

# Response of the obligate halophile fungus *Aspergillus loretoensis* to stress salinity

## Abstract

Fungi are recognized as indigenous microbes in natural hypersaline habitats. *Aspergillus* sp, among other fungi, is predominant in those environments; however, their adaptative abilities are recently studied. This study analyzes the transcriptomic response of an obligate halophile *Aspergillus loretoensis* under two salinity conditions (4% and 15% NaCl). This fungus shows stress under the low NaCl concentration tested since it overexpresses genes like SOD2 (oxidative stress and oxygen toxicity), ASG (resistance to salinity), and transmembrane transport (ZRT2, OAC1, PMA1, ZRC1, SNQ2, MCH4, YO075, SIT1). Meanwhile, at 15% NaCl, the up-regulated genes at 15% NaCl were related to osmolytes transport (STL1, HXT13, ZRT1), carbohydrate transport, and metabolism (MAL11, PK1, ITR1), all suggesting their adaptive conditions. This fungus expresses interesting metabolic enzymes with potential uses in biotechnology as invertases, isomerases, maltases, and lipases. As well it showed biosynthetic pathways related to oil degradation and antibiotic production.

**Keywords:** glycerolipids, marine fungi, gene expression, osmoregulation

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## Introduction

*Aspergillus loretoensis* was recently described as an obligate halophile species, it was found in the deep sediment of the ocean where the salinity is around 3.5% (0.6M) even when this fungus can grow at 25% (4.31 M) of NaCl, showing an optimal of 15% (2.58 M).<sup>1</sup> Halophiles “salt-loving” require salt to grow and they are classified as non-halophiles that require less than 0.2 M of NaCl but can tolerate higher salinities and are considered halotolerant; as the extremely halotolerant black yeast *Hortaea werneckii*. Then the slight halophiles that show grow at 0.2 M to 0.5 M of NaCl, characteristic of marine microorganisms. The next group is the moderate halophiles that grow at 0.5 M to 2.5 M NaCl and the extreme halophiles above 2.5 M NaCl.<sup>2</sup> Halophile fungi have been described since 1998, and more species in nature are discovered yearly.<sup>3-5</sup> To date, there have been found few obligate halophiles fungi among them; *Aspergillus baarnensis*,<sup>6</sup> *Gymnascella marismortui*,<sup>3</sup> *Wallemtia ichthyophaga*,<sup>7</sup> *A. penicillioides*, *A. unguis*,<sup>8</sup> *A. salinarum*,<sup>9</sup> *A. ruber*,<sup>10</sup> *W. muriae*,<sup>7</sup> *Gymnoascus halophilus*,<sup>11</sup> *A. salisburgensis*, *A. atacamensis*,<sup>12</sup> *A. sydowii*,<sup>13</sup> *A. montevidensis*,<sup>14</sup> *A. loretoensis*<sup>1</sup> among others. Halophile fungi are less studied than the prokaryote part; however, they are an excellent source to understand the eukaryote mechanism to bear the cell osmolarity. These organisms are good candidates as industrial enzymes, anticancer, antimicrobial compounds, antioxidants for food prevention, and cosmetics industry producers.<sup>15,2</sup> In the fungi kingdom, there are several adaptations to osmotic pressure; the main is “salt in,” where the cell accumulates high concentrations of inorganic ions to obtain osmotic balance K<sup>+</sup> ion is the most important and excludes the toxic Na<sup>+</sup> ion.<sup>16</sup> Another strategy is “organic osmolytes” (low-salt, salt-out, or compatible solute), where microorganisms use organic solutes to maintain the osmotic balance. Those solutes are polyols such as glycerol, arabitol, and mannitol, preventing the intercellular sodium below toxic levels.<sup>16,17</sup> Other osmotic protection mechanisms include changes in the cell wall thickness, pigmentation, fatty acid accumulation, amino acids, and soluble sugar production.<sup>17</sup>

*A. loretoensis* is capable of growth at 4.31 M NaCl; however, the strategies to be an obligate halophile are unknown, which is why

this study focuses on analyzing the transcriptomic response of *A. loretoensis* under two salinity conditions (4 and 15% NaCl) to find out which genes are involved in its halotolerant adaptations. This work contributes to understanding the metabolic process of *A. loretoensis* as a new species of California Gulf sea inhabitant.

## Materials and methods

### Samples and total RNA extraction

*A. loretoensis* was isolated from marine sediments at 275 m deep in Loreto Bay, Baja California Sur, México (coordinates: 25°89'14" N 111°25'09" W). The isolation methodology was described previously.<sup>18</sup> The isolate was deposited at the CM-CNRG collection from INIFAP (Colección de Microorganismos del Centro Nacional de Recursos Genéticos, Instituto Nacional de Investigaciones Agrícolas y Pecuarias, México (<https://vun.inifap.gob.mx>)), and the isotype culture to Herbario BCMEX-UABC fungi collection, with accession number 6006. The accession number at INIFAP is CM-CNRG 624 holotype culture. *A. loretoensis* also was registered at mycobank (<http://mycobank.org/page/Simple%20names%20search>) with accession number 830181, and it is associated to the Index Fungorum (<http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=830181>).

*A. loretoensis* was cultured in the dark for seven days in YPD (Yeast Extract-Peptone-Dextrose) broth at 4 and 15% NaCl at 28°C, with three replicates. Then, the mycelium was collected and immediately frozen with liquid nitrogen, lyophilized, and homogenized using a pestle and mortar. Total RNA was purified using the EZNA Fungal RNA mini kit (OMEGA®). RNA integrity was confirmed by electrophoresis (1% agarose gel and TAE 1X). Residual DNA was eliminated by RQ1 RNase-Free DNase I kit (Promega, Madison, WI, USA). RNA was quantified using a Qubit™ RNA Broad Range assay (Life Technologies, USA), and their quality was confirmed using RNA 6000 Nano Kit (Agilent Technologies, Wilmington, DE, USA). All samples had an RNA Integrity Number (RIN) > 7 and were considered for library preparation.

## Libraries construction and sequencing

TruSeq® RNASeq v2 (Illumina, San Diego, CA, USA) was used to construct the libraries. The libraries concentration was quantified with the Qubit® dsDNA Broad Range assay (Life Technologies, USA), and the cDNA fragments were verified in a 4% agarose gel. Each library's size was determined on an Agilent 2100 Bioanalyzer® using the Agilent DNA 1000 kit (Agilent Technologies, Germany). The final concentration of libraries and sequencing control (PhiX) was 10 pM and 1%, respectively. The MiSeq® System Reagent V3 kit was used for sequencing (Illumina MiSeq platform), generating pair-end reads of 76 bp.

## De novo transcriptome assembly

The quality of the raw reads obtained from the MiSeq sequencer was verified by FastQC V0.11.7.<sup>19</sup> The preprocessing of reads was carried out with the Trimmomatic v0.36.<sup>20</sup> In this phase, adapters and sequences with ambiguous 'N' bases and quality values < 30 were removed and reads with a length less than 36 bp. Before assembly, orphan reads were removed, and all left and right pair-end reads were concatenated. De novo assembly of linked reads was performed with Trinity v2.6.6.24 with default parameters.<sup>21</sup> The assembled transcriptome quality was assessed with Transrate v1.0.3,<sup>22</sup> and Trinity utilities, while its completeness with BUSCO v4.0.2 based on fungi\_odb10 database.<sup>23</sup>

## Transcripts abundance and differential expression analysis

De novo transcriptome from Transrate and reads from Trimmomatic were used to calculate the abundance of transcripts from each replicate. All reads were aligned against the indexed transcriptome using Bowtie2 v2.3.5.<sup>24</sup> The abundance was calculated using the RNA-Seq by expectation-maximization algorithm included in Trinity (RSEM).<sup>25</sup> The differential expression analysis was based on the raw counts from replicates, and the edgeR package v3.28.1.<sup>26</sup> Counts were normalized, considering the library size. Then, low abundance transcripts (CPM < 0.9) in all three replicates were removed. For statistical analysis, a binomial generalized linear model was fitted to the normalized counts for pairwise comparison between NaCl concentrations. A multiplicity correction to the control (the expected false discovery rate (FDR)) was made through the Benjamini-Hochberg method.<sup>27</sup> Transcripts with an adjusted P-value (FDR) < 0.01 and at least a two-fold change (FC > |2|) were considered differentially expressed transcripts (DET).

## Functional annotation and enrichment analysis

The functional annotation of all transcripts was made through a local BLAST with the NCBI-BLAST-2.4.0<sup>27</sup> and the Swissprot/Uniprot databases,<sup>28</sup> based on release 2020\_2. Trinotate v3.0.1 software<sup>29</sup> was used to assign the best BLAST result for each protein against the SwissProt/UniProt database and PFAM predictions domains. Gene ontology (GO) was analyzed based on the more significant categories between differentially expressed transcripts (DET), using the R packages biomaRt v2.42.1,<sup>30</sup> cluster Profiler v3.14.3,<sup>31</sup> and the gene set enrichment analysis strategy (GSEA) through the correction of

FDR < 0.05. The enricher function identified KEGG pathways from DETs lists from the cluster profile R package and edition of FDR < 0.1. Enrichments for GO and KEGG analysis considered all annotated transcripts as background. Finally, the predicted interactions between the proteins encoded by DET between treatments were modeled using the STRING v11.0 online tool.<sup>32</sup> *Saccharomyces cerevisiae* was used as a reference to set the predicted interactions based on the mean network edges confidence (0.400) for their interaction score. Nodes without connection were removed.

## Results

### Transcriptomic analysis and quality assessment

The transcriptome of *A. loretoensis* generated 12 832 262 paired-end raw reads for 4% NaCl treatment and 10 986 333 paired-end raw reads for 15% NaCl treatment. The read length was 35-76 bp, and GC content of 60% for both conditions. After the quality control of raw reads with Trimmomatic, the initial transcriptome assembly generated 15 123 transcripts with 10,507 putative genes. The median contig length was 1,525 bp with N50 of 2 897 bp and GC content of 56.60% from 28 887 258 total assembled bases. The longest isoform per putative gene median size was 1 175 bp with N50 of 2 475 bp. The assembly quality assessment with Transrate indicated that 11 354 transcripts and 7 985 putative genes were retained, representing 75 and 76%, respectively. Statistics based on all transcripts from clean assembly reported a median contig length of 1 734 bp, a GC content of 56.69%, and N50 of 2 831 bp. The median contig length was 1 540 bp with an N50 of 2 561 bp (Table I). The overall alignment rate (mapping) generated with bowtie2 between the processed reads against the assembled transcriptome was 96.81 and 97.70% for 4 and 15% NaCl treatment, respectively. On the other hand, BUSCO results generated a high integrity score, with 723 complete orthologous groups from 758, representing 95.4%. Single copies represented 70.5%, and 29.5% were duplicated.

### Functional annotation of the whole transcriptome

All transcripts were annotated with the Trinotate pipeline. The *sprot\_Top\_BLASTX\_hit* results showed an annotation level of 71.8%. (Table I) Based on the eggnoG database, 13% of transcripts were annotated. EggnoG reports annotations with two categories: ENOG and COG (Clusters of Orthologous Groups), with COG composed of twenty-five categories. The most abundant COG categories for all transcripts were carbohydrate transport and metabolism, post-translation modification, protein turnover, chaperones, amino acid transport and metabolism, and lipid transport and metabolism. Annotation from the KEGG Pathway database showed that 52.4% of transcripts had a match. Several metabolic pathways were considered essential to halophilic as glycerolipids, starch, and sucrose synthesis. Metabolic analysis shows several enzymes related to sucrose and starch metabolism where we can find invertase, maltase, and isomerase, which are important to industrial processes (Table II). Also, genes were found in antibiotics biosynthesis pathways such as streptomycin, penicillin, neomycin, hydrocarbon degradation, and vitamin and hormone producers (Table III).

**Table I** Statistical summary for the *de novo* transcriptome assembly and annotation from 4% and 15% NaCl libraries of *Aspergillus loretoensis*

Final assembly	
Total Trinity 'genes'	7,985
Total Trinity transcripts	11,354
GC (%)	56.69
Stats based on all transcript contigs	
Contig N50 (bp)	2,831

Table Continued...

<b>Final assembly</b>	
Median contig length (bp)	1,734
Average contig (bp)	2,088
Total assembled bases	23,705,699
Stats based on only longest isoform per 'gene'	
Contig N50 (bp)	2,561
Median contig length (bp)	1,540
Average contig (bp)	1,878
Total assembled bases	14,997,382
<b>Assembly quality assessment</b>	
Recovered transcripts with Transrate (%)	75.1
BUSCO completeness (%)	95.4
Overall alignment rate (%)	
4% NaCl reads	96.81
15% NaCl reads	97.7
<b>Annotation</b>	
Transcripts with coding region	10,137
Transcripts with BLAST hit (BLASTX)	8,162
Transcripts with BLAST hit (BLASTP)	6,797
Transcripts with orthologous groups (ENOG and COG)	1,474
Transcripts with KEGG pathway	5,948
Transcripts with GO terms	6,972
Transcripts belonging to protein families (pfam)	4,449

**Table 2** Enzymes involved in glycerolipid, sugar and starch metabolism

<b>Glycerolipid metabolism</b>		<b>Sucrose and starch metabolism</b>	
<b>Enzyme</b>	<b>name</b>	<b>Código de la enzima</b>	<b>Nombre</b>
ec:2.7.1.107	kinase (ATP)	ec:2.7.1.2	glucokinase (phosphorylating)
ec:3.1.3.81	diphosphate phosphatase	ec:2.7.1.1	hexokinase type IV glucokinase
ec:2.7.1.29	kinase	ec:2.7.1.4	fructokinase (phosphorylating)
ec:2.3.1.158	acyltransferase	ec:3.1.3.12	trehalose-6- phosphatase
ec:3.1.3.21	alpha-glycerophosphatase	ec:2.7.7.9	uridylyltransferase
ec:3.1.1.23	lipase	ec:3.2.1.4	endo-1-4-beta-D-glucanase
ec:1.1.1.156	2-dehydrogenase (NADP+)	ec:3.2.1.3	1-4-alpha-glucosidase
ec:3.1.1.3	lipase	ec:3.2.1.1	glycogenase
ec:2.3.1.51	O-acyltransferase	ec:5.3.1.9	isomerase
ec:3.1.3.4	phosphatase	ec:3.2.1.48	alpha-glucosidase
ec:1.1.1.2	dehydrogenase (NADP+)	ec:3.2.1.91	1-4-beta-cellobiosidase (non-reducing end)
ec:1.2.1.3	dehydrogenase (NAD+)	ec:5.4.2.2	(alpha-D-glucose-1-6-bisphosphate dependent)
ec:3.2.1.22	melibiose	ec:3.2.1.20	maltase
ec:1.1.1.21	reductase	ec:3.2.1.21	gentiobiase
		ec:2.4.1.34	synthase
		ec:3.2.1.28	trehalase
		ec:3.2.1.26	invertase
		ec:2.4.1.1	phosphorylase
		ec:2.4.1.18	branching enzyme
		ec:2.4.1.15	synthase (UDP-forming)
		ec:2.4.1.11	synthase
		ec:2.4.1.25	disproportionating enzyme
		ec:2.4.1.21	synthase (glycosyl-transferring)

**Table 3** KEGG pathways of biotechnological interest were found in the transcriptome of *Aspergillus loretoensis* under two NaCl treatments (4 and 15%)

<b>KEGG Pathway</b>	<b>Pathway ID</b>	<b>Genes found in <i>A. loretoensis</i> transcriptome</b>
<b>Antibiotics</b>		
Biosynthesis of antibiotics	map01130	129
Biosynthesis of streptomycin	map00521	5
Biosynthesis of novobiocin	map00401	3
Biosynthesis of neomycin, kanamycin and gentamicin	map00524	2
Biosynthesis of monolactams (Tabtoxinine $\beta$ -lactam)	map00261	2
Biosynthesis of carbapenem (Nortienamycin)	map00332	2
Biosynthesis of penicillin and cephalosporin	map00311	1

Table Continued...

KEGG Pathway	Pathway ID	Genes found in <i>A. loretoensis</i> transcriptome
<b>Antibiotics</b>		
<b>Hydrocarbons degradation</b>		
Degradation of toluene	map00623	3
Degradation of xylene	map00622	1
Degradation of chlorocyclohexane and chlorobenzene	map00361	1
<b>Vitamins</b>		
Metabolism of riboflavin	map00740	7
Metabolism of B6 vitamin	map00750	4
<b>Metabolism of xenobiotics</b>		
Metabolism of xenobiotics, cytochrome P450	map00980	4
<b>Hormone biosynthesis</b>		
Biosynthesis of steroids	map00100	4
Biosynthesis of steroid hormone	map00140	3
<b>Pharmaceutic metabolism</b>		
Metabolism of fluorouracil (chemotherapeutic)	map00983	5
Metabolism of azathioprine 6-Mercaptopurinan	map00983	4
Metabolism of isoniazid (tuberculosis treatment)	map00983	3
Metabolism of irinotecan (cancer treatment)	map00983	1
<b>Other compounds degradation</b>		
Degradation of benzoate	map00362	7
Degradation of naphthalene	map00626	1

Sixty-one percent of transcripts were annotated in the gene ontology database (GO), generating 48,175 hits. In the biological process category, the most abundant genes were transmembrane protein and pathogenesis. ATP and metal ion binding (zinc) were the most abundant for the molecular function. The tops of the cellular component category were the nucleus and cytosol. Transcripts from *A. loretoensis* had homology mainly with species of *Schizosaccharomyces sp.*, *Aspergillus sp.*, *Saccharomyces sp.*, and *Neurospora sp.*

### Differential expression analysis and gene enrichment

Early-stage differential expression analysis removed 1,400 transcripts with low expression through all replicates (CPM < 0.9). A comparison of replicates consistency evaluated with multidimensional scaling showed that each NaCl treatment clustered separately (Figure 1a). The standard dispersion value, an indicator of the biological coefficient of variation (BCV), was 0.34. Mathematically, the BCV is equal to the square root of typical dispersion and had a value of 0.58 (Figure 1b). Statistical comparison between 15% NaCl against 4% NaCl generated 420 DET with FDR < 0.01 and two-fold change (FC > |2|) parameters. Regarding 15% NaCl treatment, two hundred and twenty-seven transcripts were up-regulated, while hundred and ninety-three transcripts were down-regulated. DET distribution between NaCl treatments was represented on mean average (MA) and Volcano plots (Figure 1 c-d). The heatmap, constructed with DET, displayed the aggrupation of replicates within its corresponding cluster and confirmed different expression patterns between each NaCl treatment (Figure 2).

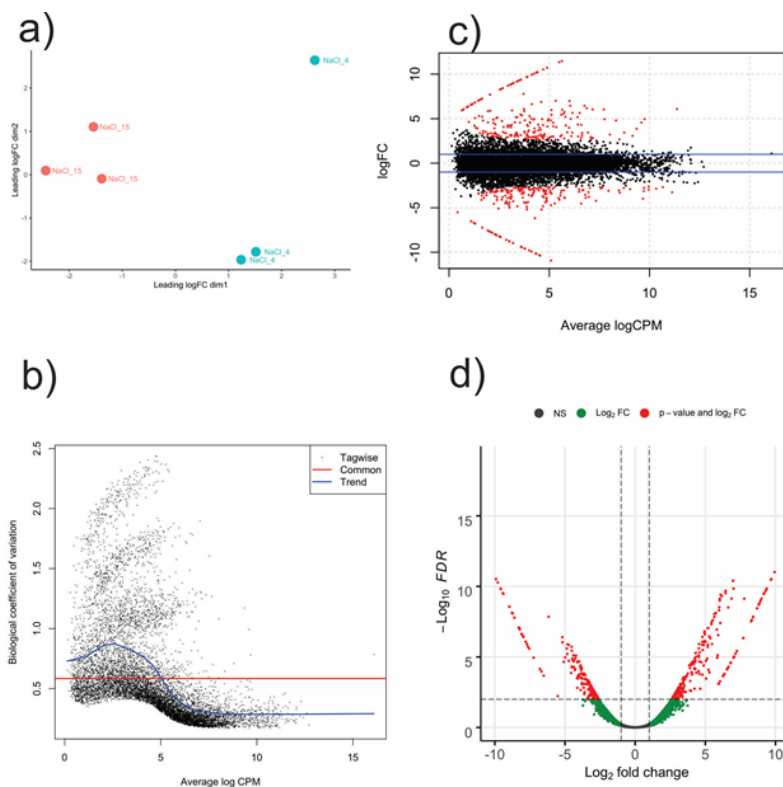
A gene enrichment analysis was done based on DET to know the overrepresented biological categories and KEGG pathways. GO enrichment profile for 4% NaCl included terms related to the mitochondrion, oxidation-reduction process, ribosome, cytoplasmic translation, protein kinase activity, and transmembrane transporter activity (Figure 3). Compared with 4% NaCl, the GO enrichment profile for 15% NaCl was broader. It included terms related to nuclear chromatin, cellular response to DNA damage stimulus, mRNA

processing, protein ubiquitination, RNA splicing, transferase activity-transferring glycosyl groups, carbohydrate metabolic process, and hydrolase activity-acting on glycosyl bonds. One aspect to highlight is that transcriptional activity was highly enriched at 15% NaCl treatment, as evidenced by the terms associated with RNA Polymerase II and methylation, probably as a response to high salinity. Regarding KEGG enrichment, the profiles included different pathways in a general way, indicating a specific response to NaCl treatments. Enriched KEGG pathways at 4% NaCl were related to nicotinate and nicotinamide metabolism, necroptosis, citrate cycle (TCA cycle), SNARE interactions in vesicular transport, and PPAR signaling pathways, among others. At 15% NaCl, the paths were related to the metabolism of sugars, amino acids, and cell size control. The most significant and abundant pathways at 15% NaCl included glucagon, hippo, neurotrophin, amino sugar, nucleotide sugar, arginine, and proline metabolism. The protein-protein network constructed in the STRING online database and based on *S. cerevisiae* confirmed the enrichment analysis results. At 4% NaCl, proteins with high confidence were related to the oxidation-reduction process and stress (HSP78, HSP30, FES1, PMA, RG11, YOR1, HYR1, MDR1), TCA cycle (SDH1 and UGA2), and carbohydrate metabolism and transport (GAL, STL1, GLC3). Proteins at 15% NaCl were related to transcription (STP15, MOT2, MED7, FCP, NRD1, ESS1), methylation and RNA processing (RSC8, NOP1, UTP6, MES1, LSM5), cell division (NUF2, MAD2, APC2, MOB1), electron transport chain (COX1, COX2, CYB2) and metabolism and transport of carbohydrates (PK1, ITR1).

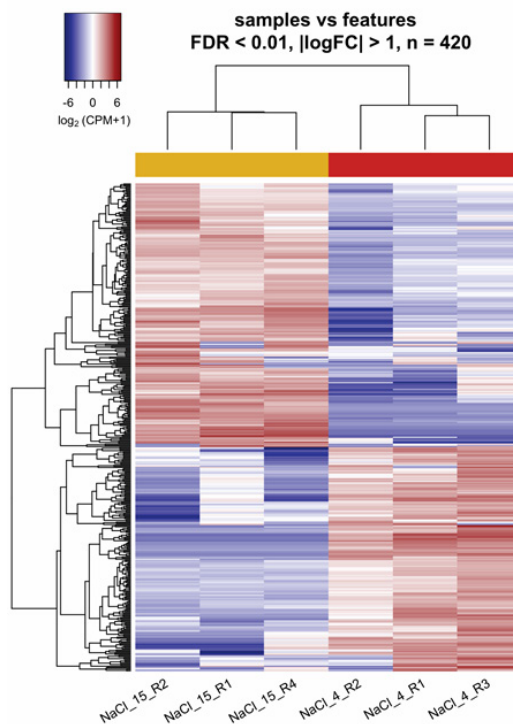
### Data availability

Data supporting this work is available at GenBank. All RNA-Seq raw reads were deposited into the Sequencing Read Archive (SRA) of NCBI with the accession numbers from SRR15465818 to SRR15465823. The BioProject ID of our data is PRJNA739890, and the BioSample accession is SAMN19773222. The Transcriptome Shotgun Assembly (TSA) project has been deposited at DDBJ/EMBL/GenBank under the accession GJLJ00000000. The version described in this paper is the first version, GJLJ01000000.

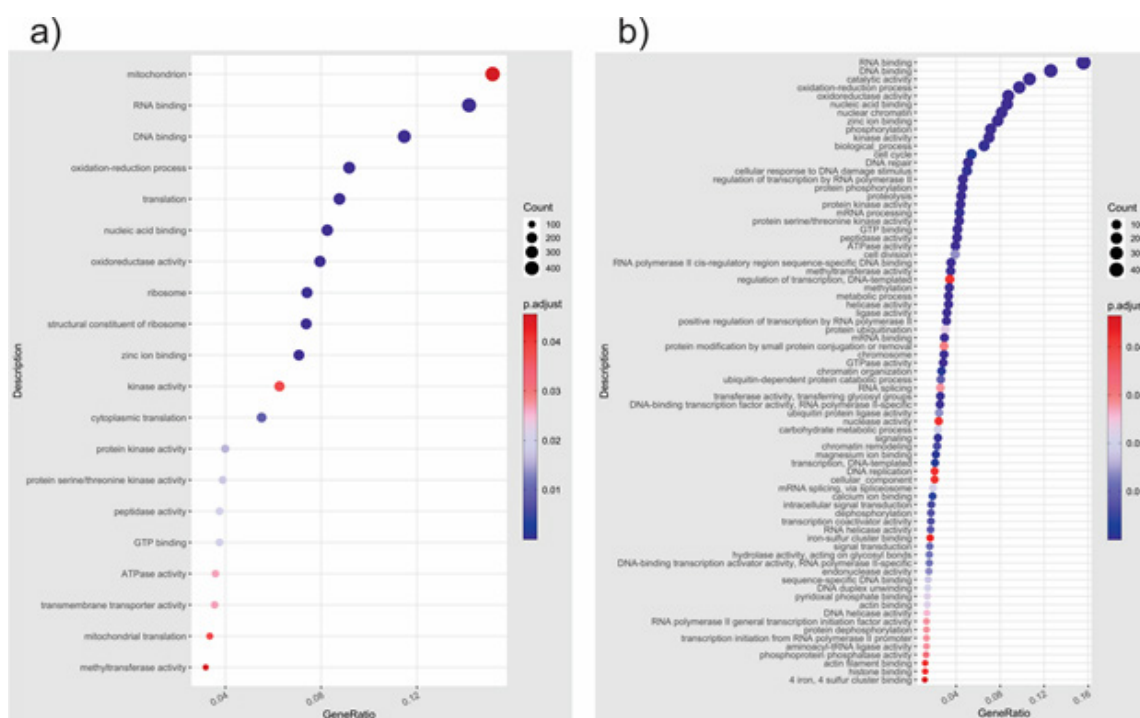




**Figure 1** Differential expression analysis with edgeR in *Aspergillus loretoensis*. 4% NaCl treatment was used as control during the comparison. A) MDS plot based on the TMM (Trimmed Mean of M-values) normalized RNA libraries. B) Scatterplot for the biological coefficient variation between NaCl treatments. C-D) MA plot (upper) and Volcano plot (lower) showing the spatial distribution of differentially expressed transcripts. 15: 15%, 4: 4%, NS, non significant; FC, fold change; FDR, false discovery rate.



**Figure 2** Clustering of the up and down-regulated transcripts from 15% NaCl vs 4% NaCl comparison in *Aspergillus loretoensis*. FDR, false discovery rate; FC, fold change; CPM, counts per million; Red: Up-regulated, Blue: Down-regulated, R, replicate.



**Figure 3** GO enrichment based on differentially expressed transcripts from a) 15% NaCl vs b) 4% NaCl comparison in *Aspergillus loretoensis*.

## Discussion and conclusion

Previously it was reported that the morphological changes that show *A. loretoensis* when it was exposed to 4%, 15%, and 25% of salinity, where the mycelia were scarce at 25% NaCl. It shows circles in the hyphae morphology; however, 15% was the optimal growth; those observations were taken in a count to choose 4 and 15% to analyze the transcriptome.<sup>1</sup>

The putative genes found in *A. loretoensis* (7 985 to 10 507 genes) are within the range of the genes reported for other *Aspergillus sp.* as *A. fumigatus* (10,087 genes), *A. nidulans* (10 937 genes), *A. niger* (14 504 genes), *A. oryzae* (12,322 genes), *A. montevidensis* (12 296 genes), *A. sydowii* (11 269 genes). Genome and transcriptome of some subgenus *Polypaecilum* have been reported, as *A. sclerotialis* (11 307 genes) with the highest gene numbers and *A. salisburgensis* with the lowest (8 895). The most halophilic fungus reported *Waleimia ichthyophaga* shows a reduction in its genome with 538 genes, contrary to all fungi mentioned before.<sup>33-34</sup> This condition is unrelated to the halophile characteristics in *Polypaecilum* subgenus. *A. loretoensis* was isolated from the sediment of the ocean. We expect that 4% salinity will be the best for this fungus however been demonstrated that 15% is the optimum for growth. The transcriptome analyses corroborate these findings with apparent differences in the gene expression related to their strategies to adapt and survive in the seawater. In the GO, it is observed that more transcripts are related to integral components of the membrane; other studies have reported the essential role that plays the features of the membrane in the osmolarity regulation as an adaptive strategy in the halophilic organism.<sup>33-35</sup> Up-regulation of the carbohydrate metabolic process at 15% of salinity includes the glycolysis pathway, where glycerol is produced between their primary metabolites and is a compatible solute to survive extreme conditions. Interestingly, at 15% NaCl, the cell responded to DNA damage stimulus, and DNA repair is increased and not to 4%, where the fungus is stressed. Besides the physiological indication, the

differential analysis indicates that *A. loretoensis* is stressed at 4% NaCl since several heat shock proteins (HSP) are up-regulated at this condition as HSP78, HSP30, and HSP70. Those proteins were reported to be related to osmotic stress in other fungi.<sup>36-38</sup> OpS6 is a stress-related protein up-regulated in this condition.

This protein is a Glutathione S-transferase-like protein that detoxifies reactive electrophilic compounds; this protein protects the cells against oxidative stress by scavenging any leaked free radical present by activating the thiol group glutathione.<sup>39</sup> In contrast, no HSP was found in the 15% NaCl condition. Transcripts of cell wall remodeling protein as Class III chitinase (CHI33) were observed in other halophilic fungi,<sup>33-35</sup> and it was overexpressed in *A. loretoensis* in 15% NaCl condition. Chitinases from this family were found in *Trichoderma sp.* under stress conditions (low or high temperatures and carbon or nitrogen starvation).<sup>40-41</sup> Several proteins involved in chitin and chitosan degradation were up-regulated, including endochitinase 1, endochitinase B, endochitinase B1, endo-chitosanase, and chitotriosidase-1. The up-regulation of these proteins was observed in the halophilic fungi *A. sydowii*.<sup>35</sup> However, a putative glycosidase YHZ7 is involved in the cell wall polysaccharide metabolic process. It seems that overexpression of related proteins protects the cell against osmotic stress. For example, *S. cerevisiae* mutants of CRH glycosidase genes reduced their survival in a high salt concentration.<sup>42</sup> On the other hand, *A. sydowii* overexpresses the *crh* glycosidase gene at 2.0 M NaCl (11.6 % NaCl).<sup>35</sup> In contrast, *A. loretoensis* at 4% NaCl overexpress proteins responsible for synthesizing new cell walls like *ecm33*, chitin synthase 8, and chitin synthase G, compared to *A. sydowii* where just one gene was found strongly downregulated in the presence of salt.<sup>35</sup>

The transporter systems in halophilic fungi regulate the cation homeostasis of alkali cation levels and eliminate toxic ion levels such as sodium.<sup>35</sup> *A. loretoensis* overexpressed at 15% NaCl several transporters as MFS (Major facilitator superfamily): MFS10, MFS A,

and ABC multidrug transporter: MDR1 and MDR2. In contrast, at 4% NaCl, *A. loretoensis* also overexpress other MFS and ABC transporters like the MFS\_sugar\_transport-like MFSB, MFS-type transporter DBAD, MFS hexose transporter KHT2, uncharacterized transporter from the MFS protein family YN43, ABC multidrug transporter MDR1, ATRF, and ABCC. The MFS transporters are not specific to molecules since they can also transport ions, carbohydrates, lipids, amino acids, and peptides. It has been reported that up to 50% of the coding genome for transporters belonging to the MFS and ABC (ATP-binding cassette) family's protein has been related to drug resistance.<sup>33</sup> MFS transporters are down-regulated on saline stress conditions in *W. ichthyophaga* and *A. salisburgensis*; however, *A. loretoensis* showed an up-regulation of different transporters at NaCl concentrations tested in this study. *A. sclerotialis* and *A. sydowii* showed similar conditions at high salt concentrations.<sup>37,39</sup> Zajc et al.<sup>33</sup> mention that this down-regulation in halophilic fungi such as *W. ichthyophaga* and *A. salisburgensis* is because of high salinity environments species belong, where competition with other species is limited. However, *A. loretoensis* probably has a more competitive habitat.

Another transporter up-regulated at 15% NaCl was the ZRT1 gene, which codes for a Zn transporter protein. Zinc transporters are not specific and respond to lower ion concentrations transporting other metals under stress conditions, suggesting that regulatory mechanisms act similarly under non-optimal conditions for the cell. Up-regulation of zinc transporters has been reported in *A. sclerotialis* under high salinity conditions.<sup>34</sup> Contrary to the yeast *Pichia kudriavzevii*, where the expression of ZRT1 has been down-regulated under saline stress conditions.<sup>43</sup> Tafer et al. mention that this regulatory mechanism under high salinity conditions is related to oxide-reduction reactions and ion exchange during high salinity.<sup>34</sup>

The high osmolarity glycerol (HOG), a map kinase pathway activated by osmotic stress, is an essential osmoregulatory mechanism in several fungi. In *A. loretoensis*, HOGA1 and PHSG genes regulated by stress-response elements and the HOG MAP kinase pathway were overexpressed at 15% NaCl. In other halophilic fungi, genes of this pathway have been found in *Hortaea werneckii* and *W. ichthyophaga*. In this species, the HOG genes activate at limiting salinities.<sup>33</sup> Genes of the HOG family were found in *A. sydowii*, but they were not expressed differentially under saline stress conditions,<sup>35</sup> and in *A. salisburgensis* a homolog of tyrosine-protein phosphatase 3 (PTP3) was found; this protein is a repressor of the HOG family.<sup>34</sup>

The STL1 gene was up-regulated at 4% NaCl, which codes for a glycerol-gate symporter protein in the plasmatic membrane. This gene has been previously reported in *W. ichthyophaga*, which acts during hyperosmotic shock, preventing the exit of the cell's glycerol; under these conditions, the channels of water-glycerol-porins Fps1 are closed.<sup>33</sup> This study finds some of the general strategies of the fungi to address the osmolarity shock in *A. loretoensis* being the glycerol biosynthesis, the principal metabolite regulator since it was up-regulated at 15% NaCl. However, at 4% NaCl, *A. loretoensis* showed stress corroborating previously reported results of their optimal growth.<sup>1</sup> We discover interesting metabolic pathways related to antibiotic biosynthesis and biodegradation of hydrocarbon molecules that could be explored in oil bioremediation in the ocean. Also, there were enzymes of biotechnological interest (invertase, isomerase, maltase, etc.) that needed further exploration since these enzymes are used in the food industry. Previously was reported the capacity of *A. loretoensis* to degrade lignin and starch,<sup>1</sup> so this characteristic is important in the paper industry. More studies are necessary to analyze the conveniences of this fungus as a producer of commercial enzymes or as an oil biodegradation organism.

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## Conflicts of interest

The author declares that there is no conflict of interest.

## References

- González-Martínez S, Galindo-Sánchez C, López-Landavery E, et al. *Aspergillus loretoensis*, a single isolate from marine sediment of Loreto Bay, Baja California Sur, México resulting as a new obligate halophile species. *Extremophiles*. 2019;23(5):557–568.
- Śliżewska W, Struszczyk-Świta K, Marchut-Mokolajczyk O. Metabolic potential of halophilic filamentous fungi-current perspective. *Int J Mol Sci*. 2022;23(8):4189.
- Buchalo AS, Nevo E, Wasser SP, et al. Fungal life in the extremely hypersaline water of the dead Sea: first records. *Proc Biol Sci*. 1998;265:1461–1465.
- Chung D, Kim H, Choi HS. Fungi in salterns. *J Microbiol*. 2019;57(9):717–724.
- Itoandon E, Shobowale O, Adams F, et al. Characterization and identification of probable halophiles. *Int J Biotech Microbiol*. 2020;2(1):1–4.
- Pitt JI, Hocking AD. New species of fungi from Indonesian dried fish. *Mycotaxon*. 1985;22(1):197–208.
- Zalar P, Sybren de Hoog G, Schroers HJ, et al. Taxonomy and phylogeny of the xerophilic genus *Wallemia* (Wallemiomycetes and Wallemiales, cl. of ord. nov.). *Antonie van Leeuwenhoek*. 2005;87:311–328.
- Nazareth S, Gonsalves V, Nayak S. A first record of obligate halophilic aspergilli from the dead Sea. *Indian J Microbiol*. 2012;52(1):22–27.
- Greiner K, Peršoh D, Weig A, et al. Phialosimplex salinarum, a new species of Eurotiomycetes from a hypersaline habitat. *IMA Fungus*. 2014;5(2):161–172.
- Kis-Papo T, Weig AR, Riley R, et al. Genomic adaptations of the halophilic Dead Sea filamentous fungus *Eurotium rubrum*. *Nat Comm*. 2014;5(3745):1–8.
- Zhou N, Zhang Y, Liu F, et al. Halophilic and thermotolerant *Gymnoascus* species from several special environments. *China Mycologia*. 2016;108(1):179–191.
- Martinelli L, Zalar P, Gunde-Cimerman N, et al. *Aspergillus atacamensis* and *A. salisburgensis*: two new halophilic species from hypersaline/arid habitats with a Phialosimplex-like morphology. *Extremophiles*. 2017;21(4):755–773.
- Cong B, Wang N, Liu S, et al. Isolation, characterization and transcriptome analysis of a novel antarctic *Aspergillus sydowii* strain MS-19 as a potential lignocellulosic enzyme source. *BMC Microbiol*. 2017;17:129.
- Ding X, Liu K, Lu Y, et al. Morphological, transcriptional, and metabolic analyses of osmotic-adapted mechanisms of the halophilic *Aspergillus montevidensis* ZYD4 under hypersaline conditions. *Appl Microbiol Biotech*. 2019;103:3829–3846.
- Park H, Jun S, Han K, et al. Diversity, application, and synthetic biology of industrial important *Aspergillus* fungi. *Adv Appl Microbiol*. 2017;100:1–42.
- Gunde-Cimerman N, Ramos J, Plemenitaš A. Halotolerant and halophilic fungi. *Mycol Res*. 2009;113(11):1231–1241.

17. Plemenitaš A, Lenassi M, Konte T, et al. Adaptation to high salt concentrations in halotolerant/halophilic fungi: a molecular perspective. *Front Microbiol.* 2014;5:199.
18. González-Martínez S, Soria I, Ayala N, et al. Culturable halotolerant fungal isolates from Southern California Gulf sediments. *Open Agriculture.* 2017;2(1):292–299.
19. Andrews S. FastQC; 2010.
20. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114–2120.
21. Grabherr MG, Haas BJ, Yassour M, et al. Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nat Biotechnol.* 2011;29(7):644.
22. Smith-Unna R, Boursnell C, Patro R, et al. TransRate: reference-free quality assessment of de novo transcriptome assemblies. *Genome Res.* 2016;26(8):1134–1144.
23. Simão FA, Waterhouse RM, Ioannidis P, et al. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics.* 2015;31(19):3210–3212.
24. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 2012;9(4):357–359.
25. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics.* 2011;12(1):1–16.
26. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010;26(1):139–140.
27. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B.* 1995;57(1):289–300.
28. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. *J Mol Biol.* 1990;215(3):403–410.
29. Apweiler R, Bairoch A, Wu CH, et al. UniProt: the universal protein knowledgebase. *Nucleic Acids Res.* 2004;32(suppl\_1).
30. Haas BJ, Papanicolaou A, Yassour M, et al. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat protocols.* 2013;8(8):1494–1512.
31. Durinck S, Moreau Y, Kasprzyk A, et al. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics.* 2005;21(16):3439–3440.
32. Szklarczyk D, Franceschini A, Wyder S, et al. STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 2015;43(D1):D447–D452.
33. Zajc J, Liu Y, Dai W, et al. Genome and transcriptome sequencing of the halophilic fungus *Wallemia ichthyophaga*: haloadaptations present and absent. *BMC Genomics.* 2013;14(1):1.
34. Tafer H, Poyntner C, Lopandic K, et al. Back to the salt mines: Genome and transcriptome comparisons of the halophilic fungus *Aspergillus salisburgensis* and its halotolerant relative *Aspergillus sclerotialis*. *Genes.* 2019;10(5):381.
35. Pérez-Llano Y, Rodríguez-Pupo EC, Druzhinina IS, et al. Stress reshapes the physiological response of halophile fungi to salinity. *Cells.* 2020;9(3):525.
36. Posas F, Chambers JR, Heyman JA, et al. The transcriptional response of yeast to saline stress. *J Biol Chem.* 2000;275(23):17249–17255.
37. Montero-Barrientos M, Hermosa R, Nicolás C, et al. Overexpression of a *Trichoderma* HSP70 gene increases fungal resistance to heat and other abiotic stresses. *Fungal Genet Biol.* 2008;45(11):1506–1513.
38. Raffiello T, Keriö S, Asiegbu FO. Role of the HaHOG1 MAP kinase in response of the conifer root and but rot pathogen (*Heterobasidion annosum*) to osmotic and oxidative stress. *PLoS One.* 2012;7(2):e31186.
39. Feng P, Shang Y, Cen K, et al. Fungal biosynthesis of the bibenzoquinone oosporein to evade insect immunity. *Proc Natl Acad Sci.* 2015;112(36):11365–11370.
40. De las Mercedes Dana M, Limón MC, Mejías R, et al. Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. *Curr Genet.* 2001;38(6):335–342.
41. Alcazar-Fuoli L, Clavaud C, Lamarre C, et al. Functional analysis of the fungal/plant class chitinase family in *Aspergillus fumigatus*. *Fungal Genet Biol.* 2011;48(4):418–429.
42. Ene IV, Walker LA, Schiavone M, et al. Cell wall remodeling enzymes modulate fungal cell wall elasticity and osmotic stress resistance. *mBio.* 2015;6(4):e00986–15.
43. Li C, Yang X, Xu Y, et al. Cadmium detoxification induced by salt stress improves cadmium tolerance of multi-stress-tolerant *Pichia kudriavzevii*. *Environ Pollut.* 2018;242:845–854.