Extremophiles (2006) 10:1–16
DOI 10.1007/s00792-005-0466-z

REVIEW

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Rhodothermus marinus: physiology and molecular biology

Received: 8 November 2004 / Accepted: 17 June 2005 / Published online: 2 August 2005

Abstract *Rhodothermus marinus* has been the subject of many studies in recent years. It is a thermohalophilic bacterium and is the only validly described species in the genus *Rhodothermus*. It is not closely related to other well-known thermophiles and is the only thermophile within the family *Crenotrichaceae*. *R. marinus* has been isolated from several similar but distantly located geothermal habitats, many of which are subject to large fluctuations in environmental conditions. This presumably affects the physiology of *R. marinus*. Many of its enzymes show optimum activity at temperatures considerably higher than 65°C, the optimum for growth, and some are active over a broad temperature range. Studies have found distinguishing components in the *R. marinus* electron transport chain as well as in its pool of intracellular solutes, which accumulate during osmotic stress. The species hosts both bacteriophages and plasmids and a functional intein has been isolated from its chromosome. Despite these interesting features and its unknown genetics, interest in *R. marinus* has been mostly stimulated by its thermostable enzymes, particularly polysaccharide hydrolysing enzymes and enzymes of DNA synthesis which may be useful in industry and in the laboratory. *R. marinus* has not been amenable to genetic analysis until recently when a system for gene transfer was established. Here, we review the current literature on *R. marinus*.

Communicated by D. A. Cowan

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Keywords *Rhodothermus marinus* · *Bacteroidetes* · Thermophile · Halophile · Thermostable enzymes

Introduction

Rhodothermus marinus is both a thermophile and a halophile (Alfredsson et al. 1988). It belongs to the newly defined phylum *Bacteroidetes*, which consists of three classes, *Bacteroidetes, Flavobacteria* and *Sphingobacteria* (Garrity et al. 2004). Class *Bacteroidetes* represents anaerobes, which are often found in high numbers in intestinal tracts of animals (Kirchman 2002). Members of the other two classes are mainly aerobic and very abundant in many freshwater and marine systems, particularly in the oceans. Many of them are important in degradation of organic material. *Cytophaga* species of class *Sphingobacteria*, in particular, digest various biopolymers, such as cellulose, chitin, pectin, starch and proteins (Reichenbach 1991). *R. marinus* belongs to the class *Sphingobacteria* and family *Crenotrichacea* (Garrity et al. 2004). The species shares many characteristics with its relatives of *Sphingobacteria*. It is aerobic, chemoorganotrophic and has been isolated from marine habitats. It also possesses many polysaccharide hydrolysing enzymes. However, *R. marinus* grows optimally at 65°C while most species in class *Sphingobacteria* grow optimally at 26–30°C. The only other thermophiles in *Sphingobacteria* belong to family *Flammeovirgaceae* and genus *Thermonema*. Interestingly, the closest relative of *R. marinus* is *Salinibacter ruber*, an extreme halophile of family *Crenotrichaceae* (Anton et al. 2002; Garrity et al. 2004).

Habitat, phylogeny and genetic diversity

A *Rhodothermus* species was first isolated from submarine alkaline freshwater hot springs at 2–4 m depth in Isafjardardjup, NW Iceland (Alfredsson et al. 1988). The

described species, *R. marinus*, grew at 54–77°C and from 0.5% NaCl to over 6% NaCl and is therefore both thermophilic and slightly but strictly halophilic. *R. marinus* was also found to be heterotrophic and obligately aerobic. Thus, *R. marinus* can only grow in a very narrow zone in the hot springs, close to their openings, determined by temperature and salt concentration as well as of content of O_2 and organic material. Since 1988, *R. marinus* has been isolated in similar habitats in distantly located geothermal environments around the world. It was found in marine hot springs at Praia de Ribeira Quente (Nunes et al. 1992a) and Ferraria (Silva et al. 2000) on the island of São Miguel in the Azores, Portugal, in Stufe di Nerone, near Naples, Italy (Moreira et al. 1996) and on the island of Monserrat in the Caribbean Sea (Silva et al. 2000). It has also been isolated from three additional geothermal sites in Iceland; from coastal springs and from a borehole effluent in Oxarfjordur, NE Iceland and from borehole effluents from a powerplant at the Blue Lagoon and from a salt factory in Reykjanes, both in SW Iceland (Petursdottir et al. 2000).

The phylogenetic position of the genus *Rhodothermus* has been studied by sequence analysis of the 16S rRNA gene of the *R. marinus* type strain DSM 4252^T (Andresson and Fridjonsson 1994). Its chromosome was found to contain a single rRNA operon with a 16S–23S intergenic region coding for $tRNA^{Ile}$ and $tRNA^{Ala}$. Sequence comparison placed *Rhodothermus* close to the root of the *Flexibacter-Cytophaga-Bacteroides* (FCB) group with affinities to green sulphur bacteria, fibrobacteria and spirochaetes (Andresson and Fridjonsson 1994). The former FCB group is now represented by the phylum *Bacteroidetes* and *Rhodothermus* belongs to family *Crenotrichaceae* along with *Crenothrix, Chitinophaga, Salinibacter* and *Toxothrix* (Garrity et al. 2004). The current location of *Rhodothermus* in the phylogenetic tree and relatedness within *Bacteroidetes* is represented in Fig. 1. The only described thermophilic species within *Bacteroidetes* belong to *Rhodothermus* and *Thermonema*. Culture independent studies at various thermal sites have nevertheless detected phylotypes assigned to *Bacteroidetes* (Hugenholtz et al. 1998; Nakagawa and Fukui 2002; Sievert et al. 2000). Extremophilic characters, besides thermophily, are found in other members of *Crenotrichaceae*. *S. ruber*, the closest relative of *R. marinus* is extremely halophilic (Anton et al. 2002) and *Toxothrix trichogenes* is a psychrophile (Hirsch 1989, 1991). The genera in *Crenothrichaceae* contain only one described species each.

In 1996, Sako et al. described a new *Rhodothermus* species, *R. obamensis*, isolated from Tachibana Bay, Japan. The description was based on 95% identity with the *R. marinus* 16S rRNA sequence and *R. obamensis* was reported to grow at 50–85°C and optimally at 80°C. However, Silva et al. (1999) examined the effect of temperature on growth of the *R. obamensis* type strain JCM 9785^T and found it to grow optimally at about 70°C and at a maximum temperature of about 77°C. They redetermined the 16S rRNA sequence of JCM 9785^T and found it to be 99.5% identical to the sequence of *R. marinus* DSM 4252^T (Silva et al. 2000). They also found a DNA–DNA reassociation value of 78%. Silva et al. (2000) therefore suggested reclassification of *R. obamensis* as a member of the species *R. marinus* and it will be referred to herein as *R. marinus* JCM 9785. *R. marinus* strains, although isolated from distantly located thermal sites, have been shown to be genetically closely related on the basis of DNA–DNA reassociation values of 68–96% (Moreira et al. 1996; Silva et al. 2000) and highly similar 16S rRNA sequences (Petursdottir et al. 2000; Silva et al. 2000).

Despite high phylogenetic similarity, considerable genetic diversity of *R. marinus* isolates has been detected by ribotyping (Moreira et al. 1996) and by analysis of electrophoretically demonstrated variation (Petursdottir et al. 2000). The latter approach revealed quite high genetic diversity (*Ht* of 0.586) when 13 loci in 81 Icelandic and two Azorean strains were examined. It also showed that *R. marinus* forms distinct populations in different geothermal locations in Iceland. Isolates from SW and NE Iceland, as well as the Azorean strains, clustered together while isolates from NW Iceland constituted a second major cluster. The study concluded that isolation and genetic drift has caused divergence in *R. marinus* and, from a high association index, that its diversity is not much affected by intraspecies recombination.

Phenotypic characters and growth

R. marinus is a gram-negative rod, about 0.5 μm in diameter and 2–2.5 μm long (Fig. 2) (Alfredsson et al. 1988). Neither spores, lipid granules, nor filaments have been seen but Nunes et al. (1992a) reported the presence of a polar flagellum. The cells have been shown to form a distinct capsule when grown on carbohydrate rich medium (Alfredsson et al. 1988). *R. marinus* isolates have very similar phenotypic and chemotaxonomic characters (Alfredsson et al. 1988; Moreira et al. 1996; Nunes et al. 1992a, 1992b; Sako et al. 1996). The subtle variation found is sporadically distributed among isolates except for the lack of pigment, which is characteristic for strains from certain sample sites (Petursdottir et al. 2000). Most strains are reddish-coloured, however, due to a carotenoid pigment (Alfredsson et al. 1988). The carotenoids of strain DSM 4253 have been found to consist of two types of free carotenoid glucosides (6%) and their acyl derivatives (94%) (Lutnaes et al. 2004). Interestingly, the major catotenoid acyl glucoside is closely related in structure to the major carotenoid of *S. ruber*. The minor carotenoid acyl glucoside is highly similar to a carotenoid detected in the thermophile *Rosiflexus castenholzii*.

The fatty acid composition of different *R. marinus* isolates was found to be very similar and to include mainly branched chains (Moreira et al. 1996; Nunes et al. 1992b;

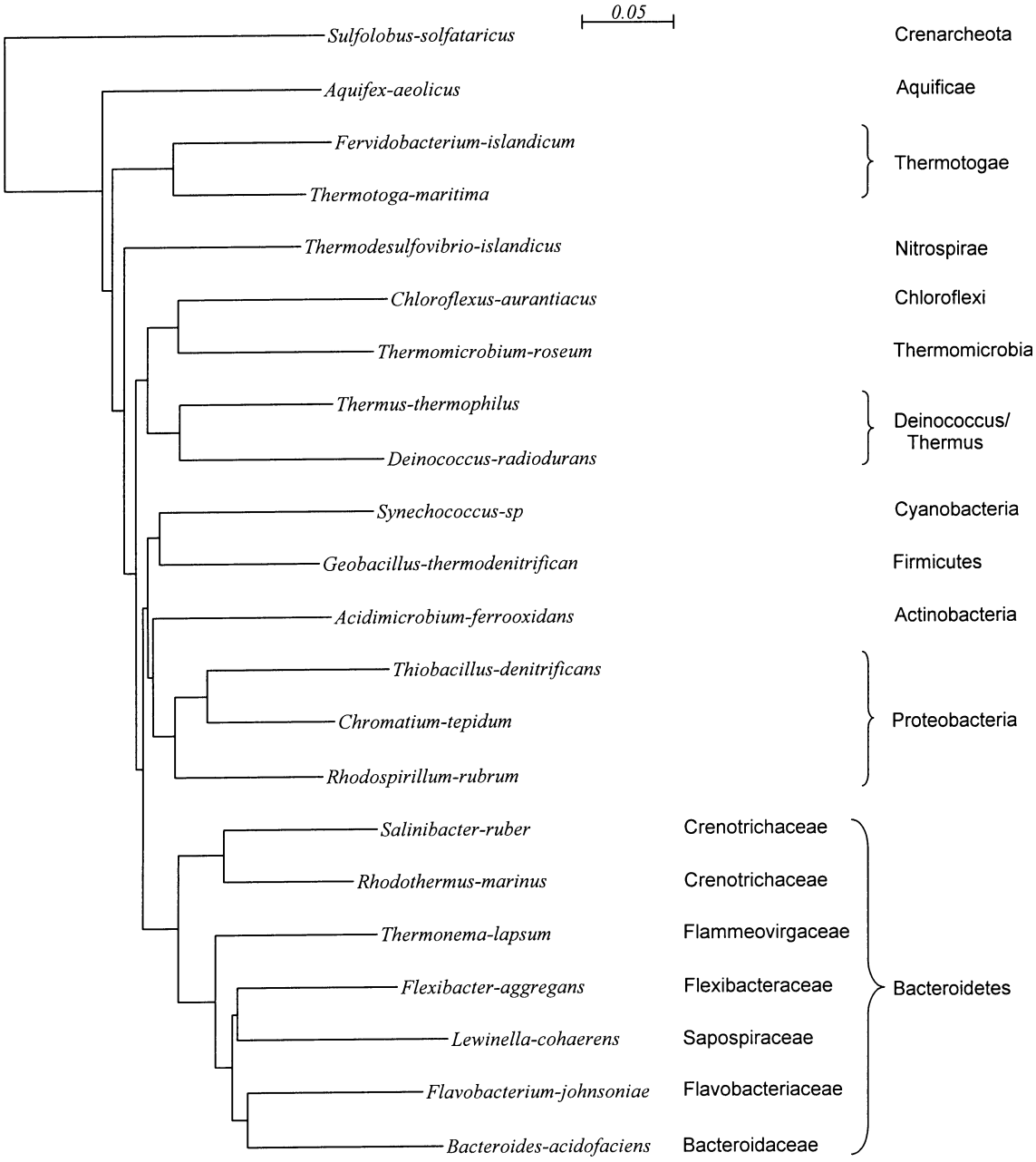

Fig. 1 The phylogenetic position of *R. marinus* showing the relatedness within the phylum of *Bacteroidetes*. *S. ruber* is the closest relative of *R. marinus*. Evolutionary distances were computed from pairwise similarities using the correction of Jukes and Cantor. Distance tree was constructed by the neighbour joining algorithm. The 16S rRNA sequences from various bacteria, mainly thermophiles, representing the main bacterial phyla were used. The archaeon *Sulfolobus solfataricus* was included as an outgroup. The sequences were retrieved from GenBank under the following accession numbers: *Sulfolobus solfataricus* (SSREN16S), *Aquifex aeolicus* (AAE309733), *Fervidobacterium islandicum* (AF434670), *Thermotoga maritima* (AJ401021), *Thermodesulfovibrio islandicus* (TIS16SRRN), *Chloroflexus aurantiacus* (CFX16SRRNA), *Thermomicrobium roseum* (THRRRDA), *Thermus thermophilus* (AY554280), *Deinococcus radiodurans* (DEIRGDA), *Synechococcus sp* (AY172835), *Geobacillus thermodenitrificans* (AB116104), *Acidimicrobium ferrooxidans* (AFU75647), *Thiobacillus denitrificans* (TDE243144), *Chromatium tepidum* (CVNRR16SA), *Rhodospirillum rubrum* (RSP16SRNAK), *Salinibacter ruber* (AF323499), *Rhodothermus marinus* (AF217495), *Thermonema lapsum* (L11703), *Flexibacter aggregans* (AB078041), *Lewinella cohaerens* (AF039292), *Flavobacterium johnsoniae* (CYTRR16SH) and *Bacteroides acidofaciens* (AB021157)

Tindall 1991). Nunes et al. (1992b) determined iso-C15, anteiso-C15, iso-C17 and anteiso-C17 as the major fatty acids in *R. marinus*. However, proportions of even-numbered and odd-numbered chains depended highly on the growth medium (Chung et al. 1993). Polar lipids were found to include phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and glycolipids as minor components (Nunes et al. 1992b; Tindall 1991). The

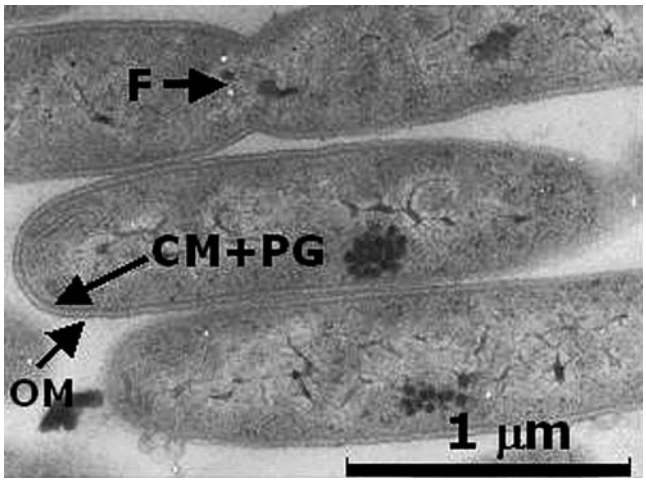

Fig. 2 An electron micrograph of *R. marinus* strain R-18. The figure shows both single cells and dividing cells with furrowing (*F*). The cell envelope is clearly visible. It consists of a cytoplasmic membrane and a peptidoglycan layer (*CMPG*) and a well separated outer membrane (*OM*)

polyamines spermidine, spermine, thermopentamine as well as a tertiary tetraamine and a quaternary pentaamine have been detected (Hamana et al. 1992). The G+C content of the *R. marinus* genome is about 64% (Alfredsson et al. 1988) and its size has been estimated at about 3.3–3.6 Mbp (Moreira et al. 1996). *R. marinus* is oxidase negative and catalase positive. It was found to be sensitive to several antibiotics, including ampicillin, chloramphenicol, lincomycin and tetracycline but resistant to aminoglycosides.

R. marinus isolates show some variation in utilization of carbon sources. Most strains use several common sugars, such as glucose, galactose, lactose, maltose, raffinose and sucrose (Alfredsson et al. 1988; Nunes et al. 1992a; Sako et al. 1996). *R. marinus* JCM 9785 utilizes several amino acids (Sako et al. 1996) while other strains are only reported to use aspartate, glutamate, asparagine and glutamine (Alfredsson et al. 1988; Nunes et al. 1992a). Optimum pH for growth is 7.0 (Alfredsson et al. 1988) and no growth has been observed at pH 5.0 or below (Gomes et al. 2000). Small amounts of formic acid, acetic acid and some alcohols were found to accumulate during growth and lower the pH of the growth medium (Kristjansson and Alfredsson 1992). The products were used up and the pH rose again at the end of the growth. However, when glucose concentration was higher than 4 g/l, the pH did not recover and a sudden drop in optical density was seen. *R. marinus* is routinely grown in Degryse medium 162 (Degryse et al. 1978) containing 0.25% yeast extract, 0.25% tryptone and 1% NaCl. Strain DSM 4252 has a maximum specific growth rate of 0.5 h^{-1} in this medium at 65°C (Alfredsson et al. 1988). It grows optimally at 65–70°C in the medium while 55°C and 77–80°C are minimum and maximum growth temperatures, respectively (Alfredsson et al. 1988; Silva et al. 1999). The strain does not grow without NaCl. The optimum NaCl concentration is about 2% and the maximum is above 6% (Alfredsson et al. 1988). However, optimal salinity is dependent on the growth temperature since 3% NaCl gave higher specific growth rate at supraoptimal growth temperatures than 1–1.5% NaCl while the reverse was true for growth at temperature of 70°C or lower (Silva et al. 1999). *R. marinus* can use different high-molecular weight carbohydrates, such as starch, xylan and locust bean gum as carbon sources and different nitrogen sources such as meat and fish peptones as well as yeast extract (Gomes and Steiner 1998). Media optimised for production of several enzymes have been reported (Gomes et al. 2000, 2003; Gomes and Steiner 1998).

Enzymes

Most of the known *R. marinus* enzymes were identified in the search for thermostable enzymes with biotechnological potentials. Thermostable polysaccharide degrading enzymes are expected to have advantages in industrial processes and such enzymes from *R. marinus* have turned out to be extremely thermostable. Many of them exhibit optimal activity at about 85°C (Table 1), which is high above the optimum temperature for growth. It should be noted that all characterized polysaccharide hydrolysing enzymes from *R. marinus* are derived from strains from NW Iceland where temperature in the hot springs fluctuates with the ebb and flow of the tides. Possession of such enzymes might enable *R. marinus* to utilize organic material in its surroundings outside the narrow zone where it can grow. However, although most of these enzymes have been found extracellularly, many of them appear to be attached to the cell surface. The cells should therefore have direct access to the degradation products. Restriction endonucleases and enzymes of DNA synthesis from *R. marinus* have also been examined for biotechnological use. These, and other known *R. marinus* enzymes which carry out their function inside the cell, have in general lower optimal temperatures than the polysaccharide hydrolysing enzymes (Table 1) and some are active over wide temperature ranges (Blondal et al. 1999; Thorbjarnardottir et al. 1995). Only a few proteins that participate in biosynthesis or other pathways in *R. marinus* have been characterized except for those involved in the respiratory chain and stress responses.

Polysaccharide hydrolysing enzymes

β-Glucanases

Genes encoding enzymes belonging to glycoside hydrolase family 16 have been isolated from *R. marinus* DSM 4253 (Spilliaert et al. 1994) and DSM 4252 (Krah et al. 1998). The gene products, designated BglA and LamR were 89.6% identical with most of the difference being in

Table 1 *R. marinus* genes that have been sequenced and whose products have been characterized after expression in an alternative host

Gene	GenBank No.	Strain	Product	Function	Topt (°C)	References
bglA	U04836	DSM 4253	β-Glucanase	β-Glucan hydrolysis	85°C	Spilliaert et al. (1994)
lamR	AF047003	DSM 4252	Laminarinase	β-Glucan hydrolysis	88°C	Krah et al. (1998)
celA	U72637	DSM 4253	Cellulase	β-Glucan hydrolysis	100°C	Halldorsdottir et al. (1998)
xyn10A	Y11564X87417	DSM 4252	Xylanase	Xylan hydrolysis	80°C	Nordberg-Karlsson et al. (1997, 1998b)
manA	X90947	DSM 4252	Mannanase	Mannan hydrolysis	85°C	Politz et al. (2000)
chiA	AY706992	DSM 4253	Chitinase	Chitin hydrolysis	70°C	Hobel et al. (2004)
polA	AF028719	ITI 518	DNA polymerase I	DNA synthesis	55°C	Blondal et al. (2001)
ligA	U10483	DSM 4253	DNA ligase	DNA ligation	55°C	Thorbjarnardottir et al. (1995)
tdk	AF028720	ITI 518	Thymidine kinase	Pyrimidine metabolism	65°C	Blondal et al. (1999)
ppc	X99379	JCM 9785	Phosphoenolpyruvate carboxylase	Pyruvate metabolism	75°C	Takai et al. (1998b)
glgB	AB060080	JCM 9785	1,4-α-Glucan branching enzyme	Starch biosynthesis	65°C	Shinohara et al. (2001)
mgs	AF173987	DSM 4252	Mannosylglycerate synthase	Mannosylglycerate biosynthesis	85–90°C	Martins et al. (1999)
mpgS	AY271294	DSM 4252	Mannosyl-3-phospho-glycerate synthase	Mannosylglycerate biosynthesis	80°C	Borges et al. (2004)
mpgP	AY271294	DSM 4252	Mannosyl-3-phospho-glycerate phosphatase	Mannosylglycerate biosynthesis	70–80°C	Borges et al. (2004)

Optimum temperatures for activity of the recombinant enzymes are listed

the N-terminal end (Krah et al. 1998). They were shown to have the same substrate specificity and were active on mixed-linkage β-glucans such as barley glucan and lichenan as well as on 1,3-β-homoglucans, such as laminarin but neither on carboxymethyl cellulose nor xylan (Krah et al. 1998; Spilliaert et al. 1994). The BglA has a putative signal sequence of 30 residues and the molecular mass of the predicted mature protein is 29.7 kDa (Spilliaert et al. 1994). Its catalytic domain was produced in *Escherichia coli* and was able to degrade laminarin and lichenan to monosaccharides and disaccharides. It had optimum activity at 85°C and a half-life of 3 h at this temperature. Activity of the recombinant LamR in *E. coli* was mainly found in a periplasmic fraction and N-terminal sequencing suggested a mature protein of 29.9 kDa (Krah et al. 1998). The LamR activity was mainly found in a fraction of cytoplasm and membranes of *R. marinus* and two active bands of 32 and 34 kDa were detected in cell extracts. The LamR showed optimum activity at 88°C. Its structure was examined by circular dichroism and fluorescence measurements, which suggested prevailing β-secondary structures and surface localization of tryptophan residues (Krah et al. 1998). Also, site-directed mutagenesis confirmed that Glu 129, Asp 131 and Glu 134, which are conserved in glycoside hydrolase family 16, were essential for catalytic activity. The LamR has been shown to perform transglycosylation during hydrolysis (Petersen et al. 2000).

An extremely thermostable cellulase, or endo-β-1,4-glucanase, with a half-life of 3.5 h at 100°C has been detected in cultivation supernatants of *R. marinus* DSM 4253 (Hreggvidsson et al. 1996). The enzyme was found to degrade carboxymethyl cellulose to glucose, cellobiose, cellotriose and a mixture of cellopentaose and larger oligosaccharides. A gene from DSM 4253, *celA*, possibly encoding the same cellulase, was sequenced and expressed in *E. coli* (Halldorsdottir et al. 1998). Sequence analysis predicted a polypeptide of 28.8 kDa with a putative signal sequence of 17 residues and placed CelA in glycoside hydrolase family 12. The enzyme produced in *E. coli* had highest measured initial activity at 100°C. Its putative signal peptide, which is of hydrophobic nature, has been shown to cause low levels of CelA production as well as cytotoxicity in *E. coli* (Wicher et al. 2001). Its deletion significantly improved production rates and independent expression of the 5′-part of the gene caused cell lysis. Wicher et al. suggested that CelA is cell associated through its hydrophobic signal peptide, which is separated from the catalytic domain by a flexible linker. The structure of the catalytic domain has been solved to a 1.8 Å resolution and is thought to represent an active conformation (Crennell et al. 2002). The CelA has a β-jelly roll fold, characteristic of other known structures of glycosyl hydrolase family 12. It is comprised of two β-sheets, which pack against each other and an α-helix. The two catalytic residues, Glu 207 and Glu 124, lie on one of the β-sheets, within an active site cleft. Structure comparison with family 12 cellulases from mesophiles suggested that an increase in ion pairs and stabilization of a mobile loop close to the active site are among factors contributing to the thermostability of CelA.

Xylanolytic enzymes

R. marinus DSM 4252 has been shown to secrete xylanolytic enzymes when grown on xylan (Dahlberg et al. 1993). Activity of an endo-1,4-β-xylanase was mainly found in culture supernatants or loosely attached to the cells and had a half-life of about 14 h at 90°C. An extracellular 1,4-β-xylosidase was also detected. It was later partially purified and shown to have optimal activity at 90°C and a half-life of 14 h at 85°C (Manelius et al. 1994). A gene encoding Xyn10A, a modular endo-1,4-β-xylanase of five domains, was later isolated from *R. marinus* DSM 4252 (Nordberg Karlsson et al. 1997). The two N-terminal domains were 88% similar and showed homology to carbohydrate-binding domains of family 4. They were followed by a domain of unknown function and a catalytic domain, which showed similarity to glycoside hydrolase family 10. The C-terminal domain has been proposed to mediate cell attachment (Abou-Hachem et al. 2003a, 2003b). The catalytic domain, of 411 amino acids has been cloned and expressed in *E. coli* (Nordberg Karlsson et al. 1998b, 1999). It exhibited optimal activity at 80°C and had a half-life of 100 min at this temperature (Nordberg Karlsson et al. 1998b). It was stabilized in the presence of calcium. The domain showed highest specific activity towards xylan but also degraded xylo-oligosaccharides and β-glucans.

The two N-terminal domains, CMB4-1 and CMB4-2, have been studied in some detail. They were proven essential for carbohydrate-binding of the full length xylanase which is specific for xylan, β-glucan and amorphous but not crystalline cellulose (Abou-Hachem et al. 2000; Nordberg Karlsson et al. 1998a). However, the presence of CMB4-1 and CMB4-2 did not improve pulp bleaching when truncated forms of the enzyme were compared (Pfabigan et al. 2002). Both modules were found to bind substrates and had the same specificity but differed in substrate affinity and stability (Abou-Hachem et al. 2000). The solution structure of CMB-2 has been determined (Simpson et al. 2002). It has a prominent cleft and consists of two β-sheets, which form a sandwich motif. The structure allowed identification of residues, in and around the cleft, which presumably interact with xylan. Residues Trp 69 and Phe 110 were identified as key ligands. The structure was shown to bind two calcium ions, which increased its unfolding temperature by approximately 23°C (Abou-Hachem et al. 2002). The locations of calcium binding sites in CMB4-2 were identified and verified by site-directed mutagenesis (Abou-Hachem et al. 2002). One calcium ion was bound with extremely high affinity and was totally buried within the structure. It is ligated by residues Glu 11 and Asp 160 and bridges the two termini of the module. The other calcium ion was located at the edge of the substrate-binding groove and is ligated by the side chain of Asp 29. Stability of the full-length enzyme was also positively affected by calcium and the third domain was shown to bind it as well (Abou-Hachem et al. 2003a). However, excess calcium reduced kinetic stability of the enzyme.

β-Mannanase

R. marinus DSM 4252 was shown to possesses a high β-mannanase activity as well as a very low β-mannosidase activity when grown on locust bean gum (Gomes and Steiner 1998). Optimal mannanase activity in culture filtrates was found at 85°C. It had a half-life of 4.2 h at 90°C and was stable over a broad pH range. A mannanase encoding gene, *manA*, was later isolated from strain DSM 4252 (Politz et al. 2000). Sequence analysis found the protein to consist of two domains. The N-terminal domain is of unknown function while the C-terminal domain showed homology to mannanases belonging to glycoside hydrolase family 26. Putative membrane-spanning helices were found on both sides of this putative catalytic domain but neither a signal peptide nor a carbohydrate-binding module was found. When produced in *E. coli*, several active protein bands of 45–60 kDa were observed. The full-length protein of 113 kDa was only observed in crude extracts of *R. marinus* but the main active fragment was about 62 kDa. Almost all mannanase activity in *R. marinus* was found in a periplasmic fraction. The recombinant ManA showed optimal activity at 85°C and 25% of the activity remained after 1 h at 90°C. The enzyme isolated from *R. marinus* had slightly higher optimal temperature and 87% of the activity remained after 1 h at 90°C. ManA was endo-acting, hydrolysed carbo-galactomannan, locust bean gum and guar gum and required oligosaccharide substrates containing at least five sugar moieties for efficient cleavage.

Chitinase

A gene encoding a chitinase of glycoside hydrolase family 18 has been isolated from the genome of *R. marinus* 4253 (Hobel et al. 2005). The deduced enzyme is of one domain of 377 amino acids. The gene was poorly expressed in *E. coli* but yields of the enzyme increased when the region encoding a putative signal peptide of 19 amino acids was deleted. Optimum activity of the enzyme was observed at 70°C. It had a half-life of 3 h at 90°C and is the most thermostable bacterial chitinase in family 18. The enzyme was shown to be endo-acting and released mainly chitobiose but acted as an exochitobiohydrolase on chitin oligomers of less than five residues.

Other polysaccharide hydrolysing enzymes

Activities of other polysaccharide hydrolysing enzymes have been detected in *R. marinus* but they are characterized to a lesser extent than the chitinase, mannanase, xylanase and the glucanases. Strain DSM 4252 was

shown to produce an α-L-arabinofuranosidase, which was induced during growth on xylan (Gomes et al. 2000). More yields of the enzyme were obtained after treatment with Triton X-100, which might indicate that it is loosely attached to the cells. The crude arabinofuranosidase showed optimal activity at 85°C and had a half-life of 8.3 h at this temperature. It was successfully used for prebleaching of softwood kraft pulp, in a crude preparation containing xylanase and mannanase.

Activity of amylase, pullulanase and α-glucosidase has been detected in five *R. marinus* strains, including DSM 4252 (Gomes et al. 2003). The culture medium for strain ITI 990 was optimised for production of the enzymes and maltose was most efficient for their induction, followed by starch, glycogen and pullulan. Optimum temperatures were 85 and 80°C for the crude amylase and pullulanase and they had half-lives of 3 h and 30 min at 85°C, respectively. Activity of β-glucosidase as well as of α-galactosidase and β-galactosidase have also been found in culture filtrates of *R. marinus* DSM 4252 (Gomes et al. 2000). Blucher et al. also reported activity of α-galactosidase in DSM 4252 and found most of it in cell fractions. The enzyme is presumably a homotetramer of 200 kDa with optimal activity at 85°C and a half-life of 2 h at 75°C. Interestingly, it hydrolysed both galactosaccharides of high and low molecular weight (Blucher et al. 2000).

Restriction endonucleases and enzymes of DNA synthesis

R. marinus strains have been screened for restriction endonucleases (Hjorleifsdottir et al. 1996) and for activity of thermostable DNA ligases and DNA polymerases (Hjorleifsdottir et al. 1997). Extracts from 43 strains were examined for restriction-activity and polymerase activity and extracts from 12 strains for ligase activity. Restriction of selected template DNA was observed after incubation with majority of the extracts (63%) (Hjorleifsdottir et al. 1996). All but two of the examined extracts possessed *Rma*I, a type II restriction endonuclease recognizing 5′-CTAG-3′ (Hjorleifsdottir et al. 1996; Ronka et al. 1991). Activity of isoschizomers of *Bst*BI or *Eco*RV were also detected in a few strains (Hjorleifsdottir et al. 1996). Activity of ligases and polymerases was measured after incubation of the extracts for 30 min at 90 and 95°C, respectively (Hjorleifsdottir et al. 1997). No ligase activity was found after the heat treatment but four extracts retained more than 10% of their DNA polymerase activity. A gene encoding a DNA polymerase I was isolated from one of the strains, ITI 518 (Blondal et al. 2001). It was cloned and expressed in *E. coli* and found to have 5′-3′ exonuclease and 3′-5′ proofreading activities in addition to polymerase activity. It had optimum activity at 55°C and a half-life of 2 min at 90°C while a truncated form lacking the 5′-3′ exonuclease domain had a half-life of 11 min at 90°C and optimum activity at 65°C. Previously, a gene from *R. marinus* DSM 4253 encoding a DNA ligase was cloned and sequenced (Thorbjarnardottir et al. 1995). The product had homology to NAD^+ ligases. It had optimum activity at 55°C and was active over a broad temperature range from 5 to 75°C. It ligated nicks in double stranded DNA and was proven effective, compared to other thermostable DNA ligases, in ligation assays which measured the rate, extent and efficiency of oligonucleotide ligations (Housby and Southern 2002; Housby et al. 2000).

Enzymes involved in metabolic pathways and biosynthesis

Citrate synthase catalyses the conversion of acetyl-CoA and oxaloacetate to citrate and CoA and is the first enzyme in the citric acid cycle. Two citrate synthase enzymes have been isolated from cell extracts of *R. marinus* DSM 4252 (Nordberg Karlsson et al. 2002). One is a dimeric protein while the other is hexameric and they are most likely encoded by separate genes. The dimeric enzyme also exhibited 2-methylcitrate synthase activity and catalysed conversion of n-propionyl-CoA and oxaloacetate to form 2-methylcitrate. The enzyme appeared to accept acetyl-CoA and *n*-propionyl-CoA equally well. Sodium propionate was not found to induce its activity, nor was *R. marinus* able to grow when it was supplied as the sole carbon source. Its role in *R. marinus* might be to metabolise propionyl-CoA generated internally. Both citrate synthases showed optimum activity around 85°C but the hexameric enzyme appeared to be more thermostable.

Phosphoenolpyruvate carboxylase (PEPC) has been purified from *R. marinus* JCM 9785 and the encoding gene was subsequently isolated and expressed in *E. coli* (Takai et al. 1997b, 1998b). The PEPC catalyses conversion of oxaloacetate and phosphate to phosphoenolpyruvate (PEP), CO_2 and H_2O. The *R. marinus* enzyme was shown to be a homotetramer of 100 kDa monomers, required divalent cations and had optimum activity at 75°C (Takai et al. 1997b). Acetyl-CoA or fructose-1,6-biphosphate and L-aspartate or L-malate were positive and negative effectors, respectively, since presence of the former compounds increased enzyme activity while the latter compounds completely inhibited it. Their presence also shifted the optimum temperature of the enzyme to 80 and 70°C, respectively. The half-life of PEPC was 26 h at 80°C (Takai et al. 1997a). Increased enzyme concentration was found to enhance thermostability as well as PEP, acetyl-CoA and monovalent ions, which were therefore identified as extrinsic thermostabilization factors (Takai et al. 1997b). A further study on thermodenaturation of PEPC concluded that extrinsic thermostabilization factors help to maintain the quaternary structure of the *R. marinus* PEPC (Takai et al. 1997a).

A gene, *glgB*, from *R. marinus* JCM 9785, was cloned and sequenced (Shinohara et al. 2001). The product

showed significant homology to known bacterial branching enzymes (α-1,4-glucan: α-1,4-glucan 6 α-glucosyltransferases), which are important in biosynthesis of starch and glycogen. The enzyme was produced in *E. coli* and *Aspergillus orzyae* and the latter host gave much higher yields. The enzyme had optimum activity at 65°C and a half-life of 16 h at 80°C. It catalysed formation of α-1,6-branch linkages and was six times more active towards amylose than amylopectin. It was not active towards glycogen. The enzyme is most likely intracellular as no signal sequence was found in its sequence of 621 residues.

Additional known *R. marinus* genes are the *tdk* and *hemB* genes, which encode thymidine kinase, a key enzyme in the pyrimidine salvage pathway (Blondal et al. 1999) and 5-aminolevulinic acid dehydratase (ALAD), which catalyses synthesis of porphobilinogen (Gudmundsdottir et al. 1999). The *tdk* gene was isolated from *R. marinus* ITI 518 and the product had homology to thymidine kinases of type II (Blondal et al. 1999). It was shown to be a tetramer of 24 kDa monomers, produced thymidine monophosphate from thymidine and ATP and was severely inhibited by dTTP. It showed activity over a broad temperature range, optimally at 65°C, and had a half-life of 15 min at 90°C. The *R. marinus* ALAD was found to contain two putative metal binding sites (Gudmundsdottir et al. 1999) characteristic of Mg^{2+} -requiring ALADs. Both sites showed strong homology with metal binding sites from plant ALADs and one of them is almost identical to the pea ALAD sequence. Other *hem* genes were not found closely linked to *hemB* but a closely linked gene is *cysS*, encoding Cysteinyl-tRNA synthase. The gene has been sequenced and used in construction of phylogenetic trees of CysRS proteins (Li et al. 1999). Annotated *R. marinus* genes that are published in databases but whose expression and characterization has not been reported, are listed in Table 2.

Respiratory chain

Bacterial respiratory chains have a modular organization and are composed of dehydrogenase complexes, quinols and terminal oxidoreductases (Gennis and Stewart 1996). Reduced quinones are reoxidized by the bc_1 complex, which transfers electrons to terminal oxidases via cytochrome *c*. In aerobic respiration, the terminal acceptor is oxygen. *R. marinus* is a strict aerobe (Alfredsson et al. 1988). Its respiratory chain has been extensively studied in the last decade and many components isolated and characterized (Pereira et al. 2004). Previously, menaquinone-7 was identified as the predominant respiratory lipoquinone in the species (Tindall 1991). The respiratory chain of *R. marinus* was shown to possess complexes analogous to bacterial complexes I and II, which reduce menaquinone (Pereira et al. 1999b). A typical complex I, NADH: menaquinone oxidore-

Table 2 Annotated *R. marinus* genes and gene fragments whose characterization after expression in an alternative host is not reported in the literature

Gene	GenBank account No.	Strain	Product	References
argH	AF045557	DSM 4253	Arginosuccinase	Unpublished
cysS	AF162864	DSM 4252	Cysteinyl-tRNA synthase	Li et al. (1999)
dnaB	AF006675	DSM 4252	DNA helicase	Liu and Hu (1997)
dnaJ	AF145250	DSM 4252	Heat shock protein DnaJ	Unpublished
dnaK	AF145251	DSM 4252	Heat shock protein DnaK	Unpublished
fd	AF515798	PRQ 62B	Ferredoxin	Pereira et al. (2002)
groESL	AF145252	DSM 4252	Heat shock proteins groes and groel	Unpublished
hemB	AF137366	DSM 4252	5-Aminolevulinic acid dehydratase	Gudmundsdottir et al. (1999)
hxk	E59412	JCM 9785	Hexokinase	Koga and Takahashi (2000)
metF	AJ249578	PRQ 62B	5,10-Methylenetetrahydro-folate reductase	Santana et al. (2001)
nag	AF173987	DSM 4252	Glucoseamine-6-phosphate deaminase	Martins et al. (1999)
polA	AF121780	JCM 9785	DNA polymerase I	Unpublished
prt	AF028721	ITI 518	Protease	Unpublished
RcoxABCD	AJ249578	PRQ 62B	Cytochrome oxidase, subunits I-IV	Santana et al. (2001)
recA	AF026690	DSM 4252	RecA	Unpublished
rrna[a]	X80994AF217494	DSM 4252	16S rRNA	Andresson and Fridjonsson (1994) and Silva et al. (2000)
rrna[a]	X95071AF217493	JCM 9785	16S rRNA	Sako et al. (1996) and Silva et al. (2000)
trpB	AY454303	DSM 4252	Tryptophan synthase, subunit B	Bjornsdottir et al. (2004)

[a]16S rRNA sequences from further *R. marinus* strains can be found under accession numbers AF217495–AF217499 and Y14143. An intergenic region coding for $tRNA^{Ile}$ and $tRNA^{Ala}$ is found within the rRNA operon

ductase or NADH dehydrogenase, was later purified and characterized (Fernandes et al. 2002). It consisted of about 14 subunits with a total molecular mass of about 800 kDa. The N-terminal ends showed no similarity with known sequences but three subunits reacted with antibodies from *Neurospora crassa* complex I. The complex contained a flavin, menaquinones and an unknown chromophore. Evidence for at least five iron-sulphur centres was found. The complex had optimal NADH dehydrogenase activity at 50°C and electron transfer from NADH to menaquinone was coupled to formation of a membrane potential.

A succinate dehydrogenase or complex II was also isolated from *R. marinus* (Fernandes et al. 2001). The enzyme consisted of three subunits of 70, 32 and 18 kDa and belongs to the succinate/menaquinone oxidoreductase family. N-terminal ends of the two larger subunits showed high similarity with flavosubunits and iron–sulphur subunits of homologous proteins, respectively. The smallest subunit showed no significant homology with known sequences and is possibly an anchor domain. Typical electronic absorption spectra for a heme protein with iron sulphur centres and flavins were detected and evidence for two B-type hemes and three iron–sulphur centres, S1, S2 and S3 was found (Fernandes et al. 2001; Pereira et al. 1999b). The enzyme had optimal succinate dehydrogenase activity at 80°C and a high affinity for succinate. The S3 centre had two conformations and an unusually high redox potential of +130 mV. The hemes also had high reduction potentials, which were pH dependent, particularly of the lower potential heme (b_L). This might imply that the b_L heme intervenes in proton uptake and direct reduction of quinone (Fernandes et al. 2001).

In contrast to typical complexes I and II, *R. marinus* does not contain a typical bc_1 complex or complex III (Pereira et al. 1999a). Its respiratory rate was unaffected by complex III inhibitors and no evidence for Rieske type centres were found (Pereira et al. 1994, 1999a, 1999b). Instead, it was shown to harbour a novel type of complex III with a unique organization of hemes (Pereira et al. 1999a). Three subunits of the complex, of 43, 27 and 18 kDa were isolated. It is a multihemic cytochrome *bc* complex and was found to contain five low-spin heme centres of B and C types in a 1:4 ratio. All the C-type hemes were in the 27 kDa subunit. The heme centres covered a broad range of redox potential of −45 to +235 mV at pH 7.0. An iron–sulphur centre of unknown function copurified with the complex. The complex was shown to be one of the major components of *R. marinus* membranes and it was produced under different growth conditions suggesting a central role in the respiratory chain. It had a quinol/cytochrome c oxidoreductase activity and is therefore a functional analogue of the typical bc_1 complex. It also had a high-potential iron sulphur protein (HiPIP) oxidoreductase activity(menaquinol/HiPIP oxidoreductase activity) (Pereira et al. 1999a).

A HiPIP type protein has been isolated from *R. marinus* (Pereira et al. 1994) while proteins of this kind have mainly been found in a restricted group of purple photosynthetic bacteria (Meyer 1994, Van Driessche et al. 2003). No HiPIP sequences are found in sequenced *Bacteroidetes* genomes (G.O. Hreggvidsson, unpublished results). This might indicate that the presence of HiPIP in *R. marinus* is due to lateral gene transfer. The *R. marinus* protein was the first HiPIP reported to be membrane bound but it has similar characteristics to soluble HiPIPs (Pereira et al. 1994). The protein was found to be a monomer of 10.5 kDa in solution and to contain an iron–sulphur centre (Pereira et al. 1999b). It had a high reduction potential of about +290 mV at pH 7.0 and was implicated in the *R. marinus* respiratory chain when it was found to be reduced by NADH or succinate in the presence of an inhibitor of terminal oxidases (Pereira et al. 1994, 1999b). By reconstituting the *R. marinus* electron transfer chain in vitro, the HiPIP was shown to carry electrons between the cytochrome *bc* complex and a terminal oxidase (Pereira et al. 1999b). No small soluble electron carriers such as copper or Rieske type proteins or cytochrome *c* have been found in *R. marinus* (Pereira et al. 1999b). Both its cytochrome *c* and HiPIP, the electron donors for terminal oxidases, are membrane bound. This might be related to the *R. marinus* growth conditions as other extremophiles are known to perform electron transfer to terminal oxidases via membrane bound components without involving the usual soluble cytochromes (Pereira et al. 1994).

The *R. marinus* respiratory chain branches at the level of terminal oxidases as in most other bacteria. Two terminal oxidases, of caa_3 and cbb_3 types, have been found in the species (Pereira et al. 1999c, 2000a, 2000b). Both had an extra redox centre compared to mitochondrial enzymes but it is not clear whether that makes them more effective at growth conditions with limiting oxygen concentrations (Pereira et al. 1999c). Both enzymes were detected under all conditions examined but the cbb_3 type might be more expressed during O_2 deficiency (Pereira et al. 2000a). The cbb_3 enzyme is the major terminal oxidase in *R. marinus*. It was partially purified and found to consist of five subunits with apparent molecular masses of 64, 57, 36, 26 and 13 kDa. It contained two low-spin heme C centres and both a low-spin and a high-spin heme B centre. Furthermore, primers designed after conserved sequences of subunit I of cbb_3 terminal oxidases were used in a successful amplification from the *R. marinus* genome. The product showed homology to terminal oxidases of the FixN type. The *R. marinus* cbb_3 terminal oxidase had oxygen reductase activity but HiPIP was a poor electron donor and the physiological electron donor is unknown (Pereira et al. 2000a).

The caa_3-type terminal oxidase has been characterized in more detail (Pereira et al. 1999c, 2000b; Sigurdson et al. 2001) and genes encoding all its subunits were

cloned and sequenced (Santana et al. 2001). They were found within an operon along with an open reading frame whose deduced sequence showed homology to a hypothetical protein from *Aquifex aeolicus*. The genes were in the order *rcoxA* (subunit II), *rcoxB* (subunit I), *rcoxC* (subunit III) and *rcoxD* (subunit IV). Sequence homology confirmed that the complex belongs to the superfamily of heme-copper oxidases. The complex contains the binuclear centre Cu_A and RcoxA has a C-type heme (Pereira et al. 1999c). The RcoxA amino acid sequence has conserved ligands for Cu_A (Santana et al. 2001). Its C-terminal part has a cytochrome *c* domain with a heme attachment site. The RcoxB has six conserved histidines which are ligands of a cytochrome a_3-Cu_B centre and cytochrome *a*. The *R. marinus* caa_3 terminal oxidase had unusually low reduction potentials and its hemes were found to be of A_s type (Pereira et al. 1999c) The enzyme catalysed complete reduction of O_2 to water (Pereira et al. 1999c). It could use HiPIP and *R. marinus* cytochrome *c* as electron carriers but had a higher turnover with the former (Pereira et al. 1999b, 1999c).

The *R. marinus* RcoxB contains most of the conserved residues considered important for proton transfer for complete reduction of oxygen at the reaction centre as well as for proton translocation (Pereira et al. 1999c; Santana et al. 2001). However, a glutamate residue corresponding to the essential Glu 278 of the *Paracoccus denitrificans* aa_3 oxidase is not present. By comparing models of the RcoxB subunit and the known structure of the *P. denitrificans* enzyme, a tyrosine residue (Y256) was proposed to substitute for the missing glutamate (Pereira et al. 1999c; Santana et al. 2001). By investigating equilibrium thermodynamics of the two structures, it has been shown that Y256 is not protonatable at physiological pH and is therefore unlikely to have the same role as the glutamate (Soares et al. 2004). It could nevertheless participate in a proton conducting pathway, as well as its consecutive serine residue, by orienting water molecules. In fact, sequence comparisons have found the YS motif in other oxidases in which the glutamate is missing and they are thought to be ancestral to the glutamate-containing oxidases (Santana et al. 2001). In spite of its lack of the glutamate residue, the *R. marinus* caa_3 terminal oxidase has been shown to translocate protons with a stoichiometry of $1H^+/e^-$ when reconstituted into liposomes (Pereira et al. 2000b).

A ferredoxin was found in soluble extracts of *R. marinus* (Pereira et al. 2002). It was small, of 13.7 kDa and its amino acid sequence showed a high similarity with other ferredoxins. It is not assumed to be an electron carrier, due to a low reduction potential, and its function is therefore unknown. The enzyme was found to be highly thermostable, with a T_m for unfolding of 102 ± 2°C at pH 7.0. Its denaturation was irreversible, presumably because of breakdown of an iron–sulphur centre. The amino acid sequence has two binding sites for iron–sulphur centres but only one was detected in the isolated protein. However, a second centre could be detected when the protein was isolated under anaerobic conditions. This could implicate the *R. marinus* ferredoxin in some kind of a regulatory mechanism related to oxygen (Pereira et al. 2002).

Stress responses

R. marinus responds to increasing salinity of the growth medium by accumulating intracellular solutes (Nunes et al. 1995; Silva et al. 1999). In addition to low levels of glutamate, glucose, trehalose, as well as of K^+, which are all common osmolytes in microorganisms, strain DSM 4252 was found to accumulate two major osmolytes. They were determined by nuclear magnetic resonance (NMR), mass spectrometry and elemental analysis to be α-mannosylglycerate and α-mannosylglyceramide (Silva et al. 1999). The former compound is a common osmolyte in thermophiles while the latter has only been encountered in *R. marinus* (Santos and da Costa 2002). Strain DSM 4252 accumulated increasing amounts of mannosylglyceramide during growth at 3–5% NaCl (Silva et al. 1999). Mannosylglyceramide was not detected in cells grown at 77.5°C but its production did not seem to be otherwise affected by growth temperature. Mannosylglycerate also accumulated in response to increasing salinity. However, its quantity depended on growth temperature as well since it increased with growth temperature at a given NaCl concentration in the range of 3–5%. Mannosylglycerate also accumulated in the absence of salt stress (1% NaCl) at 70°C and above. Accumulation of osmolytes, such as mannosylglycerate at supraoptimal temperatures in thermophiles has led to the hypothesis that they could contribute to stabilization of their proteins (Santos and da Costa 2001). Mannosylglycerate has, in fact, been shown to stabilize some enzymes against heat inactivation in vitro (Borges et al. 2002; Ramos et al. 1997).

Biosynthesis of mannosylglycerate has been examined in *R. marinus* and shown to proceed via two pathways (Martins et al. 1999). The GDP-mannose is a substrate in both and is directly derived from glucose. One is condensation of GDP-mannose with D-glycerate, a single step catalysed by mannosylglycerate synthase (MGS). The enzyme has been purified and characterized and the corresponding gene isolated. The enzyme activity was not affected by salt and its production was recently shown to be selectively induced during heat stress (Borges et al. 2004). A shift in temperature from 65 to 75°C for 2 h resulted in a 2.5-fold increase in the amount of MGS, which was determined by Western blotting. In the alternative pathway, mannosylphosphoglycerate synthase (MPGS) catalyses conversion of GDP-mannose and D-3-P-glycerate into mannosylphosphoglycerate which is converted by mannosyl-3-phosphoglycerate phosphatase (MPGP) into mannosylglycerate (Martins et al. 1999). This system required NaCl or KCl for

optimal activity. Both enzymes of the system have been characterized and the corresponding genes were sequenced and found to be contiguous (Borges et al. 2004). The C-terminus of MPGS was identified as a possible regulatory site for catalytic activity. Production of MPGS was enhanced in response to osmotic stress. A threefold increase in the amount of MPGS was detected when salinity of the growth medium was shifted from 2 to 6% NaCl for 2 h. *R. marinus* therefore responds to heat stress and osmotic stress by selectively inducing components of each of the two pathways (Borges et al. 2004). *R. marinus* is the only species known to possess two distinct pathways for synthesis of mannosylglycerate.

The heat shock response in *R. marinus* has been characterized in strain DSM 4252 (Thorolfsdottir 1999). After a shift from 65 to 77°C, the production of about 20 proteins was increased while the production of many others was reduced. Several heat shock proteins were identified by two dimensional electrophoresis and N-terminal sequencing. Strong induction of the heat shock proteins GroEL and GroES was confirmed. The major heat shock genes *groES, groEL, dnaK* and *dnaJ* were cloned in *E. coli* and sequenced (Backman 1997; Thorolfsdottir 1999). As generally seen in bacteria, the *groES* and *groEL* genes were found to be together in an operon but the *dnaK* and *dnaJ* genes, which frequently are linked (Segal and Ron 1996), were found in separate locations (Thorolfsdottir 1999). The DnaJ protein has, when compared to the DnaJ protein of *E. coli*, a deletion of 68 amino acids. This sequence, which spans the zinc finger region of DnaJ, is present in most bacterial species. The inverted repeat CIRCE was found upstream of the *groESL* operon but was not found to be associated with the *dnaK* or *dnaJ* genes (Backman 1997; Thorolfsdottir 1999). The presence of CIRCE indicates that the *groESL* operon is under control of a repressor protein (Schulz and Schumann 1996). Experiments have been reported where *R. marinus* strain JCM 9785 grown at 76°C was heat shocked at 88°C (Takai et al. 1998a). None of the major heat shock proteins was prominently induced but the expression of proteins of 35 and 30 kDa was markedly induced. The N-terminal ends of these proteins correspond to the ATP subunits of ABC transporters and domains II and III of elongation factor Tu, respectively. The significance of these findings is uncertain, but the high temperatures used for growth and induction make it likely that a heat shock response typical for this strain was not observed.

Extrachromosomal and mobile elements

Bacteriophages

Two T4-like plaque forming bacteriophages infecting *R. marinus* have been isolated (S. Hjorleifsdottir, unpublished results; Hjorleifsdottir et al. 2002). They originated in samples from NW and SW Iceland and infection was restricted to strains from the same areas (S. Hjorleifsdottir, unpublished results). The phage from the former sample, RM378, has been studied in more detail (Hjorleifsdottir et al. 2002). It is a large head and tail phage with an A2 morphology and is a member of the *Myoviridae* family. It consists of a moderately elongated head, 95-nm long and 85 nm in diameter, a connector and a tail of 150 nm. It was completely stable and infectious at temperatures up to 65°C but lost viability at higher temperatures. The genome of RM378 is fully sequenced (GenBank No. NC004735). It is a double stranded DNA molecule of 130 kb with a G + C content of 42%. Over 200 open reading frames were found in the sequence and 125 of them could encode polypeptides of at least 100 residues. The overall similarity with known genes is low but a few genes have been annotated including those exhibiting homology with genes encoding DNA polymerases, RNA ligases, polynucleotide kinases (PNKs), helicases and both 5′-3′ and 3′-5′ exonucleases. The genomes of RM378 and T4 have different arrangements and similarity of their corresponding gene products is limited. The sequence of RM378 genome is 98% identical to a partial sequence from the phage from SW Iceland (Kristjansdottir 2000).

Two RM378 enzymes, the RNA ligase and the PNK have been characterized (Blondal et al. 2003, 2005). Together they form a viral defense system to counter a suicidal antiphage defense system, which in *E. coli* is known as the anticodon nuclease system (Kaufmann 2000). Such host systems cleave tRNA molecules of infected cells in an attempt to hinder viral spreading. The PNK mends tRNA fragments into correct substrates (3′-OH and 5′-PO_4) so that RNA ligase can seal them. The RM378 RNA ligase belongs to RNA ligase family 1 as well as the T4 RNA ligase (Blondal et al. 2003). It was found to be moderately thermostable with optimal activity at 64°C. It acted on RNA oligonucleotides and on single stranded DNA with less efficiency. The PNK was also moderately thermostable with a temperature optimum of about 70°C (Blondal et al. 2005). It consists of a C-terminal kinase domain, which showed homology with the T4 kinase domain and a N-terminal posphohydrolase domain which showed limited homology with the HD hydrolase superfamily (Aravind and Koonin 1998). It possessed a 5′-kinase activity which adds γ-phosphate from ATP to a hydroxylated 5′-end of RNA or DNA (Blondal et al. 2005). It also had a 3′-phosphohydrolase activity which eliminates 2′-3′ cyclic phosphate groups from ribonucleotides leaving hydroxylated termini.

Plasmids

A cryptic plasmid, pRM21, has been isolated from *R. marinus* DSM 4253 (Ernstsson et al. 2003). Sequence analysis found the plasmid to consist of 2935 bp, 58.2% G + C (GenBank No. NC001755) and to have characteristics of iteron-containing replicons. Recognition se-

quence for the *R. marinus* restriction endonuclease *Rma*I, 5′-CTAG-3′ (Ronka et al. 1991) was not found in the plasmid sequence. A previous study found 90% of restriction positive *R. marinus* isolates from Iceland to contain *Rma*I activity (Hjorleifsdottir et al. 1996). Several open reading frames were identified in the pRM21 sequence but only one, ORF1, showed significant similarities to sequences in GenBank (Ernstsson et al. 2003). The predicted product had 33–52% similarity to replication proteins of several plasmids, the highest being to the RepA protein of the broad host-range plasmid pSa from *E. coli* (Okumura and Kado 1992) and ORF1 was therefore designated *repA*. Experiments indicated that pRM21 is not maintained in *E. coli* (Ernstsson et al. 2003) but is in 42 ± 2 copies per chromosome and stable in DSM 4253 (Bjornsdottir et al. 2005). Hybridization experiments have shown that pRM21 is not incorporated into the DSM 4253 chromosome (Ernstsson et al. 2003). Forty *R. marinus* strains, which were isolated at the same thermal site as DSM 4253 5 years later were also screened for plasmids and 15% of them were found to contain plasmids of the same molecular size and restriction pattern as pRM21. *R. marinus* isolates from other thermal sites in Iceland have not been analysed for plasmid content. Moreira et al. (1996) reported identification of plasmid bands during pulse field gel electrophoresis of genomic DNA from some *R. marinus* strains but pRM21 remains the only isolated plasmid reported in the genus *Rhodothermus*.

Inteins

The *dnaB* gene of *R. marinus* DSM 4252 has been cloned and sequenced and the product was found to contain an intein (Liu and Hu 1997). The *R. marinus* DnaB protein, which is 35% identical to the *E. coli* DnaB protein, has several conserved sequence blocks, including putative motifs for ATP and DNA binding as well as a leucine zipper motif. A sequence containing features of an intein interrupts a highly conserved region of the protein. These include seven conserved intein sequence blocks and four conserved residues, which are crucial for protein splicing. An intein of 428 amino acids and flanking extein sequences of 421 and 92 amino acids at the N-end and C-end, respectively, were predicted from sequence analysis. Furthermore, protein splicing of the intein sequence, which did not require complete extein sequences, was demonstrated in *E. coli*. The intein is identically positioned in the *R. marinus* DnaB as an intein in the DnaB of the cyanobacterium *Synechocystis* sp. PCC6803. The two-intein sequences are clearly homologous as they are 54% identical whereas the respective extein sequences are 37% identical. A difference between codon usage and G + C content was found between the *R. marinus* DnaB intein (55% G + C) and extein sequences (68% G + C) while no such difference was seen in the *Synechococcus* DnaB sequence. This indicated that acquisition of the intein occurred more recently in the former species. The two inteins are most likely related through horizontal gene transfer, or intein homing, although the path of such a transfer is unknown.

Genetic manipulation

Few methods of genetic analysis have until recently been available for use in *Rhodothermus*. Characterization of its enzymes and elucidation of biosynthetic pathways have been accomplished using biochemical and spectroscopic analysis and expression of genes in *E. coli*. Development of tools for direct genetic manipulation is important, however, as expression in a heterologous host does not always provide sufficient information on properties of gene products in their natural environment. Furthermore, such tools are required for detailed studies on gene expression. A prerequisite for the use of genetic methods is reproducible growth on solid medium and isolation of single colonies. Many *R. marinus* isolates, such as DSM 4253, have shown extensive aggregation in liquid cultures and some have shown variable plating efficiencies or failed to produce single colonies on certain media despite confluent growth (G. Eggertsson and S.H. Bjornsdottir, unpublished results). Therefore, *R. marinus* strains must be carefully chosen before applying genetic methods.

Standard methods of chemical mutagenesis have been applied to two *R. marinus* strains and several auxotrophic mutants were obtained by screening mutagenized cultures by replica plating (G. Eggertsson and S.H. Bjornsdottir, unpublished results). Strain DSM 4252 has been treated with ethyl methane-sulfonate (EMS). Treatment with EMS (7 μl/ml for 30 min) followed by penicillin enrichment (800 U/ml for 6–7 h) resulted in auxotroph frequencies of 0.2–0.3%. Mutants defective in biosynthesis of several amino acids, such as isoleucine/valine, lysine, proline, and threonine were isolated. Higher frequencies, up to 4% auxotrophs, were obtained by treating the strain with *N*-methyl- *N*′-nitro- *N*-nitrosoguanidine (NTG). Treatment with 50 and 100 μg/ml of NTG resulted in 50 and 90% killing, respectively. Optimal conditions for penicillin enrichment following treatment with NTG (100 μg/ml) were determined to be an incubation of 6 h at 65°C and two additions of penicillin (1000 U/ml), at the beginning of the enrichment and 3 h later. These conditions resulted in killing of about 3000–40,000-fold. More detailed description of NTG mutagenesis and penicillin enrichment as well as of isolation of *trpB* mutants of strain PRI 493, is reported by Bjornsdottir et al. (2005).

Methods of gene transfer are important for genetic manipulation. Phenotypic markers are necessary to monitor gene transfer and antibiotic resistance markers are normally used in bacteria. Genes encoding antibiotic resistance are most often derived from mesophilic organisms and sufficient stability of their products is therefore uncertain at the growth temperatures of thermophiles. Alternatively, auxotrophic markers can be used. A selection system for *R. marinus* has been

developed, based on complementation of a stable tryptophan auxotrophy (Bjornsdottir et al. 2005). Strain PRI 493 was chosen as a recipient because it had favourable growth characteristics and lacked a plasmid. Furthermore, no evidence for restriction activity was found in cell extracts of the strain. A shuttle plasmid was constructed from the entire pRM21 replicon and the *E. coli* vector pUC18, containing the DSM 4252 *trpB* gene constitutively expressed from the promoter of the *groESL* genes. The shuttle plasmid successfully transformed a *trpB* mutant of PRI 493 to prototrophy after electroporation. Transformation efficiency as high as $4.3 \pm 0.7 \times 10^6$ CFU per microgram of DNA was obtained. Optimal conditions for electroporation were determined at 22.5 kV/cm, 200 Ω and 25 μF. However, no transformants were obtained after chemical preparation of *R. marinus* according to several protocols. The relative copy number of the shuttle plasmid in the recipient was determined at 7 ± 1 per chromosome and it is very stable as no loss was observed after growth for 80 generations under non-selective conditions. The shuttle plasmid has now been used as the basis for construction of cloning vectors and cloning of several heterologous genes in *R. marinus* is now under way (G. Eggertsson and S.H. Bjornsdottir, unpublished results).

Future directions

Although considerable information has in recent years been gathered on the physiology and molecular biology of *R. marinus*, current knowledge is still mainly restricted to the characterization of single genes and proteins. Several of these proteins show high temperature resistance and some have a wide temperature range of activity which may reflect the highly variable temperature in the natural environment of the species. Further studies of the adaption of *R. marinus* to its natural environment would be of particular interest, including comprehensive studies of its stress responses and of individual enzymes that are active over a wide temperature range. For these and other studies of molecular genetics and cellular processes, a new experimental approach is needed. A host-vector system, which is currently being developed is expected to provide a useful tool for such studies.

Acknowledgements We thank Dr. Ana Melo, Universidade Nova de Lisboa, Oeiras, Portugal, for reviewing part of the manuscript. S.H. Bjornsdottir is a recipient of a grant from the Icelandic Research Fund for Graduate Students.

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