocyanate, a cyano-derivative less toxic than cyanide (Park and Ely, 2008). However, in the strain CECT5344 the rhodanase-encoding gene was down-regulated in nitrogen-limiting conditions but also in the presence of sodium cyanide (Table S6, Supplementary material), suggesting its lack of functionality in cyanide detoxification under these growth conditions. A large number of genes up-regulated by cyanide (sodium cyanide and jewelry wastewater) can be grouped in three different functional categories (GOStats analysis) that include copper ion binding, terminal oxidases (*cyoA*, *coxAB* and *cox11* genes), iron–sulfur cluster binding (*isc* genes and others) and pyridoxal phosphate binding (aminotransferases). This could be related to the cyanide affinity for metals and cofactors.

Genes involved in nitrate/nitrite assimilation were induced in the strain CECT5344 in response to the jewelry wastewater and nitrogen-limiting conditions, both compared against ammonium (Table S7, Supplementary material). Genes involved in bacterial inorganic nitrogen (nitrate/nitrite) assimilation have been widely described to be induced under nitrogen-limiting conditions in a great variety of Gram negative bacteria, usually through the NtrBC global nitrogen regulatory system (Luque-Almagro et al., 2011b). The nitrate-assimilating genes were also induced in the strain CECT5344 in response to the jewelry residue since it contains small amounts of nitrite (mM range) that may act as an inducer. It is worth nothing that the polyhydrohyalkanoate structural and regulatory *phaI* gene was also up-regulated in cells grown with the jewelry wastewater and under nitrogen limiting conditions. The *phaI* gene is clustered together other genes involved in polyhydroxyalkanoates metabolism in *P. pseudoalcaligenes* CECT5344, which has been recently described as a cyanide-degrading bacterium with by-product (polyhydroxyalkanoates) formation capacity. Therefore, the strain CECT5344 could be used in bioremediation of industrial residues containing cyanide, while concomitantly generates by-products like polyhydroxyalkanoates with a biotechnological added value (Manso et al., 2015).

DNA microarrays of *P. pseudoalcaligenes* CECT5344 in response to sodium cyanide, the jewelry wastewater and nitrogen-limiting conditions, all three compared against ammonium, showed several up-regulated genes (Table 2; Table 8, Supplementary material) including the *nitC* gene that codes for the nitrilase NitC essential for cyanide assimilation in the strain CECT5344 (Estepa et al., 2012), the GCN5-*N*-acetyltransferase gene and other genes of unknown function that constitute a single transcriptional unit with the *nitC* gene. Also induced in cells grown in all three media were genes coding for GntR–MocR and pyridoxal phosphate (PLP)-dependent aminotransferases, biotin transport and synthesis, and the ABC-type cyanate transporter (*cynABD*) and cyanase (*cynS*) required for cyanate assimilation. The GntR–MocR proteins are transcriptional regulators containing a DNA-binding HTH motif and an aminotransferase domain. In MocR-like proteins, PLP is required as cofactor for both aminotransferase and regulation capacities. The most relevant evidence comes from *Streptomyces venezuelae* PdxR, which is involved directly in the regulation of pyridoxal phosphate synthesis (Rigali et al., 2002). However, relationship between cyanide, nitrogen starvation and the coenzymes PLP and biotin is still unkmown. Cyanate and cyanide assimilation/detoxification are separate pathways in *P. pseudoalcaligenes* CECT5344, since a mutant strain defective in the cyanase *cynS* gene uses cyanide and it is able to grow with this nitrogen source at a similar rate to that presented by the wild type strain (Luque-Almagro et al., 2008). However, cyanide and cyanate are indirectly connected, as suggests by the induction of the cyanate transporter and the cyanase genes in the cyanide-containing media. It has been postulated that in the presence of cyanide electrons blockage occurs in the respiratory electron transfer chain as a result of cyanide-inhibition of terminal oxidases and, as a consequence, free radicals and reactive oxygen species are produced. Oxidation of cyanide by these reactive oxygen species can lead to the formation of cyanate (Sarla et al., 2004). Interestingly, the nitrilase *nitC* gene was up-regulated in cells grown in cyanide-containing media (NaCN and CN-WW) and also in nitrogen starvation conditions. Furthermore, the *nitC* gene expression pattern was similar to that shown by genes involved in assimilation of other nitrogenous compounds, such the *nas* genes (nitrate/nitrite assimilation) and the *gln*/*glt* genes (ammonia assimilation), suggesting that the *nitC* gene functions as part of an assimilatory pathway to use organic nitriles as nitrogen source for growth. Therefore, cyanide-assimilation conditions may provoke nitrogen-limitation responses, as occurs when nitrate and other alternative nitrogen sources are used. This finding has been described in a previous proteomic analysis (Luque-Almagro et al., 2007) and it is also supported by the present microarray study and additional microarray data described in other bacteria (Silberbach et al., 2005), showing induction of genes encoding glutamine synthetase, nitrate reductase and ammonium transport. Considering other nitrilase genes, the *nit3* gene was induced exclusively by sodium cyanide whereas the *nit1* and *nit4* genes were induced with sodium cyanide and cyanide-containing jewelry wastewater. The fact that these nitrilases genes (*nit1*, *nit3* and *nit4*) were not induced in *P. pseudoalcaligenes* CECT5344 cells grown under nitrogen star-

vation conditions, in contrast to the *nitC* gene, suggests a role for these three nitrilases in detoxification of cyanide rather than in assimilation of this nitrogenous compound. Although studying the neighborhood of a specific gene can be useful to predict a possible gene function, this is not the case of the nitrilases *nit1* and *nit3* since they display surrounding genes of unknown function. However, the nitrilase *nit4* gene is clustered together other genes that were induced by sodium cyanide and the jewelry residue, such as the *cioB* gene for cyanide-insensitive respiration (Wibberg et al., 2014). The *aguB* gene (BN5_0258) also codes for a member of the nitrilase/C–N hydrolase superfamily that is not considered a proper nitrilase (Podar et al., 2005). This gene was specifically repressed in cells grown with the jewelry wastewater. It is worth nothing that these five putative nitrilase–superfamily proteins of *P. pseudoalcaligenes* CECT5344 do not share significant sequence homology (Luque-Almagro et al., 2013).

5. Concluding remarks

DNA microarrays of *P. pseudoalcaligenes* CECT5344 in response to cyanide confirmed the essential role of several genes previously established by mutational analysis, including the nitrilase *nitC*-encoding gene for cyanide assimilation, and the *cioAB* genes for cyanide insensitive respiration. The induction by cyanide of these genes, which are essential for the strain CECT5344 to survive in the presence of cyanide, validates the DNA microarrays data presented in this work. Furthermore, under nitrogen starvation conditions were induced several genes like *glnK*, *ntrB* and *amtB* previously described in different bacteria to be induced when the nitrogen concentration in the media is scarce. DNA microarray data have been also validated by qPCR analysis of four nitrilases genes differentially expressed in *P. pseudoalcaligenes* CECT5344 according to DNA microarrays. Several genes up-regulated by cyanide (sodium cyanide and the cyanide-containing jewelry wastewater) might have a relevant role in cyanide assimilation/detoxification, which will require future work in order to optimize bioremediation of industrial wastes containing high concentration of cyanide. It is worth highlight that is of special interest to further investigate the link between cyanide degradation and carbon metabolism, especially with polyhydroxyalkanoates production. The relevance of metal extrusion systems in detoxification of the jewelry wastewater has been also revealed in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.09.032.

References

Akcil, A., Mudder, T., 2003. Microbial destruction of cyanide wastes in gold mining: process review. Biotechnol. Lett. 25, 445–450.
Bellapadrona, G., Ardini, M., Ceci, P., Stefanini, S., Chiancone, E., 2010. Dps proteins prevent Fenton-mediated oxidative damage by trapping hydroxyl radicals within the protein shell. Free Rad. Biol. Med. 48, 292–297.
Crosa, J.H., Walsh, C.T., 2002. Genetics and assembly line enzymology of siderophore biosynthesis. Microbiol. Mol. Biol. Rev. 66, 223–249.
Dash, R.R., Gaur, A., Balomajumder, C., 2009. Cyanide in industrial wastewaters and its removal: a review on biotreatment. J. Hazard. Mater. 163, 1–11.
Esclapez, J., Pire, C., Camacho, M., Bautista, V., Martínez-Espinosa, R.M., Zafrilla, B., Vegara, A., Alcaraz, L.A., Bonete, M.J., 2015. Transcriptional profiles of *Haloferax mediterranei* based on nitrogen availability. J. Biotechnol. 193, 100–107.
Estepa, J., Luque-Almagro, V.M., Manso, I., Escribano, M.P., Martínez-Luque, M., Castillo, F., Moreno-Vivián, C., Roldán, M.D., 2012. The nit1C gene cluster of *Pseudomonas pseudoalcaligenes* CECT5344 involved in assimilation of nitriles is essential for growth on cyanide. Environ. Microbiol. Rep. 4, 324–326.
Gaupp, R., Ledala, N., Somerville, G.A., 2012. Staphylococcal response to oxidative stress. Front. Cell. Inf. Microbiol. 2, 33, http://dx.doi.org/10.3389/fcimb.2012.00033.
Gerike, U., Hough, D.W., Russell, N.J., Dyall-Smith, M.L., Danson, M.J., 1998. Citrate synthase and 2-methylcitrate synthase: structural, functional and evolutionary relationships. Microbiology 144, 929–935.
Gupta, N., Balomajumder, C., Agarwal, V.K., 2010. Enzymatic mechanism and biochemistry for cyanide degradation: a review. J. Hazard. Mater. 176, 1–13.
Gutiérrez, P., Michael, Y.L., Osborne, J., Pomerantseva, E., Liu, Q., Gehring, K., 2005. Solution structure of the carbon storage regulator protein CsrA from *Escherichia coli*. J. Bacteriol. 187, 496–3501.
Hervás, A.B., Canosa, I., Santero, E., 2008. Transcriptome analysis of *Pseudomonas putida* in response to nitrogen availability. J. Bacteriol. 190, 416–420.
Huertas, M.J., Sáez, L.P., Roldán, M.D., Luque-Almagro, V.M., Martínez-Luque, M., Blasco, R., Castillo, F., Moreno-Vivián, C., García-García, I., 2010. Alkaline cyanide degradation by *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor. Influence of pH. J. Hazard. Mater. 179, 72–78.
Jünemann, S., 1997. Cytochrome bd terminal oxidase. Biochim. Biophys. Acta 1321, 107–127.
Kumar, M.S., Mishra, R.S., Jadhav, S.V., Vaidya, A.N., Chakrabarti, T., 2011. Simultaneous degradation of cyanide and phenol in upflow anaerobic sludge blanket reactor. J. Environ. Sci. Eng. 53, 277–280.
Luque-Almagro, V.M., Blasco, R., Huertas, M.J., Martínez-Luque, M., Moreno-Vivián, C., Castillo, F., Roldán, M.D., 2005a. Alkaline cyanide biodegradation by *Pseudomonas pseudoalcaligenes* CECT5344. Biochem. Soc. Trans. 33, 168–169.
Luque-Almagro, V.M., Huertas, M.J., Martínez-Luque, M., Moreno-Vivián, C., Roldán, M.D., García-Gil, J., Castillo, F., Blasco, R., 2005b. Bacterial degradation of cyanide and its metal complexes under alkaline conditions. Appl. Environ. Microbiol. 71, 940–947.
Luque-Almagro, V.M., Huertas, M.J., Roldán, M.D., Moreno-Vivián, C., Martínez-Luque, M., Blasco, R., Castillo, F., 2007. The cyanotrophic bacterium *Pseudomonas pseudoalcaligenes* CECT5344 responds to cyanide by defence mechanism against iron deprivation, oxidative damage and nitrogen stress. Environ. Microbiol. 9, 1541–1549.
Luque-Almagro, V.M., Huertas, M.J., Sáez, M.P., Martínez Luque-Romero, M., Moreno-Vivian, C., Castillo, F., Roldan, M.D., Blasco, R., 2008. Characterization of the *Pseudomonas pseudoalcaligenes* CECT5344 cyanase, an enzyme that is not essential for cyanide assimilation. Appl. Environ. Microbiol. 74, 6280–6288.
Luque-Almagro, V.M., Blasco, R., Martínez-Luque, M., Moreno-Vivián, C., Castillo, F., Roldán, M.D., 2011a. Bacterial cyanide degradation is under review: *Pseudomonas pseudoalcaligenes* CECT5344, a case of an alkaliphilic cyanotroph. Bio. Chem. Soc. Trans. 39, 269–274.
Luque-Almagro, V.M., Gates, A.J., Moreno-Vivián, C., Ferguson, S.J., Richradson, D.J., Roldán, M.D., 2011b. Bacterial nitrate assimilation: gene distribution and regulation. Bio. Chem. Soc. Trans. 39, 1838–1843.
Luque-Almagro, V.M., Merchán, F., Blasco, R., Igeño, M.I., Martínez-Luque, M., Moreno-Vivián, C., Castillo, F., Roldán, M.D., 2011c. Cyanide degradation by *Pseudomonas pseudoalcaligenes* CECT5344 involves a malate: quinone oxidoreductase and an associated cyanide-insensitive electron transfer chain. Microbiology—SGM 157, 739–746.
Luque-Almagro, V.M., Acera, F., Igeño, M.I., Wibberg, D., Roldán, M.D., Sáez, L.P., Hennig, M., Quesada, A., Huertas, M.J., Blom, J., Merchán, F., Escribano, M.P., Jaenicke, S., Estepa, J., Guijo, M.I., Martínez-Luque, M., Macías, D., Szczepanowski, R., Becerra, G., Ramírez, S., Carmona, M.I., Gutiérrez, O., Manso, I., Pühler, A., Castillo, F., Moreno-Vivián, C., Schlüter, A., Blasco, R., 2013. Draft whole genome sequence of the cyanide-degrading bacterium *Pseudomonas pseudoalcaligenes* CECT5344. Environ. Microbiol. 15, 253–270.
Manso, M.I., Ibáñez, M.I., de la Peña, F., Sáez, L.P., Luque-Almagro, V.M., Castillo, F., Roldán, M.D., Prieto, M.A., Moreno-Vivián, C., 2015. *Pseudomonas pseudoalcaligenes* CECT5344, a cyanide-degrading bacterium with by-product (polyhydroxyalkanoates) formation capacity. Microb. Cell Fact. 14, 77–88.
Nies, D.H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. 27, 313–339.
Park, S., Ely, R.L., 2008. Candidate stress genes of *Nitrosomonas europaea* for monitoring inhibition of nitrification by heavy metals. Appl. Environ. Microbiol. 74, 5475–5482.
Park, S., Ely, R.L., 2009. Whole-genome transcriptional and physiological responses of *Nitrosomonas europaea* to cyanide: identification of cyanide stress response genes. Biotechnol. Bioeng. 102, 1645–1653.
Podar, M., Eads, J.R., Richradson, T.H., 2005. Evolution of a microbial nitrilase gene family: a comparative and environmental genomic study. BMC Evol. Biol. 5, 42–54.
Quesada, A., Guijo, M.I., Merchán, F., Blázquez, B., Igeño, M.I., Blasco, R., 2007. Essential role of cytochrome bd-related oxidase in cyanide resistance of *Pseudomonas pseudoalcaligenes* CECT5344. Appl. Environ. Microbiol. 73, 5118–5124.
Rigali, S., Derouaux, A., Giannotta, F., Dusart, J., 2002. Subdivision of the Helix-Turn-Helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies. J. Biol. Chem. 277, 12507–12515.

Sambrook, J., Russel, D.W., 2001. A laboratory manual. In: Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sarla, M., Pandit, M., Tyagi, D.K., Kapoor, J.C., 2004. Oxidation of cyanide in aqueous solution by chemical and photochemical process. J. Hazard. Mater. 116, 49–56.

Silberbach, M., Hüser, A., Kalinowski, J., Pühler, A., Walter, B., Krämer, R., Burkovski, A., 2005. DNA microarray analysis of the nitrogen starvation response of *Corynebacterium glutamicum*. J. Biotechnol. 119, 357–367.

Smith, K.F., Bibb, L.A., Schmitt, M.P., Oram, D.M., 2009. Regulation and activity of a zinc uptake regulator, Zur, in *Corynebacterium diphtheria*. J. Bacteriol. 191, 1595–1603.

Thieme, D., Grass, G., 2010. The Dps protein of *E. coli* is involved in copper homeostasis. Microbiol. Res. 165, 108–115.

Wibberg, D., Luque-Almagro, V.M., Igeño, M.I., Bremges, A., Roldán, M.D., Merchán, F., Sáez, L.P., Guijo, M.I., Manso, M.I., Macías, D., Cabello, P., Becerra, G., Ibáñez, M.I., Carmona, M.I., Escribano, M.P., Castillo, F., Sczyrba, A., Moreno-Vivián, C., Blasco, R., Pühler, A., Schlüter, A., 2014. Complete genome sequence of the cyanide-degrading bacterium *Pseudomonas pseudoalcaligenes* CECT5344. J. Biotechnol. 175, 67–68.