The α-amylase gene amyH of the moderate halophile Halomonas meridiana: cloning and molecular characterization

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Two types of Tn1732-induced mutants defective in extracellular amylase activity were isolated from the moderate halophile Halomonas meridiana DSM 5425. Type I mutants displayed amylase activity in the periplasm, and were unable to use any of the carbon sources tested, including starch and its hydrolysis product maltose. The type II mutant was affected in the gene responsible for the synthesis of the extracellular α-amylase. This gene (amyH) was isolated by functional complementation of mutant II and sequenced. The deduced protein (AmyH) showed a high degree of homology to a proposed family of α-amylases consisting of enzymes from Alteromonas (Pseudoalteromonas) haloplanktis, Thermomonospora curvata, streptomycetes, insects and mammals. AmyH contained the four highly conserved regions in amylases, as well as a high content of acidic amino acids. The amyH gene was functional in the moderate halophile Halomonas elongata and, when cloned in a multicopy vector, in Escherichia coli. AmyH is believed to be the first extracellular-amylase-encoding gene isolated from a moderate halophile, a group of extremophiles of great biotechnological potential. In addition, H. meridiana and H. elongata were able to secrete the thermostable α-amylase from Bacillus licheniformis, indicating that members of the genus Halomonas are good candidates for use as cell factories to produce heterologous extracellular enzymes.

Keywords: α-amylase, halophile, Halomonas

INTRODUCTION

Moderately halophilic bacteria are micro-organisms which grow optimally in media containing 3–15% NaCl (Ventosa et al., 1998). They constitute a complex group of micro-organisms adapted to thrive in hypersaline environments. Apart from their ecological importance, moderately halophilic bacteria have great potential for use in biotechnology. They accumulate high cytoplasmic concentrations of compatible solutes that may be used as osmoprotectants and stabilizers of enzymes and cells (Galinski, 1993), and some of them are used for the degradation of polluting industrial residues or toxic chemicals and for enhanced oil-recovery processes (Ventosa & Nieto, 1995; Ventosa et al., 1998). Moreover, moderately halophilic bacteria produce extracellular salt-tolerant enzymes of great interest for biotechnological processes (Onishi et al., 1991; Ventosa et al., 1998). Among these enzymes, amylases, which catalyse the cleavage of the α-1,4 linkage of starch, yielding short linear maltodextrins, have many commercial applications, particularly in the food and detergent industries. The use of amylases from halophilic bacteria in industrial processes would have the advantage of the enzymes having optimal activities at high salt concentrations (Kamekura, 1986; Ventosa & Nieto, 1995).

While there have been numerous reports on extracellular amylases from non-halophilic bacteria, very limited information is available on amylases from halophilic species. Amylolytic activities have been reported in the...
moderately halophilic bacteria *Acinetobacter* sp. (Onishi & Hidaka, 1978), *Nesterenkonia halobia* (Onishi, 1972b; Onishi & Sonoda, 1979), *Micrococcus varians* subsp. *halophilus* (Kobayashi et al., 1986), and other *Micrococcus* isolates (Khire, 1994; Onishi, 1972a). However, molecular characterization of these amylases is lacking. Apart from their biotechnological interest, the characterization of genes encoding amylase activity will be invaluable in elucidating their regulatory and secretion mechanisms, and the structure-function relationship of extracellular enzymes with optimal activity at high salt concentrations.

For this study we selected the moderate halophile *Halomonas meridiana* DSM 5425, which produces an extracellular α-amylase that has been recently characterized biochemically (Coronado et al., 2000). The enzyme was optimally active at 10% NaCl, although a remarkable activity was detected up to 30% salts, making it very attractive for a molecular characterization. Here we describe the isolation, cloning and sequencing of the α-amylase gene. The heterologous expression in *Halomonas* of the *Bacillus licheniformis* amyLI gene encoding a thermostable extracellular α-amylase is also reported.

### METHODS

#### Bacterial strains, plasmids, media and culture conditions.

Spontaneous rifampicin-resistant mutants isolated from the culture collection strains *Halomonas meridiana* DSM 5425 (James et al., 1990) and *H. elongata* ATCC 33173 (Vreeland et al., 1980) were used. These halophiles were routinely grown in a saline medium (SW) with a final concentration of 2% (w/v) total salts (SW-2) supplemented with 0.5% (w/v) yeast extract (Difco) (Nieto et al., 1989). When required, SW medium with 5% salt (SW-5) was used. The pH of the SW media was adjusted to 7.2 with KOH. M63 supplemented with 5% NaCl and 0.5% (w/v) soluble starch (Sigma) as the sole carbon source was used as minimal medium for the culture of halophiles. When necessary, other carbon sources were used at a final concentration of 0.5% (w/v). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989). Solid media contained 1.7% purified agar (Difco). Yeast extract (0.5% w/v), a saline medium (SW) with a final concentration of 2% (w/v) yeast extract (Difco) (Nieto et al., 1989). Other media and culture conditions were as recommended by the manufacturers (Sigma, 1989). The pH of the media was adjusted to 7.2 with KOH. M63 supplemented with 5% NaCl and 0.5% (w/v) soluble starch (Sigma) as the sole carbon source was used as minimal medium for the culture of halophiles. When necessary, other carbon sources were used at a final concentration of 0.5% (w/v). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989). Solid media contained 1.7% purified agar (Difco). Yeast extract (0.5% w/v), a saline medium (SW) with a final concentration of 2% (w/v) yeast extract (Difco) (Nieto et al., 1989). Other media and culture conditions were as recommended by the manufacturers (Sigma, 1989). The pH of the media was adjusted to 7.2 with KOH. M63 supplemented with 5% NaCl and 0.5% (w/v) soluble starch (Sigma) as the sole carbon source was used as minimal medium for the culture of halophiles. When necessary, other carbon sources were used at a final concentration of 0.5% (w/v). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989). Solid media contained 1.7% purified agar (Difco). Yeast extract (0.5% w/v), a saline medium (SW) with a final concentration of 2% (w/v) yeast extract (Difco) (Nieto et al., 1989). Other media and culture conditions were as recommended by the manufacturers (Sigma, 1989). The pH of the media was adjusted to 7.2 with KOH. M63 supplemented with 5% NaCl and 0.5% (w/v) soluble starch (Sigma) as the sole carbon source was used as minimal medium for the culture of halophiles. When necessary, other carbon sources were used at a final concentration of 0.5% (w/v). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989). Solid media contained 1.7% purified agar (Difco). Yeast extract (0.5% w/v), a saline medium (SW) with a final concentration of 2% (w/v) yeast extract (Difco) (Nieto et al., 1989). Other media and culture conditions were as recommended by the manufacturers (Sigma, 1989). The pH of the media was adjusted to 7.2 with KOH. M63 supplemented with 5% NaCl and 0.5% (w/v) soluble starch (Sigma) as the sole carbon source was used as minimal medium for the culture of halophiles. When necessary, other carbon sources were used at a final concentration of 0.5% (w/v).

#### Library construction.

An *H. meridiana* gene bank was constructed in the broad-host-range cosmid pVK102. *H. meridiana* genomic DNA was partially digested with HindIII, and DNA fragments in the size range 23–30 kb were separated in sucrose gradients and cloned into the HindIII site of pVK102. *In vitro* packaging of the recombinant molecules was performed with a commercially available extract (Amersham) as recommended by the manufacturer. Aliquots of the packaging reaction mixture were used to infect cells of *E. coli* HB101 that were then plated onto LB agar plates containing tetracycline to select recombinant clones. Randomly selected clones were then cultivated in liquid medium to contain DNA inserts ranging from 20 to 30 kb in size.

#### DNA sequencing.

DNA sequencing was performed by MWG-Biotech using an automatic DNA sequencer (LiCor). DNA
sequence was analysed with the GCG Sequence Analysis Software Package (Genetics Computer Group) and the BLAST program of the National Center for Biotechnology Information (NCBI). Protein analyses and alignments were performed by using the ProtParam program from the ExPASy (Expert Protein Analysis Systems) of the Swiss Institute of Bioinformatics and the CLUSTAL program of EBI (European Bioinformatics Institute), respectively. 

RESULTS

Isolation and analysis of *H. meridiana* mutants defective in amylase production

Mutants of *H. meridiana* without extracellular amylolytic activity were isolated by transposon mutagenesis treatment using Tn1732, a transposon of demonstrated efficiency in the genus Halomonas (Cánovas et al., 1997; Kunte & Galinski, 1995).

The Amy− mutants were identified as those unable to show amylase activity when grown on SW-2 plates supplemented with starch. Out of 5850 Km抵抗 colonies screened, 13 did not show clear haloes when iodine solution was added to the plates. These Amy− mutants were named I to XIII. To confirm the transposon insertion in these mutants, chromosomal DNA of each strain was digested with *Sal*I (which does not cut Tn1732) and an internal fragment of Tn1732 was used as a probe. All mutants were shown to have a unique insertion of Tn1732 in a DNA fragment of approximately 15 kb (type I mutants), except mutant II (type II), which contained the transposon within a fragment ranging from 6 to 11 kb in size (data not shown).

To determine if any of the isolated mutants was defective in amylase synthesis, the amylolytic activity in their different cell fractions was tested and compared to that of the wild-type strain (Table 1). In the wild-type strain, most of the activity was associated with the supernatant, but activity was also detected in the periplasmic fraction. As expected, none of the mutants showed extracellular amylolytic activity. However, type I mutants showed amylase activity in the periplasmic fraction (although this was lower than that of the parental strain), suggesting that these mutants were able to synthesize the enzyme. In contrast, amylase activity was not detected in the periplasmic fraction of mutant II. Amylolytic activity was not detected in cell extracts of either type of mutants or the wild-type strain (Table 1).

The ability of the mutants and the wild-type strain to grow in minimal medium M63 supplemented with different carbon sources was also assayed (Table 1). As expected, only the wild-type strain was able to grow with starch as the sole carbon source. Apart from starch, mutant II grew on the same compounds as the wild-type strain. In contrast, type I mutants did not grow on any of the carbon sources tested, including maltose, which was shown to be one of the main end products of the *H. meridiana* amylase activity on starch (Coronado et al., 2000). The pleiotropic phenotype exhibited by type I mutants strongly suggested that Tn1732 insertion in these strains was affecting a regulatory gene governing both amylase secretion and carbon source utilization. This led us to choose mutant II for further molecular characterization.

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<th>Table 1. Amylase activity and growth in M63 with different carbon sources of the <em>H. meridiana</em> wild-type strain and Amy− mutants</th>
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† The numbers indicate the percentage of amylase activity in the different cellular fractions.

Mutant II is affected in the α-amylase synthesis gene

To identify the gene disrupted by Tn1732 in mutant II, the region flanking the left end of the transposon insertion in this mutant was isolated and sequenced. A preliminary hybridization experiment using Tn1732 as a probe against mutant DNA independently digested with several restriction enzymes showed that a Dral site is located very close to the Tn1732 insertion in mutant II. Chromosomal DNA of mutant II was digested with Dral and PstI, and ligated to EcoRV/PstI-digested pKS. The ligation mixture was used to transform *E. coli* DH5α. From several Km抵抗 Amp抵抗 colonies, plasmid pMJC27 was isolated, which carried a 2.2 kb Dral/EcoRV-PstI insert (Fig. 1a). A restriction analysis of this plasmid showed a 1.85 kb EcoRI-PstI region, corresponding to the left end of Tn1732, and a 0.3 kb Dral/EcoRV-EcoRI fragment of DNA from mutant II, which was sequenced. A computer-assisted search in the databases revealed a high degree of homology to the genes encoding α-amylases from different microorganisms (data not shown). This clearly demonstrated that mutant II was defective in the gene encoding the α-amylase from *H. meridiana*.

Isolation of the gene encoding the α-amylase

The α-amylase gene was cloned by functional complementation of mutant II. A cosmid library containing approximately 15000 clones was prepared as described
Fig. 1. (a) Isolation of the DNA flanking the left end of the transposon Tn1732 in the Amy− mutant II of H. meridiana. (b) Restriction maps of the overlapping plasmids pMJC21 to 23, isolated by functional complementation of mutant II. Below are shown the common 10 kb HindIII region carrying the amylase gene of H. meridiana (amyH) cloned in two different vectors, and the plasmids pH5152 and pH5183 used for sequencing. The ability of the plasmids to confer amylase activity upon H. meridiana mutant II, H. elongata and E. coli is indicated on the right (+/−, extracellular amylase activity/lack of such activity on SW-2 plates, or growth/no growth on M63 medium plus starch as the sole carbon source; N.D., not determined). Restriction sites: B, BamHI; D, DraI; E, EcoRI; EV, EcoRV; H, HindIII; P, PstI; Sc, SacI.

in Methods and introduced into the Amy− mutant II for detection of Amy+ colonies. Out of 3250 KmR transconjugants screened on SW-2 starch-containing plates, 12 colonies developed a clear halo and were able to grow in M63 medium with starch. Three overlapping cosmids restoring amylase activity to mutant II were purified from these colonies and named pMJC21, pMJC22 and pMJC23 (Fig. 1b). Interestingly, transconjugants of E. coli or the moderate halophile H. elongata (which does not synthesize amylase) containing these cosmids did not show amylase activity (Fig. 1b).

The 10–3 kb HindIII region common to the three cosmids was subcloned into the expression vector pML123 in both orientations, giving the plasmids pMJC24 and pMJC25, and in pKS, to give pMJC26 (Fig. 1b). When introduced in mutant II or H. elongata, pMJC24 and pMJC25 conferred the ability to produce haloes on SW-2 + starch plates, and to grow on M63 + starch, indicating that the H. meridiana amylase gene had been successfully cloned. However, they did not confer amylase activity upon E. coli. In contrast, E. coli carrying pMJC26 developed a clear halo on LB + starch plates (Fig. 1b). These differences in gene expression may be due to the amyH gene dosage conferred by the different cloning vectors (pVK102, pML123, pKS) used.

Sequence analysis of the α-amylase gene (amyH)

Hybridization analysis using the 0.3 kb DNA insert of pMJC27 as a probe against pMJC26 localized the amyH gene in a 0.7 kb DraI-EcoRV fragment, included in a larger 6.25 SacI-EcoRV region (Fig. 1b). Two SacI-DraI segments of pMJC26, of 4.1 kb and 2.3 kb, were subcloned in pKS, generating pH5182 and pH5183, respectively (Fig. 1b). These plasmids were used for sequencing. A total of 1.6 kb DNA spanning the 0.7 kb DraI-EcoRV fragment was sequenced (EMBL accession no. AJ239061). A primer complementary to the 5′ end of pH5183 was generated and used to sequence the DNA including the DraI site with pMJC26 as a template. This ruled out the possibility of a second DraI site at the junction between pH5182 and pH5183. Computer-assisted analysis of the sequence revealed the presence of one ORF that corresponded to the amyH gene. The gene starts with a TTG codon at position 201 and ends with a TAA codon at position 1572. It encodes a 457-residue
protein with a deduced molecular mass of 50 kDa. Database searches revealed the protein encoded by amyH to have extensive sequence similarity to α-amylases from Gram-negative and Gram-positive bacteria (Fig. 2). The best alignment (55% identity) was obtained with the thermolabile α-amylase from the Antarctic psychrophilic Alteromonas (Pseudoalteromonas) haloplanktis A23 (Feller et al., 1992), followed by the α-amylases from the facultatively anaerobic Aeromonas hydrophila (53% ; Chang et al., 1993), Bacillus sp. (49% ; EMBL accession no. AB006823), the actinomycte Thermomonospora curvata (48% ; Petricek et al., 1992) and Streptomyces griseus (48% ; Vigal et al., 1991). Moreover, a considerable degree of homology (about 48% identity) was found with α-amylases from insects, such as Tribolium castaneum (Hickey et al., 1987), Aedes atropalpus (EMBL accession no. U01209) and Drosophila melanogaster (Inomata et al., 1995), and mammals, such as mouse (Hagenbuchle et al., 1980).

The α-amylase distribution, net charge and theoretical isoelectric point of AmyH were analysed and compared with the α-amylases of the non-halophilic bacteria included in Fig. 2. The α-amylase from the haloalkaliphilic archaeon Natronococcus sp. (Kobayashi et al., 1994) and the serine protease from the halophilic archaean Natrialba asiatica (Kamekura et al., 1992) were also included for comparison. To our knowledge, these are the only two extracellular enzymes from halophilic micro-organisms whose primary structure has been determined. As shown in Table 2, basic and acidic amino acids were present in approximately equivalent numbers in the enzymes from the non-halophilic micro-organisms, with net charges ranging from +2 (for the A. haloplanktis amylase) to −14 (for the Bacillus sp. amylase) and theoretical isoelectric points ranging from 8.12 (for the A. haloplanktis amylase) to 5.63 (for the S. griseus amylase). In contrast, the three enzymes from halophiles showed a markedly higher percentage of acidic residues, with negative net charges ranging from −32 (for the H. meridiana amylase) to −81.5 (for the Natronococcus amylase) and theoretical isoelectric points below 5. All these data strongly suggest that the enzymes from the moderate halophile H. meridiana and the two halophilic archaeca are predominantly acidic proteins.

Since the H. meridiana amylase is an extracellular enzyme, it would be expected to possess an amnio-
terminal signal to enable its translocation across the cytoplasmatic membrane (von Heijne, 1984). Analysis of the predicted amino acid sequence of the amylase gene identified a putative signal peptide comprising the first 20 codons of the amylase precursor protein.

Detection of the α-amylase in SDS-polyacrylamide gels

To visualize the amyH-encoded protein, supernatant fractions were prepared from the wild-type and mutant II strains, and proteins were electrophoretically separated in an SDS/10% polyacrylamide gel (Fig. 3). After silver staining, a protein band with an apparent molecular mass of 49 kDa was detected in the supernatant of the wild-type strain. As expected, this protein was absent from the supernatant of mutant II. The electrophoretic estimation of the molecular mass of the α-amylase agrees well with the value predicted from the deduced primary structure of the mature protein.

Heterologous expression of the Bacillus licheniformis α-amylase gene in the moderate halophiles H. meridiana and H. elongata

One of the most promising applications of moderate halophiles is their use as cell factories to produce heterologous extracellular enzymes. To test whether members of the genus Halomonas could secrete a heterologous amylase, the amyLI gene from Bacillus licheniformis (Gray et al., 1986), encoding a thermostable α-amylase, was subcloned into the expression vector pML122 under the control of the neomycin-resistance gene promoter, giving the plasmid pMJC28 (Fig. 4). Exconjugants of both H. meridiana mutant II and H. elongata carrying this plasmid showed extracellular amylase activity on SW-2 plates, and grew on M63 medium with starch as the sole carbon source; these results indicated that the secretion machinery of these moderately halophilic strains was able to recognize and properly cleave the signal peptide of the Bacillus α-amylase, and to secrete the enzyme to the extracellular medium.

DISCUSSION

Recently, we reported the production and biochemical characterization of an extracellular amylase by the moderately halophilic bacterium H. meridiana (Coronado et al., 2000). The amylase exhibited activity at high salinity (up to 30% salts) with an optimum at 10% salt, pH 7.0 (being stable in alkaline conditions) and 37 °C. The main end products of starch hydrolysis were maltose and maltotriose, indicating an α-amylase activity (Coronado et al., 2000). The activity of this enzyme at high salinity and the absence of genetic studies of enzymes from this group of extremophilic micro-organisms of great biotechnological potential led us to investigate it in more detail.

The cloning and characterization of the H. meridiana amyH gene in this study will enable investigations of the molecular adaptations required by the enzyme to be active at high salt concentrations. Moreover, the gene has a potential application for the construction of expression and secretion vectors for the production of heterologous proteins by moderate halophiles (Ventosa et al., 1998).

Amino acid sequence comparison revealed that AmyH
The AmyH protein contains the four highly conserved regions in amylase enzymes (Nakajima et al., 1986). The invariant amino acid residues are also all conserved in the AmyH sequence. Some of these residues in the consensus sequences have been determined to play a role in the amylolytic activity. The sites responsible for consensus sequences have been determined to play a role in invariant amino acid residues are also all conserved in enzymes from extremophiles it is important to use addition, for the production of stable salt-tolerant regions in amylase enzymes (Nakajima et al., 1995). The AmyH protein contains the four highly conserved 

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REFERENCES


belongs to the already proposed family of α-amylases composed of the enzymes from Alteromonas haloplanktis, Thermomonospora curvata, streptomyces, insects and mammals (Janecek, 1994). Janecek (1994) suggested a separate evolutionary origin of this group from the two other groups of amylases from plants, fungi and yeasts. Therefore, our results include in the same branch of evolution the two α-amylases from extremophilic bacteria Alteromonas haloplanktis and H. meridiana. The enzymes from Alteromonas haloplanktis and Aeromonas hydrophila were assigned to family 13 of Henrissat’s classification of glycosyl hydrolases (Henrissat & Bairoch, 1993). Therefore, on the basis of amino acid homology, AmyH may be considered also a member of this family.

Although the AmyH protein excreted by H. meridiana shared a high homology with the α-amylases shown in Fig. 2, it displays optimal activities at high salt concentration (Coronado et al., 2000). Extracellular enzymes produced by halophilic micro-organisms have to be adapted to high salinity. It has been suggested that at least part of this adaptation involves an abundance of acidic residues (Lanyi, 1974) that, as judged by the examination of the first crystal structures of proteins from a halophilic organism (Haloarcula marismortui) are distributed over the protein surface (Elcock & McCammon, 1998). In agreement with this, the amylases from the moderate halophile H. meridiana and the halophilic archaeon Natronococcus sp., as well as the serine protease from the halophilic archaeon Natrualba asiatica, were shown to be very acidic proteins. This fact could partially explain the halotolerance exhibited by these enzymes. However, the molecular basis of the halotolerance of the characterized amylase is probably much more complex and is currently under investigation.

The ability of moderate halophiles to grow under extreme salt conditions makes them potentially useful for the production of heterologous proteins. An additional advantage is that most of them have very simple nutritional requirements, being able to use a wide range of compounds as the sole source of carbon and energy (Kushner & Kamekura, 1988; Ventosa et al., 1998). In addition, for the production of stable salt-tolerant enzymes from extremophiles it is important to use halophilic micro-organisms as hosts, since the correct protein folding at high salt concentrations, specific post-translational modification, and protein secretion in extreme conditions are essential for a correct enzyme function. The correct heterologous expression and secretion by Halomonas of the thermostable α-amylase from B. licheniformis is a promising starting point for the use of moderately halophilic bacteria as cell factories.


