GENETIC AND PHENOTYPIC DIVERSITY OF *Azorhizobium doebereinerae* STRAINS

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ABSTRACT

Phenotypic studies based on cultural data and the analysis of total protein profiles have shown that *Azorhizobium doebereinerae* strains isolated from nodules of *Sesbania virgata* leguminous plants are highly similar. To identify better tests to discriminate among these strains, we examined genetic, using BOX-PCR, and phenotypic characteristics, such as growth at different temperatures, salt concentrations and pH values and resistance to various antibiotics. Strains representing the main genera of nitrogen-fixing nodulating bacteria were included as references in these tests. Strains isolated from *S. virgata* exhibited a high degree of genetic diversity indicated by a low similarity of BOX-PCR profiles: 36%. BR 5401^T, which was isolated from the same host but from other region, only had 26% similarity with the other *A. doebereinerae* strains. The phenotypic tests with the largest discriminative power were antibiotic sensitivity followed by temperature and salt tolerance. The pH tests did not distinguish the strains. For the antibiotic tests, all of the strains were sensitive to rifamycin, nalidixic acid, gentamicin and sulfonamides and resistant to vancomycin, amoxicillin and ampicillin, but they exhibited variable behaviour towards other antibiotics. Five out of eleven *A. doebereinerae* strains were tolerant to 40ºC temperature, and most of these strains grew in the presence of 256.5 mM NaCl.

KEYWORDS: Tolerance to stresses, legume nodulating bacteria, *Sesbania virgata.*

DIVERSIDADE GENÉTICA E FENOTÍPICA DE ESTIRPES DE *Azorhizobium doebereinerae*

RESUMO

Estudos fenotípicos baseados em dados culturais e em análise dos perfis de proteínas totais têm mostrado que as estirpes isoladas de nódulos da leguminosa *Sesbania virgata* apresentam alta similaridade entre si. Na procura de testes mais discriminatórios para estirpes isoladas de *S. virgata*, utilizou-se análises genéticas, por meio do BOX-PCR e fenotípicas, como o crescimento em diferentes temperaturas, concentrações salinas, valores de pH e a resistência a diferentes tipos de antibióticos. Estirpes representantes dos principais gêneros de bactérias fixadoras de nitrogênio nodulíferas foram incluídas como referência nestes testes. Observou-se um alto grau de diversidade genética entre as estirpes isoladas de *Sesbania virgata*, os quais agruparam-se entre si a 36% de similaridade e à BR 5401^T, isolada do mesmo hospedeiro, com apenas 26% de similaridade. Nos testes fenotípicos a maior capacidade discriminatória foi de resistência a antibióticos seguida de tolerância a temperatura e salinidade. O pH não discriminou as estirpes. Em relação aos antibióticos, todas as estirpes foram sensíveis aos antibióticos rifamicina, ácido nalidíxico, gentamicina e sulfonamidas e resistentes a vancomicina, amoxilina e ampicilina. Em relação aos outros antibióticos, as estirpes apresentaram comportamento variável. Cinco das 11 estirpes de A. doebereinerae cresceram na temperatura de 40ºC e a maioria dessas estirpes cresceu até a concentração de 256,5 mM de NaCl.

PALAVRAS-CHAVE: Tolerância a estresses, Bactérias nodulíferas em leguminosas, *Sesbania virgata*.

INTRODUCTION

A considerable number of leguminous species have the ability to establish symbiosis with nitrogen (N_2)-fixing nodulating bacteria in legumes (NFNBL), commonly known as rhizobia. This symbiosis is of great economic and ecological importance because it provides part or all the nitrogen needed for plant development. Among the species that establish symbiosis with NFNBL, the genus *Sesbania* stands out because of its strong potential for applications, such as in the recovery of soils degraded by erosion and as a green manure. Within this genus, *S. virgata* is a native species of shrub and it has been used for many purposes (MOREIRA et al., 2006).

Studies have shown that effective nodulation of *S. virgata* only occurs when inoculated with NFNBL strains of the *Azorhizobium doebereinerae* species. On the other hand, strains of *A. doebereinerae* only establish effective symbiosis when inoculated into *S. virgata*, indicating that a high symbiotic specificity exists between these two species and thereby making it difficult to distinguish among these strains (MOREIRA et al., 2006). In addition to the homogeneity of their symbiotic properties, strains isolated from *S. virgata* have total protein profiles that are highly similar to each other (MOREIRA et al*.,* 2006; FLORENTINO & MOREIRA, 2009).

Genotyping and phenotyping techniques are used in bacterial diversity studies. Some genotyping techniques use specific primers, such as those for BOX,

ERIC and REP sequences. These sequences allow for the discrimination between bacterial strains of the same species. Of these three techniques, BOX is able to generate the most complex pattern of fragments (VERSALOVIC et al*.,* 1994) resulting in a highest discriminatory resolution. In the case of NFNBL, phenotypic characteristics such as growth under different pH values, salt concentrations and temperatures or in the presence of various antibiotics, can differentiate strains and be used as initial criteria to select inoculating strains adapted to certain conditions (FLORENTINO et al*.,* 2010).

Thus, the aim of this study was to assess the ability of genotyping (BOX-PCR) and phenotyping (growth under different pH values, temperatures and salt concentrations and in the presence of various antibiotics) techniques to differentiate among bacterial strains isolated from *S. virgata* nodules derived from soil samples collected in the same region - Três Pontas city, MG, and to compare them with type and reference strains of other rhizobia genera.

MATERIAL AND METHODS

We studied 11 strains that when grown in 79 media containing bromothymol blue and at pH 6.8, had culture characteristics that were similar to *A. doebereinerae*, such as rapid growth, alkalisation of the medium and scarce production of exopolysaccharides. These strains were isolated from nodules formed in *S.virgata* after inoculation of different suspensions of soil samples collected from areas in the Três Pontas city where *S. virgata* leguminous plants were present (FLORENTINO *&* MOREIRA, 2009). These strains were authenticated in the original host *S. virgata* and hence their identification as *A. doebereinerae* due to the specificity of this symbiosis. Additionally, we included standard or reference strains, which represent the main genera of NFBNL. The identification of these strains, cultural characteristics, host of origin, location from where the soil samples were collected relative to the *S. virgata* stem and pH value of those soil samples are presented in table 1.

TABLE 1. Identification, origin (host, location and soil pH) of *Azorhizobium doebereinerae* strains isolated from *Sesbania virgata* nodules and origin of type and reference strains of other rhizobia genera

Strains	**Origin of strains**	
	Host species	**Location and soil pH**
UFLA 01-637 UFLA 01-681 UFLA 01-682 UFLA 01-683	*Sesbania virgata*	Soils collected near the stem of *Sesbania virgata* in the municipality of Três Pontas; pH = 7.60
UFLA 01-622 UFLA 01-623 UFLA 01-684 UFLA 01-686 UFLA 01-689	*Sesbania virgata*	Soils collected in the projection of the canopy of *Sesbania virgata* in the municipality of Três Pontas; pH = 7.47
UFLA 01-685 UFLA 01-688	*Sesbania virgata*	Soils collected at 4 meter distance from the stem of *Sesbania virgata* in the municipality of Três Pontas pH = 7.50
ORS 571^{T} - *Azorhizobium caulinodans*	*Sesbania. rostrata*	Senegal, Africa
BR 5401^{T} - *A. doebereinerae*	*Sesbania virgata*	Rio de Janeiro
CIAT 899^{T} - *Rhizobium tropici*	*Phaseolus vulgaris*	Colombia
UFLA 03-84 - *Bradyrhizobium sp*	*Vigna unguiculata*	Rondônia
BR 3804 - *Mesorhizobium plurifarium*	*Chamaecrista ensiformis*	Espírito Santo
BR 6806 – *Sinorhizobium* sp. (*Ensifer adhaerens*)	*Pithecellobium dulce*	Ceará
BR 3405 - *Burkholderia sabiae*	*Mimosa caesalpiniifolia*	Brazil
LMG 19424^{T} - *Cupriavidus taiwanensis*	*Mimosa pudica*	Taiwan

The genetic diversity of the strains was analysed using the Rep-PCR technique with the BOX primer (5'-CTACGGCAAGGCGACGCTGACG-3') (VERSALOVIC et al*.,* 1994) and following the methodology described by FLORENTINO et al*.* (2010).

We assessed the genetic diversity of the strains by observing the presence or absence of polymorphic bands in the gel. The data were grouped using the UPGMA (Unweighted Pairgroup Mean Arithmetic Method) algorithm and the Jaccard coefficient through the BioNumerics software V6.5 (Applied Maths, Sint-Martens-Latem, Belgium).

The strains were evaluated for the ability to grow at different pH (4.0, 5.0, 6.0, 8.0 and 9.0), temperatures (27, 35, 40, 45 and 50ºC), salt concentrations (1.71, 51.5, 85.5, 171.0, 256.5, 290.0, 342.0, 513 and 684 mM) and antibiotic resistance (μgL^{-1}): amoxicillin - AMO (10), ampicillin - AMP (10), gentamicin - GEN (10), azitromicin - AZI (15), clarithromycin - CLA (15), erythromycin - ERI (15), nalidixic acid - NAL (30), chloramphenicol - CLO (30), kanamycin - KAN (30), rifamycin - RFM (30), vancomycin - VAN (30) e sulfonamides - SUL (300). The growth of the bacteria, washing the cells and exposure to various treatments was in accord with procedures described in details by FLORENTINO et al*.* (2010).

All of the tests were performed in triplicate and assessed for the presence or absence of bacterial growth in all replicates. A matrix of these parameters was set up using the values 1 (presence) and 0 (absence) to indicate the growth phenotype. The matrix data were grouped using the unweighted pair-group mean arithmetic method (UPGMA) algorithm and the Jaccard coefficient of the NTSYS-pc program, version 2.10.

RESULTS AND DISCUSSION

Figure 1 shows the bands profiles in an agarose gel after PCR using the BOX primer for the strains isolated from *S. virgata* and the reference strains used in this study (Table 1). Using this technique, we observed a high polymorphism of bands among the bacterial strains (Figure 1). The strains isolated from *S. virgata* could be grouped together with 36% similarity. From the reference strains, the BR5401^{T} strain (*A. dobereinerae*), which was isolated from *S. virgata* and is the type strain of *A. doebereinerae*, exhibited the highest similarity (approximately 24%) with the 11 strains isolated from *S. virgata* (Figure 1).

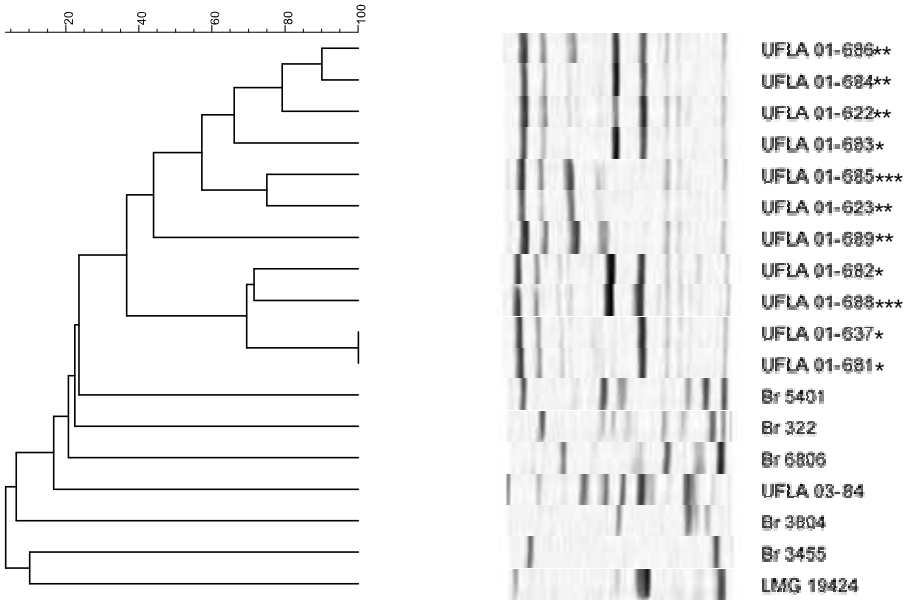

FIGURE 1. Dendrogram showing the genetic similarity of strains isolated from *S. virgata* and the reference strains used in this study based on BOX-PCR profiles. Origin of strains: *Soil collected near the stem of *Sesbania virgata*; **Soil collected in the projection of the canopy of *Sesbania virgata*; ***Soil collected at four meters distance from the stem of *Sesbania virgata.*

Based on these data, we found that the BOX-PCR technique provides the high level of resolution necessary to determine the genetic diversity among strains isolated from *S. virgata* of the city of Três Pontas, MG. Other authors (FLORENTINO et al*.,* 2010, GUIMARÃES et al. 2012, JARAMILLO et al. 2013) have reported the high discriminatory power of the BOX primer among strains of other species of NFBNL. There was a tendency for strains isolated from soil samples collected from certain locations relative to the stem of *S. virgata* (Table 1) to have a greater genetic similarity to each other (Figure 1). Bacteria isolated from soil samples collected near the stem of *S. virgata*, UFLA 01-637 and UFLA 01-681 had 100% similarity to each other. On the other hand, UFLA 01-622, UFLA 01-684 and UFLA 01-686, derived from soil samples collected in the canopy projection of *S. virgata* plants had 80% similarity to each other. In a study using the REP-PCR technique, it was also possible to observe the genetic variability among strains identified as *A. doebereinerae* that were isolated from *S. virgata* from different regions of the states of Rio de Janeiro and Minas Gerais. However, strains isolated from the same region had high genetic similarity (MOREIRA et al*.,* 2006). Nevertheless, when compared with the REP-PCR technique used by MOREIRA et al*.* (2006), the BOX-PCR technique used in this work has a greater ability to discriminate among strains isolated from *S. virgata* from the same region. Other authors (FLORENTINO et al*.,* 2010) have also reported the relationship between the site of origin and genetic diversity. It may explain the greater

genetic diversity observed in this work for the BR 5401^{T} strain of the *Azorhizobium doebereinerae* species, which was isolated from *S. virgata* in the state of Rio de Janeiro (Table 1), when compared with strains isolated from *S. virgata* in the city of Três Pontas, MG.

The assessed phenotypic characteristics (growth at different pH values, temperature, and salt concentrations and resistance to antibiotics) were used to create distinct groups among the studied strains (Figure 2). The 11 isolates from *S. virgata* formed a single group and were approximately 81% similar. This group was closely related to the strains of the *Azorhizobium* genus, *A. doebereinerae* (BR 5401T) and *A. caulinodans* (ORS 571T), which were 78% and 74% similar, respectively. These *Azorhizobium* strains formed distant groups from the other reference strains (Figure 2).

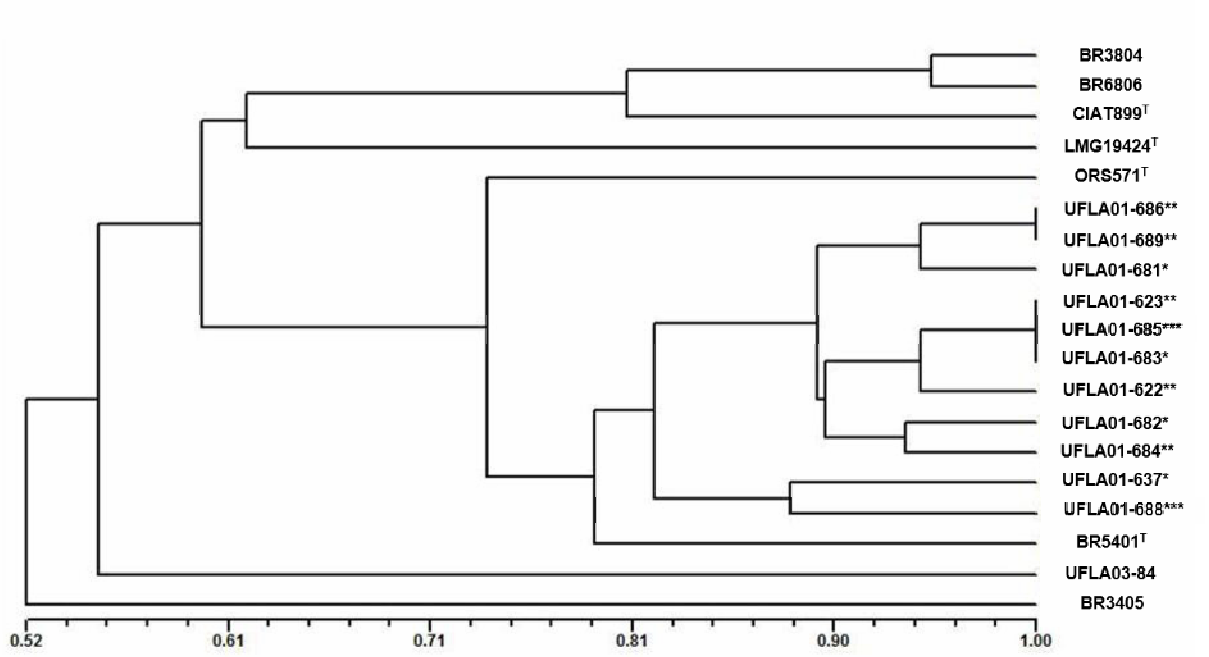

FIGURE 2. Dendrogram showing the phenotypic similarity (growth at different pH values, temperature, and salt concentrations and resistance to antibiotics) of strains isolated from *S. virgata* and the reference strains used in this study based on NTSYS-pc software. Origin of strains: *Soil collected near the stem of *Sesbania virgata*; **Soil collected in the projection of the canopy of *Sesbania virgata*; ***Soil collected at four meters distance from the stem of *Sesbania virgata.*

The different pH values used in this study (4.0, 5.0, 6.0, 6.8, 7.0, 8.0 and 9.0) were unable to distinguish among the strains isolated from *S. virgata*, which did not grow at pH values of 4.0 and 5.0. In this case, the sensitivity to these pH values may relate to the pH of the soil of origin, which was observed to have pH values above 7.0 (Table 1). The two type strains of the *Azorhizobium* genus, BR 5401^{T} (*A.*

doebereinerae) and ORS 571^T (*A. caulinodans*), and BR 3405 (*Burkholderia sabiae*) also did not exhibit growth at these pH values. The other reference strains of the *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Cupriavidus* genera grew at all pH values. Among these, the *R. tropici* strain CIAT 899^T has been widely reported in the literature to be highly tolerant to acidity (GRAHAM et al., 1994).

Searching for tolerant strains to high temperatures in tropical areas is important because the surface layer of soils (0-20 cm) may reach high temperatures. Thus, studies have been developed to select for inoculating strains that are tolerant to high temperatures for crops such as cowpea (FLORENTINO et al*.,* 2010) and soybean (RAHMANI et al., 2009) plants. For *Sesbania virgata* strains growth was observed at 27, 28 and 35℃, and no growth was obse rved at 45 and 50℃ for all of these strains. Nonetheless, at 40ºC, five strains isolated from *S. virgata*, UFLA 01-681, UFLA 01-682, UFLA 01-684, UFLA 01-683 and UFLA 01-686 exhibited growth, which differed from the BR 5401^T type strain (*A. doebereinerae*) that was isolated from the same host but did not exhibit growth at this temperature. Among the other reference strains, only the UFLA 03-84 (*Bradyrhizobium* spp.) and ORS 571^T (*A. caulinodans*) strains did not grow when cultured in at 40 ºC, which agrees with the results obtained by FLORENTINO et al. (2010).

Of the 11 strains tested, 10 tolerated the same salt concentrations as the *A. doebereinerae* type strain (BR 5401^T), 256 mM. Only UFLA 01-682 strain tolerated 290 mM NaCl, which was similar to the behaviour of the *A. caulinodans* type strain (ORS 571^T). *Cupriavidus taiwanensis* LMG 19424^T was the only one, among type and reference strains of other rhizobia genera, that displayed growth at the highest salt concentrations, 342 mM. However, the UFLA 03-84 (*Bradyrhizobium* sp.) and BR 3405 (*Burkholderia sabiae*) strains displayed growth only at the lowest salt concentration, 85.5 mM. The CIAT 899^T, BR 3804 and BR 6806 strains, which are representatives of the *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* genera, respectively, displayed growth in media containing up to 290 mM NaCl. Some studies have shown that the ability to grow at high salt concentrations is related to the greater amount of exopolysaccharides produced by NFBNL. However, in the present study, the strain that exhibited the highest tolerance to salinity, LMG 19424^T, produces a scarce amount of exopolysaccharides, indicating that it must use other mechanisms for growth at high salt concentrations. In general, NFBNL strains are able to grow in different levels of salinity. This fact can be observed within the same genus and/or species of NFBNL (ELBOUTAHIRI et al., 2010). Salt tolerance is usually related to the synthesis of other compounds, such as trehalose, which prevents water loss by the bacterial cell, thus allowing growth at high salt concentrations (MADIGAN et al., 2011).

The antibiotic resistance test provided the best distinction among strains isolated from *S. virgata*, which formed five resistance groups. Group I contained three strains, UFLA 01-681, UFLA 01-684, and UFLA 01-689, which exhibited resistance to VAN, ERI, AMO, AMP and CLO. Group II contained strains UFLA 01-622, UFLA 01-623, UFLA 01-685, UFLA 01-686, and UFLA 01-688, which exhibited resistance to VAN, ERI, CLA, AMO, AMP and CLO. Group III contained only UFLA 01-637 strain, which exhibited resistance to VAN, ERI, AMO, and AMP. Group IV contained UFLA

01-682 strain, which exhibited resistance to VAN, AMO, AMP, and CLO. Group V contained only UFLA 01-683 strain, which exhibited resistance to VAN, AMO, and AMP. Among the strains of the *Azorhizobium* genus, *A. doebereinerae* (BR 5401^T) was resistant to five antibiotics, VAN, AMO, NAL, AMP and SUL, and *A. caulinodans* (ORS 571^T) exhibited resistance to eight antibiotics, VAN, ERI, AZI, CLA, AMO, AMP, GEN and CLO. From these results, the main difference observed in relation to strains isolated from *S. virgata* was the resistance to NAL, displayed by the BR 5401^T strain. The main difference among *A.doebereinerae* strains with *A.caulinodas* strain was resistance to GEN, which was displayed by the ORS 571^T strain. The remaining strains isolated from *S. virgata* were sensitive to these two antibiotics (NAL and GEN). However, analysis of the profiles for all of the antibiotics used showed that BR 5401^T had a greater similarity to the strains isolated from *S. virgata* than ORS 571^T. All of the strains isolated from *S. virgata* were resistant to VAN, AMO and AMP and sensitive to KAN, RFM, AZI, NAL, SUL and GEN. The resistance to the VAN, AMO and AMP antibiotics may be explained by their limited spectrum because they mainly act against Gram-positive bacteria, although some Gram-negative bacteria may also exhibit sensitivity to these compounds (MADIGAN et al*.,* 2011). AMO and AMP are antibiotics synthesised from the antibiotic penicillin and may occur in soil primarily because of veterinary use (REGITANO et al*.,* 2010). In contrast, the antibiotics to which the strains remained sensitive mainly inhibit the biosynthesis of proteins (KAN, AZI and GEN) and nucleic acids (RFM and SUL) or interfere with the activity of the DNA gyrase enzyme, which is involved in packaging DNA during the replication process (NAL) (MADIGAN et al*.,* 2011). In general, the behaviour of the strains upon exposure to the different analysed conditions was unrelated to the location from which the soil samples were collected relative to the *S. virgata* stem (Table 1).

The variability of antibiotic resistance was also observed for the reference strains. It is worth mentioning that UFLA 03-84 (*Bradyrhizobium* sp.) was resistant to all antibiotics tested. The CIAT 899^T (*R. tropici*) and LMG 19424^T (*C. taiwanensis*) strains were grouped next to strains isolated from *S. virgata*. The BR 3804 (*M. plurifarium*) and BR 6806 (*Sinorhizobium* sp.) strains behaved similarly but, together with BR 3405 (*B. sabiae*), were grouped the furthest from the strains isolated from *S. virgata*.

CONCLUSIONS

Type and reference strains of genera *Azorhizobium, Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium (Ensifer), Burkholderia, and Cupriavidus w*ere quite different regarding BOX-PCR profiles and all phenotypic characteristics.

The BOX-PCR analysis provided good discriminatory power among *Azorhizobium doebereinerae* strains isolated from *S. virgata* nodules in the same region. For the phenotypic tests, the discriminatory power was greatest for antibiotic resistance, followed by temperature and salinity (which had equivalent power) and lastly by pH.

Among these strains, UFLA 01-682 stood out for its tolerance to high temperature (40ºC) and salt concentration (290 mM).

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