The genome of the Antarctic polyextremophile *Nesterenkonia* sp. AN1 reveals adaptive strategies for survival under multiple stress conditions

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Running Head: Polyextremophily of Antarctic *Nesterenkonia*

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Abstract

Nesterenkonia sp. AN1 is a polyextremophile isolated from Antarctic desert soil. Genomic analyses and genome comparisons with three mesophilic *Nesterenkonia* strains indicated that the unique genome fraction of *Nesterenkonia* sp. AN1 contains adaptive features implicated in the response to cold stress including modulation of membrane fluidity as well as response to cold-associated osmotic and oxidative stress. The core genome also encodes a number of putative cold stress response proteins. RNA-Seq-based transcriptome analyses of *Nesterenkonia* sp. AN1 grown at 5 ºC and 21ºC showed that there was significant induction of transcripts that code for antioxidants at 5 ºC, demonstrated by the upregulation of *sodA*, *bcp* and *bpoA2*. There was also overexpression of universal stress protein genes related to *uspA*, along with genes encoding other characterised cold stress features. Genes encoding the two key enzymes of the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (AceB) were induced at 5 ºC, suggesting possible adaptation strategies for energy metabolism in cold habitats. These genomic features may contribute to the survival of *Nesterenkonia* sp. AN1 in arid Antarctic soils.

Key words: Genomics, Transcriptomics, Polyextremophile, Psychrotolerant, Antarctic soil, *Nesterenkonia*

Introduction

Terrestrial Antarctica is the coldest environment on Earth (Barrett *et al.*, 2006, Cary *et al.*, 2010, Cowan *et al.*, 2014). Much of the continent is covered permanently by ice, and it is estimated that ice- free soils constitute only 0.32 - 0.4 % of the continental landmass (Chan *et al.*, 2013, Hopkins *et al.*, 2006, Ugolini and Bockheim, 2008). These soils are characterised by a wide range of harsh physico-geochemical conditions including extreme aridity, low temperature, extremes of pH, high salinity, high ultra-violet irradiation and nutrient deficiency (Aislabie *et al.*, 2006, Chan *et al.*, 2013). Despite the harsh conditions of Antarctic soils, they harbour large and diverse microbial communities (Bottos *et al.*, 2014, Cary *et al.*, 2010), predominantly in niches shielded from direct impact of environmental stresses (Cowan *et al.*, 2014). These microorganisms have evolved adaptive features to counteract physicochemical constraints that are placed on their cellular function by cold and associated stresses (De Maayer *et al.*, 2014, Mesbah and Wiegel, 2008). Some of the consequences of cold stress include decreased membrane fluidity and disruption of cellular transport, decreased rates of enzyme activity and restraints on the regulation of replication, transcription, translation and posttranslational processes (D'Amico *et al.*, 2006, De Maayer *et al.*, 2014). Low temperature also increases the vulnerability of microorganisms to the toxic effects of reactive oxygen species (Chattopadhyay, 2006, Medigue *et al.*, 2005).

The genus *Nesterenkonia* is broadly comprised of mesophilic moderate haloalkaliphiles in the family Micrococcaceae (Stackebrandt *et al.*, 1995). *Nesterenkonia* spp. are generally aerobic, catalase positive, chemo-organotrophic and haloalkaliphilic (Collins *et al.*, 2002, Li, 2005, Stackebrandt *et al.*, 1995). They are usually coccoid or rod-shaped, with or without branching, non-spore forming, and non-encapsulated (Li *et al.*, 2005, Stackebrandt, 1995). The genomic DNA is characterized by a high G+C content of 64% to 72% (Li *et al.*, 2005).

At present, thirteen species have been validly described (Parte, 2013). Strains have been isolated from cotton and paper mills (Luo *et al.*, 2008, Luo *et al.*, 2009), fermented seafood (Yoon *et al.*, 2006), as well as the faeces of an AIDS patient (Edouard *et al.*, 2014).Several strains have been isolated from extreme environments such as hyper-saline and soda lakes (Collins *et al.*, 2002, Delgado *et al.*, 2006), hot deserts and saline soils (Li *et al.*, 2005, Li *et al.*, 2004, Li *et al.*, 2008). The association of some *Nesterenkonia* strains with extreme niches highlights their potential as sources of biologically active molecules such as halophilic α-amylases and thermostable xylanases (Kui *et al.*, 2010, Shafiei *et al.*, 2012) and as models for understanding microbial adaptation strategies in harsh environments.

Nesterenkonia sp. AN1 is the first reported member of the genus isolated from Antarctic desert soil (Nel *et al.*, 2011). The cells are coccoid, non-motile, non-spore forming and form orange-to red pigmented colonies on solid media (Nel, 2009). The strain shows phenotypic characteristics of a haloalkaliphile (grows optimally at 0-15% wt/vol NaCl and pH 9-10) and is also psychrotolerant (optimal growth temperature ~21 ºC) (Nel *et al.*, 2011).

As has been observed in other bacterial isolates from cold desert soils (Dsouza *et al.*, 2014, Dsouza *et al.*, 2015), *Nesterenkonia* sp. AN1 survives temperatures well below the optimum growth temperature of psychrophiles (15 ^{o}C) but has an optimal growth temperature of 21 ^{o}C, and is hence classified as ‘psychrotolerant’ (Nel *et al.*, 2011). On the basis of fitness, Vincent, 2000 categorised microorganisms living in Antarctica into specialist and generalist. The former are adapted to survive optimally under the Antarctic ambient environmental conditions. The generalists are further classified into two types, those surviving sub-optimally (Type I) and those capable of adjusting between optimal and suboptimal growth (Type II) depending on the prevailing environmental condition (Vincent, 2000). The properties of *Nesterenkonia* AN1 suggest that this organism can be classified as a ‘generalist’ Type 1.

In an effort to gain insight into the survival mechanisms of *Nesterenkonia* sp. AN1, the genome of this strain was recently sequenced (Aliyu *et al.*, 2014). Here we attempt, by means of genomic, comparative genomic and transcriptomic analyses, to elucidate the genetic determinants of the adaptive strategies employed by *Nesterenkonia* sp. AN1 for survival in the cold desert soils of Antarctica.

Materials and Methods

Genome sequences and comparative genomics

The draft genome sequence of *Nesterenkonia* sp. AN1 and those of three mesophilic *Nesterenkonia* strains were included in this study (Table 1). The tRNA coding sequences in the draft genomes of the four *Nesterenkonia* strains were predicted using ARAGORN (Laslett and Canback, 2004). The genomes were structurally annotated using the FgenesB open reading frame (ORFs) prediction application (Solovyev and Salamov, 2011). The genomes were also scanned for genomic islands (GIs) using IslandViewer 3 (Dhillon *et al.*, 2015). For comparison purposes, the protein coding sequences (CDSs) sets for each genome were standardized through a pairwise local BLASTP analysis using Bioedit v 7.2.5 (Hall, 1999). Amino acid composition of the predicted proteins in the four strains was computed using Bioedit v 7.2.5 (Hall, 1999). A reciprocal blast best hit (RBBH) approach was used to determine orthologs between the translated CDS sets for each pairs of strains (Moreno-Hagelsieb and Latimer, 2008). Orthologs were assigned on the basis of amino acid identity and sequence coverage cut-off values of >30% and >70%, respectively. Orthologous CDSs that are present in all four strains were assigned to the core genome. The remaining CDSs common to only three or two strains and those present in only one strain were assigned to the appropriate accessory genome fractions. Functional annotation was subsequently computed by localised BLASTP analysis against the multiple alignment of the Actinobacteria non-supervised orthologous groups (actNOG) obtained from the Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG v3.0) database (Powell *et al.*, 2012).

Transcriptome analysis

Growth conditions

Nesterenkonia sp. AN1 was isolated from soil samples obtained from Miers Valleys, a maritime valley of the McMurdo Dry Valley region of eastern Antarctica as described (Nel *et al.*, 2011). The isolate was grown in triplicate in 20 ml modified Luria-Bertani (mLB) medium that comprised of 10 mg/mL tryptone, 5 mg/mL yeast extract and 50 mg/mL NaCl adjusted to pH 8.5. The culture was incubated in a rotatory shaker (180 rpm) at 21 ºC until an optical density (OD_{600}) of 0.5 was obtained. Cells were pelleted by centrifugation (6010 x g, 5 minutes) at 21^{o}C. The pellets were re-suspended in 1.5 ml of mLB broth. 0.25 µl of the cells was transferred in triplicate to freshly prepared 50 ml of mLB broth. These were incubated in a rotatory shaker (180 rpm) at the optimum growth temperature of 21 ºC and the test temperature of 5 ºC to an exponential growth phase; absorbance value (OD_{600}) of 0.5 (approximately similar cell numbers for the two temperature regimes). The cultures attained the OD600 value of 0.5 in ~ 24 hours and ~ 106 hours for the 21 ºC and 5 ºC, respectively (Supplementary Figure S1). Based on data from previous growth experiments (Nel *et al.*, 2011) which have been validated, 21 ºC is the optimal growth temperature. Our growth studies showed that *Nesterenkonia* sp. AN1 grew well at 5 ºC, but that slow growth rates at temperatures below this would constrain subsequent expression comparisons against the strain grown at the optimum temperature.

RNA preparation

Total RNA was extracted from two biological replicate samples of each treatment condition. The bacterial cultures (20 ml from each replicate) were pelleted (6010 x g, 5 minutes) at 5 ºC or 21 ºC based on the treatment conditions. The cell pellets were re-suspended in 1 ml TRI Reagent® (Zymo Research Corp., USA). RNA extraction was performed using the Direct-zol™ RNA Mini Prep kits (Zymo Research Corp., USA) according to the manufacturer's instructions with some modifications. Briefly, the cells were re-suspended in 1ml TRI Reagent® and then transferred to ZR Bashing Bead^{TM} Lysis Tubes. The cells were lysed using a PowerLyser^{TM} (MO Bio Lab. Inc., USA) at 2,000 rpm for one minute. The homogenate was centrifuged at (6010 x g, 1 minute) and the supernatant transferred into RNAse-free 1.5ml tubes DNase I treatment and subsequent RNA purification were performed as per the manufacturer's protocol. Preliminary quality assessment on of the extracted RNA was performed using NanoDrop. The RNA was preserved frozen at - 80 ºC.

Illumina sequencing

RNA sequencing was performed using the Illumina MiSeq platform at Inqaba Biotech, South Africa. The basic workflow involved selective enrichment of mRNA by depleting the ribosomal RNA using Ribo-Zero^{TM} rRNA removal kit (Epicentre, USA) and RNA quality assessment using Agilent Bioanalyzer ^{TM}. Illumina paired - end libraries were prepared according to the manufacturer's instructions. The samples were multiplexed and sequenced in a single lane of Illumina Miseq platform (600 cycles with an expected data yield of 1.2GB).

RNA-Seq analysis

The paired end sequencing read datasets were analysed using the CLC Genomics Workbench 7.5, using the draft genome of *Nesterenkonia* sp. AN1 as a reference. The reads were mapped against the reference genome using the default mapping options. Differentially expressed genes were assessed using the Empirical analysis of differentially expressed genes (EDGE) tool with a stringent filter absolute fold change of > 1.5, false discovery rate (FDR) < 0.05 and Bonferreni correction for multiple testing $p < 0.05$. The RNA-Seq data have been deposited in the NCBI GEO repository (Barrett *et al.*, 2013) under the accession number GSE71132. Functional annotation of the overexpressed genes was done using clusters of orthologous genes (COG) (Tatusov *et al.*, 2003), assessed via WebMGA (Wu *et al.*, 2011), in combination with an in house adapted multiple alignment of actNOG obtained from the Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG v3.0) database (Powell *et al.*, 2012). The differentially expressed genes were assessed for gene ontology (GO) term enrichment using the web-based GO enrichment toolkit GOEAST (Zheng and Wang, 2008). The upregulated and downregulated transcripts associated with GO terms were tested separately against the *Nesterenkonia* sp. AN1 GO annotated gene set (background) using the default setting in GOEAST (Zheng and Wang, 2008).

Results and Discussion

General genome features of Antarctic *Nesterenkonia* sp. AN1

The draft genomes of four *Nesterenkonia* strains are publicly available (Table 1). The genomes range in size from 2.59 to 3.04 Mb, with G + C contents of 62.2-71.5%. *Nesterenkonia* sp. AN1 has the largest genome (~3.04 Megabases) with a mean G + C content of 67.4% and encodes 2,853 proteins (Aliyu *et al.*, 2014).

Genomic features that may distinguish psychrophilic microorganisms from their mesophilic relatives include the number and variety of transfer RNA species as well as amino acid usage (De Maayer *et al.*, 2014). In general, proteins and enzymes in psychrophilic bacteria show a reduction in those amino acids which impose conformational restrictions and a corresponding increase in those associated with increased conformational flexibility required for enzyme functioning in cold environments (Siddiqui *et al.*, 2013). Comparison of amino acid usage between several psychrophiles and mesophiles revealed that amino acids which do not preferentially form helices, such as alanine (A), aspartic acid (D), glycine (G), serine (S) and tyrosine (T), are preferred in psychrophiles (Metpally and Reddy, 2009). Conversely, those favouring helix formation, such as glutamine (E), phenylalanine (F), lysine (K) and leucine (L), are avoided (Metpally and Reddy, 2009). Analysis of the amino acids composition of *Nesterenkonia* sp.AN1 proteins revealed high proportions of both the preferred amino acids A, D, G, S and T and the unfavoured amino acids E and L (Figure 1a). Similar patterns of amino acid usage were observed in the three mesophilic *Nesterenkonia* strains (Figure 1a). Comparative genomic analyses of temperate and Antarctic *Arthrobacter* strains also revealed high frequencies of A, G, L and V in both groups (Dsouza *et al.*, 2015). The abundance of both preferred and unfavoured amino acids in the proteome of psychrotolerant bacteria isolated from Antarctic soils might be an important strategy for coping with the wide daily and seasonal fluctuations in temperature reported in the Antarctic McMurdo Dry Valley area (Aislabie *et al.*, 2006, Dreesens *et al.*, 2014).

Microorganisms adapted to low temperatures typically contain a higher number and diversity of tRNA species, an adaptation which serves to counteract the slow mobilization of tRNA species to translation sites (Math *et al.*, 2012, Satapathy *et al.*, 2010, Sharp *et al.*, 2005). Comparative

analysis of tRNA species, predicted using ARAGORN v1.2.361 (Laslett and Canback, 2004), revealed that *Nesterenkonia* sp. AN1 has a higher number of tRNA genes (fifty five) than its mesophilic counterparts (between forty-seven and fifty) (Table 1). The most common tRNA species in all four *Nesterenkonia* strains were $tRNA^{Ala}$, $tRNA^{Glu}$, $tRNA^{Gly}$, $tRNA^{Lys}$, $tRNA^{Arg}$ and $tRNA^{Ser}$ (Figure 1b). A pyrrolysine tRNA ($tRNA^{Pyl}$) was exclusively predicted in *Nesterenkonia* sp. AN1 but absent in the mesophilic strains. Pyrrolysine is the 22^{nd} proteinogenic amino acid, encoded by the stop codon UAG, and is essential for methanogenesis from methylamines (Ambrogelly *et al.*, 2007, Have *et al.*, 2013, Gaston *et al.*, 2011b). This amino acid, which was initially thought to be restricted to the archaeal genus *Methanosarcina* and only one bacterium *Desulfitobacterium hafniense*, has recently been identified in six archaeal and six bacterial genomes (Gaston *et al.*, 2011a, Srinivasan *et al.*, 2002).

The *Nesterenkonia* sp. AN1 genome reveals several adaptive strategies against multiple stresses

Cold stress in Antarctic desert soils is invariably associated with the effects of desiccation and sometimes with extremes of pH, high UV radiation, nutrient deficiency and salinity (De Maayer *et al.*, 2014, Tehei and Zaccai, 2005). A number of adaptive genes have been linked to the different environmental stresses associated with cold environments (Berger *et al.*, 1996, Gao *et al.*, 2006, Medigue *et al.*, 2005, Methe *et al.*, 2005, Overbeek *et al.*, 2014, Riley *et al.*, 2008). Analysis of the draft genome of *Nesterenkonia* sp. AN1 revealed a total of 189 putative stress response genes. These include 50 genes linked to cold stress response, 47 genes for oxidative stress response and 59 genes for osmotic stress response (Supplementary Table S1).

Cold stress response

An important component of the cold stress adaptive strategies of microorganisms is the rapid production of cold-inducible proteins (Phadtare *et al.*, 1999). These are classified into two groups: cold shock proteins (CSPs) which are rapidly and transiently induced after a downshift in temperature, and cold acclimation proteins (CAPs) which are continuously expressed throughout the period of low temperature exposure (Phadtare, 2004, Phadtare *et al.*, 1999). The genome of *Nesterenkonia* sp. AN1 encodes twenty putative cold shock and acclimation proteins (Supplementary Table S1). These include orthologs of the cold shock proteins CspA and CspC. CspA and other cold-inducible CspA-like proteins have been shown to act as RNA chaperones which destabilise mRNA secondary structures, thereby enhancing translation efficiency at low temperatures (Chaikam and Karlson, 2010, Rabus *et al.*, 2004). The genome also contains genes which encode the secondary cold shock proteins polyribonucleotide nucleotidyltransferase (PNPase), ribosome-binding factor A (RbfA), translation initiation factor (Inf2) and transcription elongation protein (NusA), which are typically induced via transcription antitermination (Bae *et al.*, 2000). Transcription antitermination, which is mediated by CspA, prevents the formation of hairpin loops in nascent transcripts and enhances the production of intact transcripts (Bae *et al.*, 2000, Chaikam and Karlson, 2010, Phadtare *et al.*, 2007). The secondary cold shock proteins are known to be essential for survival of some bacteria at low temperature (Rath *et al.*, 2009). For instance, in the psychrotolerant bacterium *Yersinia enterocolitica* W22703, mutants disrupted in the PNPase gene (*pnp*) failed to grow at 5°C (Goverde *et al.*, 1998).

The *Nesterenkonia* sp. AN1 genome also codes for the CspA-like protein CapA, reported to be rapidly synthesised and remain overexpressed after persistent exposure to cold (Berger *et al.*, 1997). Continuous expression of CapA during prolonged cold exposure is proposed to be one of the main distinguishing factors between psychrophiles and their mesophilic counterparts (Phadtare *et al.*, 1999).

Modulation of membrane fluidity is an essential adaptation strategy in cold environments. It involves increased production of unsaturated fatty acids, modification of fatty acid branched chains and shortening of fatty acyl chains (D'Amico *et al.*, 2006, Feller, 2003, Feller and Gerday, 2003). The *Nesterenkonia* sp. AN1 genome codes for nineteen proteins involved in fatty acid biosynthetic pathways (Supplementary Table S1). These include FabG and FabH, which catalyse the onset of the reduction reaction in fatty acid biosynthesis, the condensation of fatty acids and the synthesis of branched fatty acids (Hoang *et al.*, 2002, Methe *et al.*, 2005). The genome also codes for an orthologs of 1-acyl-sn-glycerol-3-

phosphate acyltransferase (PlsC), which catalyses conversion of intermediates in phospholipid synthesis, and 3-ketoacyl-(acyl-carrier-protein) reductases, which enhances the production of polyunsaturated lipids (Hoang *et al.*, 2002, Methe *et al.*, 2005).

Pigments play a major role as modulators of membrane fluidity at low temperatures (Chintalapati *et al.*, 2004, Dieser *et al.*, 2010, Morgan-Kiss *et al.*, 2006). A temperature-dependent increase in the biosynthesis of the C-50 carotenoid bacterioruberin and related glycosides has been reported in the psychrophilic bacterium *Arthrobacter agilis* (Fong *et al.*, 2001). The genome of *Nesterenkonia* sp. AN1 contains eleven genes with putative roles in carotenoid biosynthesis (Supplementary Table S1). These include *crtB* (phytoene synthase), *crtEb* (lycopene elongase), *crtN* (phytoene dehydrogenase) *and crtYef* (C50 carotenoid epsilon cyclase) which, on the basis of orthology, are predicted to be involved in the production of decaprenoxanthin and lycopene (Klassen, 2010).

Oxidative stress response

Various metabolic processes result in endogenous build-up of toxic hydrogen peroxide and other forms of reactive oxygen species (ROS) (Bayr, 2005). The increased solubility of gasses at low temperatures makes microorganisms inhabiting cold environments more prone to the toxic effects of ROS (Chattopadhyay, 2006, D'Amico *et al.*, 2006).

The second largest group of putative stress adaptation genes (24.4% of the total predicted stress genes) in the *Nesterenkonia* sp. AN1 genome can be linked to oxidative stress response (Supplementary Table S1). Putative antioxidants encoded by *Nesterenkonia* sp. AN1 include two catalases (KatB; KatE), two superoxide dismutases (SodA; SodC), a thiol peroxidase (Bcp) as well as thioredoxin and thioredoxin reductase (TrxA and TrxB). Superoxide dismutase catalyses the conversion of superoxide anion to hydrogen peroxide which subsequently undergoes disproportionation through a reaction that is catalysed by catalases and peroxidases (Ebara and Shigemori, 2008, Moustafa *et al.*, 2010). TrxA and TrxB, while maintaining the intracellular thiol-disulfide balance, also scavenge reactive oxygen species (Ballal and Manna, 2010). The *Nesterenkonia* sp. AN1 genome also codes for an ortholog of methionine sulfoxide reductase (MsrA) which reduces the vulnerability of methionine to ROS (Weissbach *et al.*, 2005). Seven putative dioxygenases were also identified in the genome of *Nesterenkonia* sp. AN1. The presence of multiple dioxygenases in the marine Antarctic *Pseudoalteromonas haloplanktis* TAC125, the genome of which encodes sixteen dioxygenases, has been predicted to play a role in resistance against ROS damage (Medigue *et al.*, 2005).

Osmo-protection

Microbial response to osmotic stress involves two major mechanisms: the salt-in strategy, involving the uptake of inorganic ions to balance the salinity gradient created by the high salt concentration in the external environment, and the accumulation of compatible solutes which are either synthesised or taken up from the environment (Mesbah and Wiegel, 2008, Oren, 2008, Oren, 2009). Compatible solutes play a dual role in stress response as both osmolytes and cryo-protectants (Welsh, 2000). The genome of *Nesterenkonia* sp. AN1 encodes a repertoire of proteins involved in both strategies (Supplementary Table S1). The genome contains two loci coding for the Ktr potassium ion uptake system implicated in the salt-in mechanism (Corratgé-Faillie *et al.*, 2010). Similarly, the genome encodes transporters for glycine/betaine, choline/betaine and proline, all of which are known osmo-protectants (Le Rudulier *et al.*, 1984). In addition, several genes involved in the endogenous synthesis of compatible solutes are present. These include the trehalose biosynthesis genes *otsA*, *otsB*, and *treS*, known to be cold-inducible and essential for low temperature survival of *Escherichia coli* (Kandror *et al.*, 2002).

General stress response

Beside specific factors for cold, osmotic and oxidative stress response and tolerance, the *Nesterenkonia* sp. AN1 genome encodes a large number of other stress-related proteins, which can be considered as elements of a general stress response system (Supplementary Table S1). At least nineteen genes putatively involved in SOS response and DNA repair systems were identified. The genome also encodes seven paralogs of the universal stress protein, UspA, which has been linked to cold acclimation (Garnier *et al.*, 2010). Seven UspA-like proteins were also reported in the genome of the obligate psychrophiles *Psychromonas ingrahamii* (Riley *et al.*, 2008).

Comparative genomics identifies unique stress response proteins in *Nesterenkonia* sp. AN1

Comparative analysis of the protein coding genes of closely related organisms from different environments is a valid approach for identifying adaptive determinants (MacLean *et al.*, 2009, Qin *et al.*, 2014). The protein-coding sequences (CDSs) identified in the *Nesterenkonia* sp. AN1 draft genome were pairwise-compared with those of three mesophilic *Nesterenkonia* strains using a reciprocal best BLAST hit (RBBH)approach (Moreno-Hagelsieb and Latimer, 2008). A total of 4,818 non-orthologous CDSs were encoded in the genomes of the four strains. Of these, 1,332 (~ 28 %) are shared by all four strains (Figures 2), constituting ~47% of the total CDSs in *Nesterenkonia* sp. AN1 and between ~ 52 and 56% in the other strains. Consistent with its larger genome size, *Nesterenkonia* sp. AN1 encodes the largest number of accessory CDSs (1,520).

Of the 2,852 CDSs in *Nesterenkonia* sp. AN1, approximately 90% could be classified according to the EGGNOG non-supervised orthologous groups functional categories (Powell *et al.*, 2012). A high proportion of proteins assigned to the poorly categorised class are encoded in the accessory genome fractions of *Nesterenkonia* sp. AN1 and the other strains. Conversely, the core CDSs are dominated by those involved in central metabolic processes. Approximately 58 % of the 189 putative stress adaptation genes identified in *Nesterenkonia* sp. AN1 are also present in genomes of the three mesophilic *Nesterenkonia* strains (Supplementary Table S1). These include nineteen of the twenty identified cold shock and cold acclimation proteins, eighteen of the nineteen DNA repair features and a large proportion of the osmotic and oxidative stress response proteins. This would suggest that members of the genus *Nesterenkonia* are inherently resilient to environmental stresses and potentially capable of adapting to multiple stress conditions. Comparisons of Antarctic strains of *Arthrobacter* and *Paenibacillus* with their temperate counterparts also revealed that most of these features are common to both groups (Dsouza *et al.*, 2014, Dsouza *et al.*, 2015).

The genome of *Nesterenkonia* sp. AN1 does, however, encode a large number of unique proteins potentially involved in oxidative, osmotic and general stress responses (Figure 3). These include the carotenoid proteins phytoene dehydrogenase (CrtN) and lycopene elongase (CrtEb) as well as six copies of 3-oxoacyl-[acyl-carrier protein] reductase (FabG), all of which may be associated with modulation of membrane fluidity at low temperature (Chintalapati *et al.*, 2004). The osmotic stress features unique to *Nesterenkonia* sp. AN1 include one of the two loci coding for the Ktr potassium uptake system, which resides in a 6.6 Kb genomic island that was detected using IslandViewer 3 (Dhillon *et al.*, 2015) and several genes encoding glycine/betaine/proline transport systems (Kappes *et al.*, 1999, Mykytczuk *et al.*, 2013). Similarly, unique oxidative stress tolerance features include genes encoding three putative dioxygen scavengers protocatechuate 3, 4-dioxygenase beta chain (PcaH), protocatechuate 3, 4-dioxygenase alpha chain (PcaG) and 4-hydroxyphenylpyruvate dioxygenase (HPPH) (D'Argenio *et al.*, 1999, Raspail *et al.*, 2011). Unique genomic elements also include general stress genes coding for the three sigma B regulatory proteins (RsbV, RsbW, RsbU) and four of the seven copies of the universal stress response protein (UspA) gene (Foster, 2005, Garnier *et al.*, 2010, Marles-Wright and Lewis, 2007, Utratna *et al.*, 2014).

RNA-Seq analyses of *Nesterenkonia* sp. AN1 transcriptome at 5 ºC and 21 ºC highlight potential novel mechanisms for low temperature growth

To provide additional insight into the adaptations of *Nesterenkonia* sp. AN1 to cold stress, the global RNA in *Nesterenkonia* sp. AN1 grown at 5 ºC and 21 ºC was sampled, sequenced and compared. RNA sequencing using Illumina MiSeq 600 chemistry produced 1,089,506 to 1,321,644 paired-end reads per sample, yielding ~ 651 Mb of data. Alignment of the reads to the *Nesterenkonia* sp. AN1 draft genome revealed that a total of 2,796 (> 97%) of the predicted *Nesterenkonia* sp. AN1 genes were expressed under both experimental conditions.

Analysis of the differentially expressed gene profiles revealed that 164 and 152 genes were significantly (FDR < 0.05) upregulated and downregulated, respectively, when *Nesterenkonia* sp. AN1 was grown at 5 ºC (Supplementary Table S2). Among the upregulated genes, there was an over-representation of genes involved in cell cycle control, cell division, chromosome partitioning, cell membrane biogenesis, posttranslational modification, protein turnover, chaperones and signal transduction (Figure 4). In contrast, the downregulated genes were dominated by those involved in information storage and processing, particularly those of the functional class DNA replication, recombination

and repair. Similarly, genes involved in amino acid, lipid and inorganic ion transport and metabolism were downregulated at 5 ºC. Fifty-five genes, representing ~31% of the genes upregulated at 5 ºC, were classified in the poorly characterized category.

Most of the genes coding for stress adaptation proteins (183 genes; 97% of the total adaptation genes) were expressed under both experimental conditions (5 ºC and 21 ºC). Of these, 155 were constitutively expressed (i.e., expressed at the same level under both conditions). Twenty-eight of the predicted adaptive genes were differentially expressed, with twelve downregulated and sixteen upregulated at 5 ºC. Surprisingly, the majority of the cold shock and acclimation features were constitutively expressed, with the *cspA* gene being downregulated at 5 ºC, suggesting that *cspA* is not cold-induced in *Nesterenkonia* sp. AN1. By contrast, *cspA*, has been shown to be upregulated transiently upon temperature downshift in *E. coli* (Polissi *et al.*, 2003). Similarly, the antioxidant thioredoxin gene *trxA* was downregulated at 5 ºC. This may, however, be compensated for by the significant upregulation of *trxC*, which produces thioredoxinTrx2, with an equivalent antioxidant function (Zeller and Klug, 2006). Additionally, there was significant upregulation at 5 ºC of *sodA* and *bcp* which encode the reactive oxygen species scavengers superoxide dismutase SodA and thiol peroxidase Bcp, respectively (Moustafa *et al.*, 2010). The induction of these antioxidants could be a strategy to deal with a possible spike in endogenous reactive oxygen species, resulting from the induction of molybdenum cofactor biosynthesis genes *moaA* and *moaB* (Mehta *et al.*, 2014, Regulski *et al.*, 2008), both of which were significantly induced at 5 ºC. Molybdoenzymes are commonly associated with catalytic activities that lead to the production of reactive oxygen species (Mendel and Bittner, 2006, Neumann and Leimkühler, 2010).

Four of the seven copies of *uspA*, three of which are unique among the *Nesterenkonia* spp. to AN1, are upregulated at 5 ºC. The *uspA* genes of the psychrotrophic bacterium *Lactococcus piscium* CNCM I-4031 have been reported to be cold-inducible (Garnier *et al.*, 2010). UspA and UspA-like proteins have also been reported to be associated with responses to several other stresses including carbon starvation, and osmotic and oxidative stresses (Phadtare and Inouye, 2001, Seifart Gomes *et al.*, 2011). Although these genes were induced in response to cold, other stress-related roles for the *Nesterenkonia* sp. AN1 *uspA* genes cannot be excluded.

To assess whether specific molecular mechanisms were associated with the differentially expressed genes, gene ontology (GO-term) enrichment analysis was performed using GOEAST (Zheng and Wang, 2008). The results showed that three and five molecular functions were significantly ($p \leq 0.05$) upregulated and suppressed at 5 ºC, respectively (Figure 5). There was significant ($p \leq 0.05$) induction of "antioxidant activity" (GO: 0016209) at 5 ºC. This was enriched by superoxide dismutase (*sodA*) and perioxiredoxin (*bcp*) genes which are prominent scavengers of ROS (Imlay, 2008, Moustafa *et al.*, 2010). Furthermore, two genes, coding for citrate lyase beta subunit (Mcl2) and isocitrate lyase (ICL), associated with oxo-acid-lyase activity (GO: 0016833) were significantly induced at 5 ºC. Isocitrate lyase has been shown to be upregulated in the psychrophilic bacteria, *Colwellia maris* (Watanabe *et al.*, 2002b, Watanabe *et al.*, 2002a) and *Psychrobacter cryohalolentis* K5 (Bakermans *et al.*, 2007) in response to cold. The malate synthase gene (*aceB*) was similarly upregulated. ICL and AceB are key enzymes of the glyoxylate cycle, present only in those microorganisms that are capable of utilising C_2 compounds (e.g. acetate and ethanol) or fatty acids as sole carbon sources for energy production (Berg *et al.*, 2002, Kunze *et al.*, 2006). Low temperature induction of these genes suggests a mechanism for energy acquisition by *Nesterenkonia* sp. AN1 in the nutrient-deficient soils of the Antarctic Dry Valleys.

Conclusions

Nesterenkonia sp. AN1 is the first reported cold-active member of the genus *Nesterenkonia*. The genome of this polyextremophilic bacterium encodes a repertoire of features linked to the stresses typical of its Antarctic edaphic origin. These include several proteins involved in osmotic and oxidative stress responses, modulation of membrane fluidity as well as cold stress and cold acclimation proteins. Comparison of the *Nesterenkonia* sp. AN1 genome with those of three mesophilic members of the genus showed that while some adaptive features were encoded on the strain-specific fraction of the genome, the majority of these features are also shared with its mesophilic counterparts, suggesting an inherent

resilience to environmental stresses for the genus. Evaluation of the transcripts of *Nesterenkonia* sp. AN1 grown at 5 ºC showed that a substantial number of the transcripts, some of which have not been previously characterised, were cold-inducible. These uncharacterised cold-induced transcripts may encode novel candidate cold adaptation proteins. Taken together, the combined data revealed the presence of pervasive adaptation strategies which likely determine niche specialization of *Nesterenkonia* sp. AN1 and its survival in the highly dynamic arid soils of Antarctica.

Acknowledgements

The authors gratefully acknowledge financial support from the National Research Foundation-South African National Antarctic Research Programme (NRF-SANAP) (Award No. 80256) and the Genomics Research Institute (GRI) University of Pretoria. HA acknowledges bursary support from the NRF.

Conflicts of interest

The authors have no conflict of interest to declare

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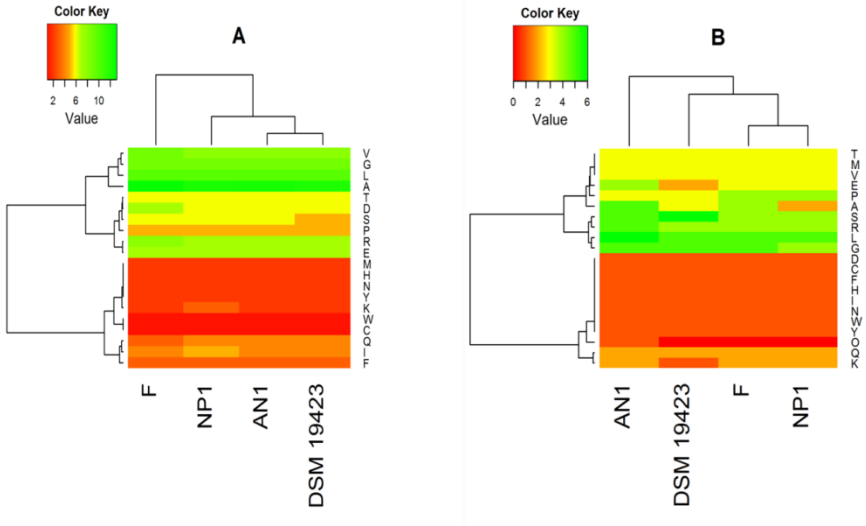

Figure 1 Heat maps showing (A) Comparison of amino acids proportions in proteins of four strains of *Nesterenkonia*. (B) Abundance of tRNA encoding genes in four strains of *Nesterenkonia*. A –Y represent the standard amino acids single letter codes. The colour key indicates the percentage of each of the amino acids (A) and each of the tRNA species (B) for each of the amino acids A-Y.

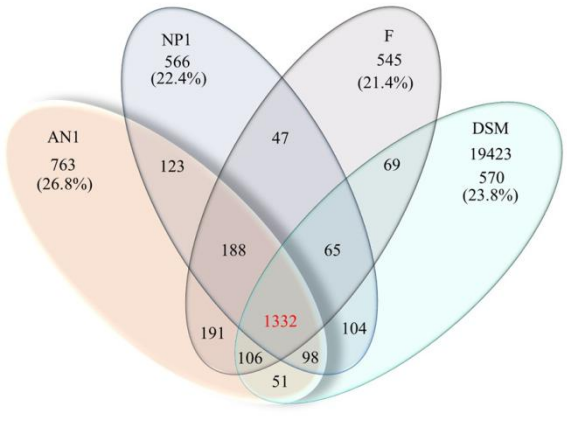

Figure 2 Comparative genomic analysis of four strains of Nesterenkonia based on orthologous relationships among the predicted proteins. The Venn diagram represents the pangenome of the four strains; *Nesterenkonia* sp. AN1, *Nesterenkonia* sp. NP1, *Nesterenkonia* sp. F and *N. alba* DSM 19423, respectively

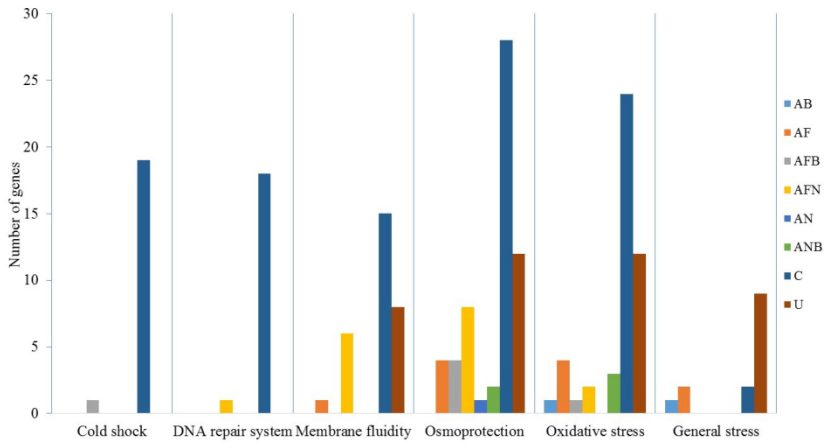

Figure 3 Number of adaptive genes associated with the different pan-genome fractions of *Nesterenkonia* strains. The letter U denotes the: *Nesterenkonia* sp. AN1 unique genome fraction; C represents the core genome fraction; A: *Nesterenkonia* sp. AN1, B: *N. alba* DSM 19423, N: *Nesterenkonia* sp. NP1 and F: *Nesterenkonia* sp. F

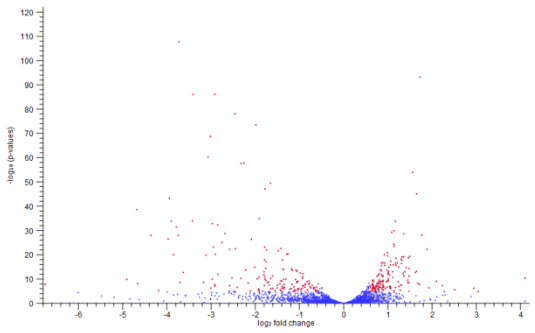

Figure 4 Volcano plot showing transcripts expression profile in *Nesterenkonia* sp. AN1 grown at 5 ºC and 21 ºC. Differentially expressed genes at 5 ºC are represented by the red dots, while blue dots are those genes which are constitutively expressed under both conditions. The negative and positive fold change values indicate the downregulated and upregulated transcripts, respectively.

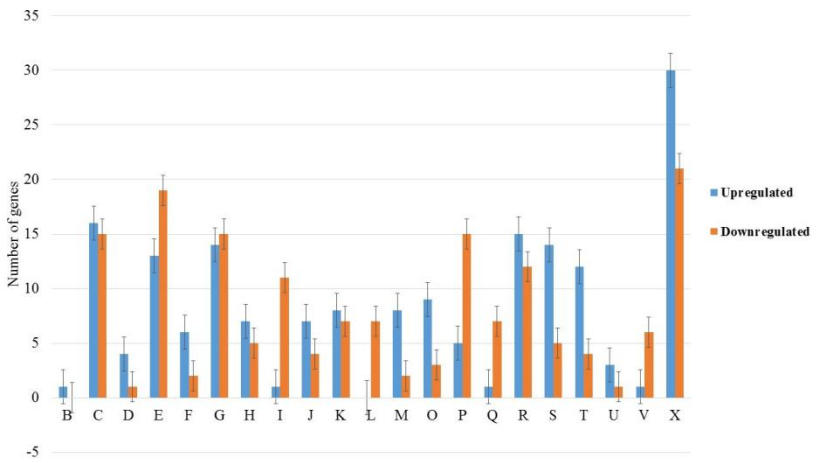

Figure 5 "EGGNOG classification of differentially expressed genes in *Nesterenkonia* sp. AN1 grown at 5 °C. The bars represent number of overexpressed transcripts. The COG categories in EGGNOG are defined as follows: INFORMATION STORAGE AND PROCESSING; translation, ribosomal structure and biogenesis (J), transcription (K), replication, recombination and repair (L) and chromatin structure and dynamics (B). CELLULAR PROCESSES AND SIGNALING; cell cycle control, cell division, chromosome partitioning (D), defence mechanisms (V), signal transduction mechanisms (T), cell wall/membrane/envelope biogenesis (M), intracellular trafficking, secretion, and vesicular transport (U) and posttranslational modification, protein turnover, chaperones (O). METABOLISM; energy production and conversion (C), carbohydrate (G), amino acid (E), nucleotide (F), coenzyme (H), lipid (I), inorganic ion (P) transport and metabolism and secondary metabolites biosynthesis, transport and catabolism (Q). POORLY CHARACTERIZED; general function prediction only (R) and function unknown (S)". The overexpressed gene whose functions are unknown are indicated by X.

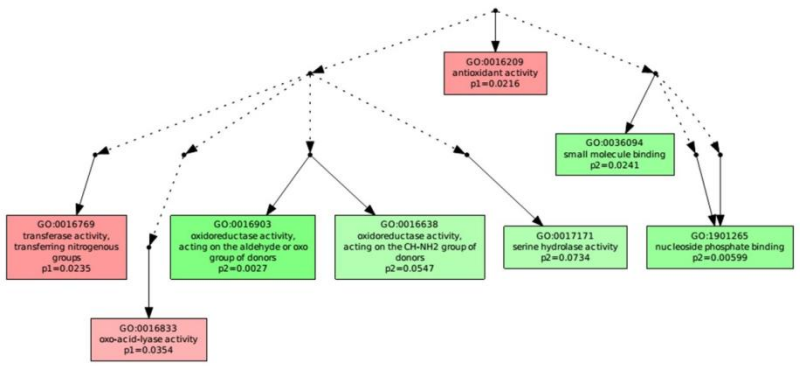

Figure 6 The GOEAST graph of significantly enriched molecular functions for differentially expressed genes in *Nesterenkonia* sp. AN1 grown at 5 ºC and 21 ºC. Red and green boxes represent enriched GO-terms for the upregulated and downregulated genes, respectively

Table 1. Genomic characteristics of the four compared *Nesterenkonia* strains. Their isolation sources as well as the optimal growth temperatures (Topt) are given. The number of contigs in the final assembly, predicted genome sizes, G+C contents (%), number CDSs and tRNA species encoded on the genomes are indicated.

Strain ID	GenBank ID	Isolation source	Topt	Contigs	Size (Mb)	G + C (%)	CDSs	tRNA

AN1	JEMO00000000	Antarctic dry Soil	21 ºC	41	3.05	67.4	2852	55
DSM 19423	ATXP00000000	Cotton pulp mill	42 ºC	36	2.59	63.8	2395	50
F	AFRW00000000	Hyper-saline lake	-	138	2.81	71.5	2543	47
NP1	CBLL000000000	Human faeces	37 ºC	175	2.67	62.2	2523	48