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

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Two types of Tn*1732*-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

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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). When appropriate, the antibiotics gentamicin (30 μg ml^{-1}), kanamycin (100 μg ml^{-1}), rifampicin (25 μg ml^{-1}) and tetracycline (125 μg ml^{-1}) for the halophiles and ampicillin (150 μg ml^{-1}), gentamicin (20 μg ml^{-1}), kanamycin (50 μg ml^{-1}) and tetracycline (15 μg ml^{-1}) for *E. coli* were added to the media. When needed, SW and LB media were supplemented with 0·5 % (w/v) soluble starch. In all cases cultures were incubated at 37 °C.

The expression vectors pML122/123 (Gm^r; Labes *et al*., 1990), and the plasmid vector pBluescript II KS (Amp^r; Stratagene) were used for cloning. The broad-host-range cloning cosmid vector pVK102 (Km^r Tc^r; Knauf & Nester, 1982) was used to construct a genomic library of *H. meridiana* DSM 5425. Derivatives of pML123 and pVK102 were transferred from *E. coli* to *Halomonas* strains by triparental matings using pRK600 (Kessler *et al*., 1992) as helper plasmid.

Preparation of cell fractions. *H. meridiana* and mutant strains were grown in SW-5 medium supplemented with 0·5 % (w/v) starch at 37 °C for 24 h. The supernatant was collected after centrifugation at 10000 r.p.m. and concentrated by centrifugation through Centricon and Centripep columns (Amicon). To obtain the periplasmic fraction, the method described by Harold & Heppel (1965) was followed. The cell extracts were obtained by ultrasonic treatment (Vibra-cell). If the amylase activity was not determined immediately, the samples were stored at −20 °C after addition of 1 mM $CaCl_2$ and 10 μg ml^{-1} of the protease inhibitor PbSc (PefablocSC, Roche).

Amylase activity. The presence of amylolytic activity on plates was routinely determined following the method described by Cowan (1991), using SW-2 medium supplemented with 0·5 % (w/v) soluble starch. After incubation at 37 °C for 7 d, the plates were flooded with 0·3 % I_2/0·6 % KI solution: a clear zone around the growth indicated hydrolysis of starch. To determine the amylase activity in the different cell fractions, samples were assayed by automated reading in microtitre plates. Ten-microlitre samples were mixed with 50 μl amylase reagent from a diagnostic kit (Sigma) into microtitre plate vials, and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 25 μl 1 M Na_2CO_3 solution. Enzymic activity was measured by following the formation of reaction products at 450 nm using an EL312e Microplate Reader (BIO-TEC Instruments).

Gel electrophoresis. The method of Laemmli (1970) was used for SDS-PAGE. Proteins were stained with a silver nitrate solution (0·2 %) (Sambrook *et al*., 1989). High-MW protein markers (RPN 756, Amersham) were used as standards.

DNA manipulations. DNA manipulations and isolation of plasmid DNA were performed by standard procedures (Sambrook *et al*., 1989). Southern blot analyses were carried out by using digoxigenin-labelled probes according to the instructions of the manufacturer (Roche).

Transposon mutagenesis. Transposon mutagenesis was performed by conjugal transfer of pSUP102-Gm::Tn*1732* from *E. coli* SM10 (Simon *et al*., 1983; Ubben & Schmitt, 1986) to a spontaneous Rif^r mutant of the *H. meridiana* wild-type strain. Matings were carried out by mixing the donor and recipient cultures at a ratio of 1:4 (100 μl donor, 400 μl recipient). After centrifugation, the pellet was washed with SW-2, resuspended in 100 μl SW-2 and placed on a 0·45 μm pore filter on SW-2 solid medium. After overnight incubation at 37 °C, cells were resuspended in 20 % (v/v) sterile glycerol and, after appropriate dilutions, plated on SW-2 medium with rifampicin and kanamycin at a density resulting in about 100–200 colonies per plate. For selection of *H. meridiana* transconjugants with the Amy^- phenotype, colonies from these master plates were transferred with sterile toothpicks to SW-2 + Rif +Km plates containing 0·5 % (w/v) soluble starch. The amylase activity on the plates was detected as described above.

Library construction. An *H. meridiana* gene bank was constructed in the broad-host-range cosmid pVK102. *H. meridiana* genomic DNA was partially digested with *Hin*dIII, and DNA fragments in the size range 23–30 kb were separated in sucrose gradients and cloned into the *Hin*dIII 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 found 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

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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 Tn*1732*, 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^r 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 Tn*1732*) and an internal fragment of Tn*1732* was used as a probe. All mutants were shown to have a unique insertion of Tn*1732* 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 Tn*1732* 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.

Table 1. Amylase activity and growth in M63 with different carbon sources of the *H. meridiana* wild-type strain and Amy⁻ mutants

	Wild-type DSM 5425	Type I mutants	Type II mutant
Amylase activity*			
Supernatant	76	0	0
Periplasm	21	15	0
Cell extract	0	0	0
Growth in M63 medium†			
Starch	+	−	−
Maltose	+	−	+
Glucose	+	−	+
Sucrose	+	−	+
Fructose	+	−	+
Glutamic acid	+	−	+
Proline	+	−	+

*The numbers indicate the percentage of amylase activity in the different cellular fractions.

†Strains were incubated in M63 supplemented with 5% NaCl and the different compounds as the sole carbon source.

Mutant II is affected in the α-amylase synthesis gene

To identify the gene disrupted by Tn*1732* 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 Tn*1732* as a probe against mutant DNA independently digested with several restriction enzymes showed that a *Dra*I site is located very close to the Tn*1732* insertion in mutant II. Chromosomal DNA of mutant II was digested with *Dra*I and *Pst*I, and ligated to *Eco*RV/*Pst*I-digested pKS. The ligation mixture was used to transform *E. coli* DH5α. From several Km^r Amp^r colonies, plasmid pMJC27 was isolated, which carried a 2·2 kb *Dra*I/*Eco*RV-*Pst*I insert (Fig. 1a). A restriction analysis of this plasmid showed a 1·85 kb *Eco*RI-*Pst*I region, corresponding to the left end of Tn*1732*, and a 0·3 kb *Dra*I/*Eco*RV-*Eco*RI 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

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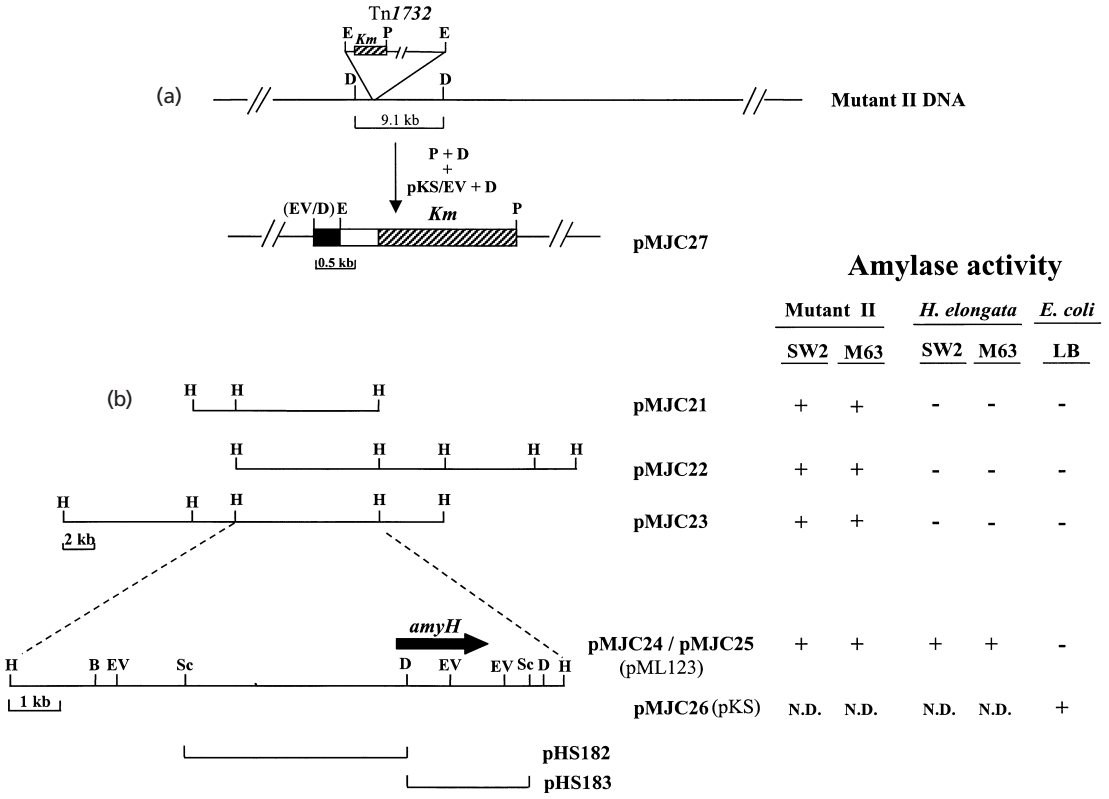

Fig. 1. (a) Isolation of the DNA flanking the left end of the transposon Tn*1732* in the Amy⁻ mutant II of *H. meridiana*. (b) Restriction maps of the overlapping plasmids pMJC21 to 23, isolated by functional complementation of mutant II. Below are shown the common 10 kb *Hin*dIII region carrying the amylase gene of *H. meridiana* (*amyH*) cloned in two different vectors, and the plasmids pHS182 and pHS183 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, *Bam*HI; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Sc, *Sac*I.

in Methods and introduced into the Amy⁻ mutant II for detection of Amy⁺ colonies. Out of 3250 Km^r 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 *Hin*dIII 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 *Dra*I-*Eco*RV fragment, included in a larger 6·25 *Sac*I-*Eco*RV region (Fig. 1b). Two *Sac*I-*Dra*I segments of pMJC26, of 4·1 kb and 2·3 kb, were subcloned in pKS, generating pHS182 and pHS183, respectively (Fig. 1b). These plasmids were used for sequencing. A total of 1·6 kb DNA spanning the 0·7 kb *Dra*I-*Eco*RV fragment was sequenced (EMBL accession no. AJ239061). A primer complementary to the 5′ end of pHS183 was generated and used to sequence the DNA including the *Dra*I site with pMJC26 as a template. This ruled out the possibility of a second *Dra*I site at the junction between pHS182 and pHS183. 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

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Microorganisms	Region I	Region II	Region III	Region IV
Halomonas	**AAGVDVYADAVINHVA** 109	**GVGGIRIDAAKHMAP** 201	**FQEVIDL** 224	**VFTDNHDN** 285
Alteromonas	**AAGVDIYVDTLINHMA** 115	**GVKGFRFDASKHVAA** 205	**FQEVIDQ** 228	**VFVDNHDN** 289
Aeromonas	**AAGVKVYADAVFNHMA** 116	**GVAGFRIDAAKHMAP** 205	EVIGAGG 229	**VFVDNHDP** 289
Bacillus	**AAGVKVIADAVINHMT** 141	**GVDGLRIDAVKHIAA** 232	IVQEVIR 256	**VFVDNHDT** 321
Thermomonospora	EAGVKIYVDAVINHMT 133	**GVAGFRIDAAKH**IPE 226	**FQEVIA**- 256	**VFVVNHDT** 314
Streptomyces	**AAGVKVVADSVINHMA** 123	**GVDGFRIDAAKH**MPA 212	K**QEAI**HG 236	**VFVDNHDT** 297

Fig. 2. Partial alignments of the deduced amino acid sequences of the α-amylase precursor encoded by the *amyH* gene of *H. meridiana* with homologous proteins from *Alteromonas haloplanktis*, *Aeromonas hydrophila*, *Bacillus* sp., *Thermomonospora curvata* and *Streptomyces griseus*. The braces indicate the four regions highly conserved in α-amylases. The asterisks and the black dot denote the amino acid residues potentially involved in Ca^{2+} and chloride binding, respectively. The sites responsible for substrate binding proposed by Matsuura *et al.* (1984) are indicated by stars. Identical amino acid residues are showed in bold. Amino acid positions are numbered to the right of the alignment.

Table 2. Amino acid composition, charge, and theoretical isoelectric point of several amylases and one serine protease from halophilic and non-halophilic micro-organisms

Micro-organism	Extracellular enzyme	Acidic residues (%)	Basic residues (%)	Net charge	pI
*Halomonas meridiana**	Amylase	12·4	5·5	−32	4·65
Alteromonas haloplanktis	Amylase	7·9	8·4	+2	8·12
Aeromonas hydrophila	Amylase	9·5	8·6	−2	6·43
Bacillus sp.	Amylase	7·2	6·2	−14	6·20
Thermomonospora curvata	Amylase	9·2	7·6	−10	6·04
Streptomyces griseus	Amylase	9·7	7·8	−11	5·63
Natronococcus sp.†	Amylase	22·6	6	−81·5	4·12
Natrialba asiatica†	Serine protease	11·5	4·7	−49	4·11

* Moderately halophilic micro-organism.

† Extremely halophilic micro-organisms.

protein with a deduced molecular mass of 50 kDa. Database searches revealed the product 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 actinomycete *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 amino acid distribution, net charge and theoretical isoelectric point of AmyH were analysed and compared with the amylases of the non-halophilic bacteria included in Fig. 2. The amylase from the haloalkaliphilic archaeon *Natronococcus* sp. (Kobayashi *et al.*, 1994) and the serine protease from the halophilic archaeon *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 archaea are predominantly acidic proteins.

Since the *H. meridiana* amylase is an extracellular enzyme, it would be expected to possess an amino-

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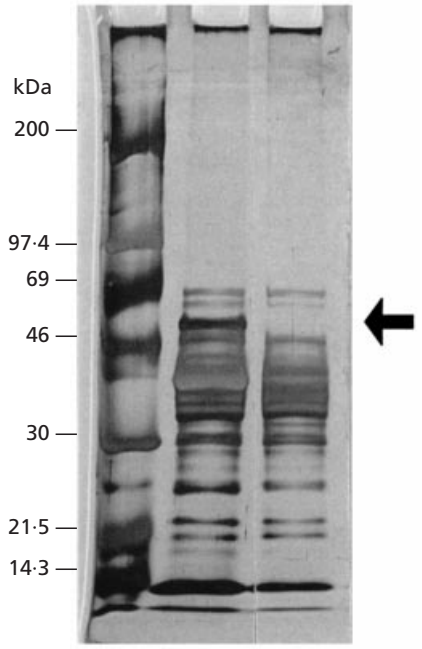

Fig. 3. Detection of the extracellular α-amylase from *H. meridiana* on an SDS-polyacrylamide gel. The wild-type (lane 1) and mutant II (lane 2) strains were grown in SW-5 medium + 0·5 % starch. The supernatants were collected by centrifugation, concentrated, and extracellular proteins were electrophoretically separated on an SDS/12 % polyacrylamide gel and visualized by silver staining. The position of the AmyH protein is indicated by an arrow on the right, and the molecular masses of marker proteins on the left.

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

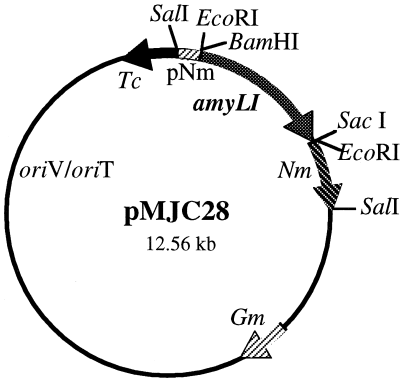

Fig. 4. Structure of pMJC28, in which the *B. licheniformis amyLI* gene is under the control of the neomycin-resistance gene promoter. This plasmid conferred amylase activity upon *H. meridiana* mutant II and *H. elongata*.

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

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belongs to the already proposed family of α-amylases composed of the enzymes from *Alteromonas haloplanktis*, *Thermomonospora curvata*, streptomycetes, 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.

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 substrate binding proposed by Matsuura *et al*. (1984) for Taka-amylase A from *Aspergillus oryzae* are present in the *H. meridiana* amylase. Furthermore, the residues Asn106 and His198 reported to be involved in Ca^{2+} binding (Machius *et al*., 1995) are fully conserved. In addition, the two strictly conserved residues likely to be involved in chloride binding (Arg192 and Asn281) are also present in AmyH.

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 *Natrialba 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.

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