CHAPTER ONE

INTRODUCTION

1.1. GENERAL INTRODUCTION

Many microorganisms thrive in diverse marine and terrestrial habitats at the extremes of temperatures. Based on the temperature at which they grow and carry out diverse metabolic processes, microorganisms are generally categorised into three major groups, namely:

a) Psychrophiles, which inhabit parts of oceans at temperatures of approximately 4°C or less (Feller *et al.*, 1997; Abe and Horikoshi, 2001). Although some psychrophiles grow well at temperatures as low as -10°C, e.g. in microscopic pockets of water at the ice-sea water interphase (Stanier *et al.*, 1995; Lengeler *et al.*, 1999), their optimal growth temperature is below 20°C and some are killed by a brief warming to room temperature (Lengeler *et al.*, 1999).

b) Mesophiles, which represent the majority of microorganisms, grow optimally between 20 and 42°C. They inhabit almost all terrestrial environments and many have been isolated from water bodies such as wells, springs, rivers and lakes (inclusive of salt lakes), marine environments, as well as soil and air (Stanier *et al.*, 1995; Lengeler *et al.*, 1999).

c) Thermophiles, which inhabit warm through extremely hot environments. Whereas thermophiles refer to microorganisms growing at temperatures up to 70°C, hyperthermophiles have growth temperature optima above 70°C (Stanier *et al.*, 1995; Lengeler *et al.*, 1999). The hyperthermophiles also include those organisms that had previously been grouped as caldo-active with a maximum growth temperature above 70°C (Williams, 1975), as well as barothermophiles (now referred to as piezophiles) that inhabit high-pressure deep-sea vents, and are capable of growing at 100°C and above (Clive, 1990; Abe and Horikoshi, 2001).

Many microorganisms, however, cannot be readily grouped into one of the above categories, as their growth temperatures overlap or span across these demarcations. Such organisms have, in many instances, been described as either psychrotolerant/thermotolerant or cold-tolerant (Lengeler *et al.*, 1999). While the former description has been applied mainly to mesophiles, the latter is applicable to thermophiles that defy the established lower limits of growth and grow at temperatures typical of mesophiles.

1.2. THERMOPHILIC ENVIRONMENTS

The environments typically inhabited by thermophiles include the upper layer of the soil surface that is heated in warm summer temperatures (Lengeler *et al.*, 1999), engineered systems operated at high temperatures (e.g. laundry heaters) (Clive, 1990), compost heaps (Maheshwani *et al.*, 2000), marine thermal vents (Lengeler *et al.*, 1999), the Earth's crust (Summit and Baross, 1998), ultra-deep mines and deep basalt aquifers (Baker *et al.*, 2003), and terrestrial hot springs. Hot springs are widely distributed in different parts of the world and are found in Yellowstone National Park (USA) (Nold and Ward, 1995; Brock, 1998; Ward, 1998), New Zealand (Saul *et al.*, 1999) and Uganda (Gislason *et al.*, 1994), as well as in the volcanic areas of Italy (Pienemann-Simon *et al.*, 1995), Iceland (Sonne-Hansen and Ahring 1999) and Japan (Koga *et al.*, 1998).

1.2.1. Hot springs

Hot springs are formed as a consequence of volcanic activity or due to the movement of the Earth's crust and tectonically active sites. The pressure created by these events leads to the upward mobilisation of heated water, which escapes in the form of geysers and fumaroles (Simoneit *et al.*, 2000). The heated water contains a range of dissolved minerals (Fe, Mn, etc.) and traces of gases (H_2S, CO_2, H_2, CO) (Clive, 1990; Cowan, 1992; Lengeler *et al.*, 1999; Reysenbach and Cady, 2001). The chemistry of terrestrial hot springs varies greatly and is mainly dependent on the properties of the parent or source rock. Also, the temperatures of hot springs range from barely tolerable to the human hand to that of boiling water. Due to temperature gradients being present in the out-flow channel of the hot springs, different microbial communities can establish themselves in these channels (Lengeler *et al.*, 1999).

The East African Rift Valley System (EARS) displays great geothermal activity (Gislason *et al.*, 1994; Darling, 1998; Simoneit *et al.*, 2000). It is divided into the "Eastern Rift", which stretches through Kenya and Ethiopia up to far-southern Tanzania, and the "Western Rift", which stretches through the Tanganyika trough southwards through western Uganda (Fig. 1.1). In Uganda, hot springs are mainly found in the western, south-western and north-western regions and surveyed areas include Kibiro (around Lake Albert), Katwe and Buranga (in Semuliki National Park) (Fig. 1.2) (Gislason *et al.*, 1994).

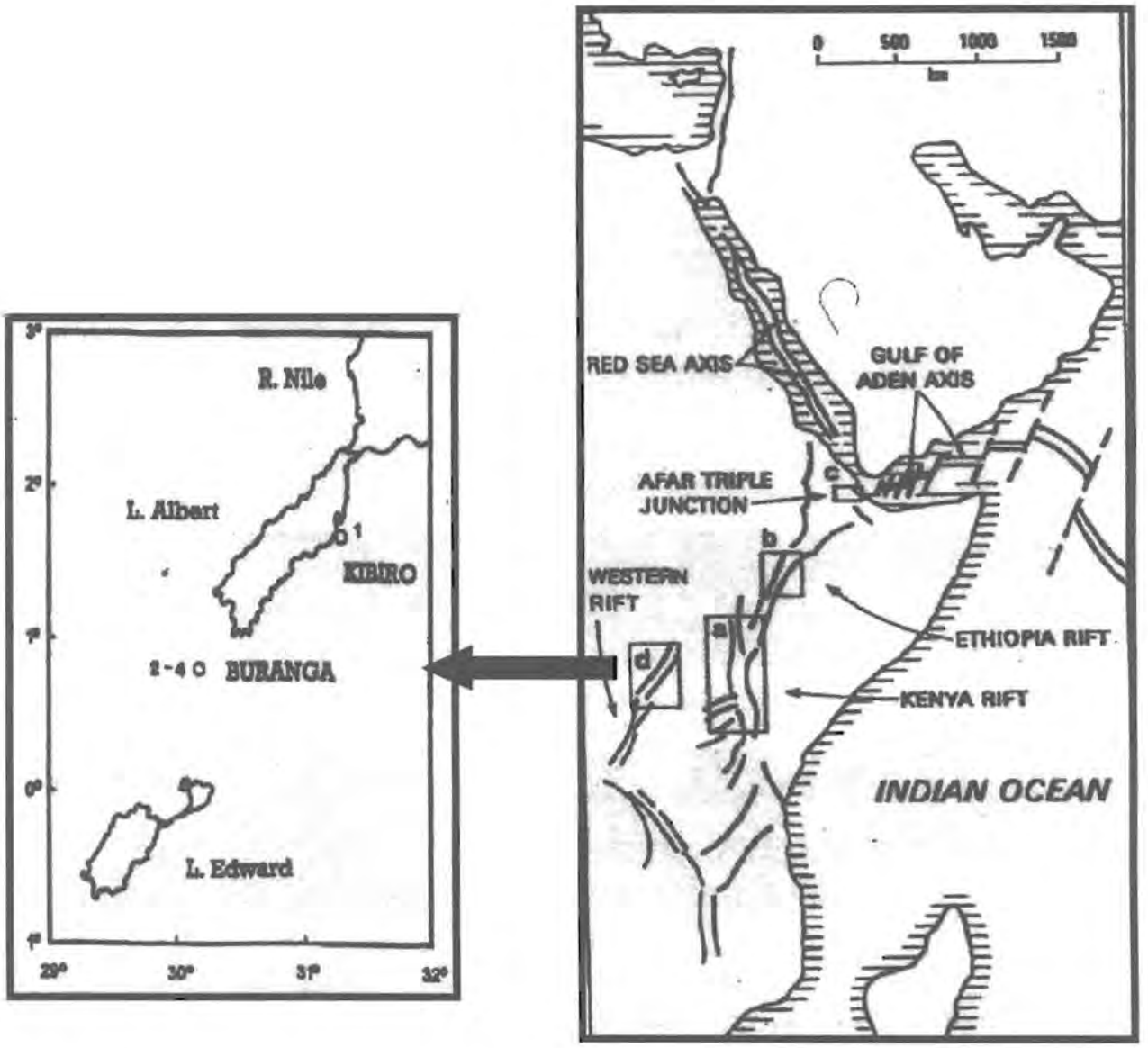

Fig. 1.1 Map of the East African Rift Valley System. The boxes (a-d) are indicative of areas displaying high geothermal activity. (a) Kenya, (b) Ethiopia, (c) Djibouti and (d) Uganda. An enlargement of the geothermal region of Uganda (d) is shown to the left. (*Adapted from Darling, 1998)*

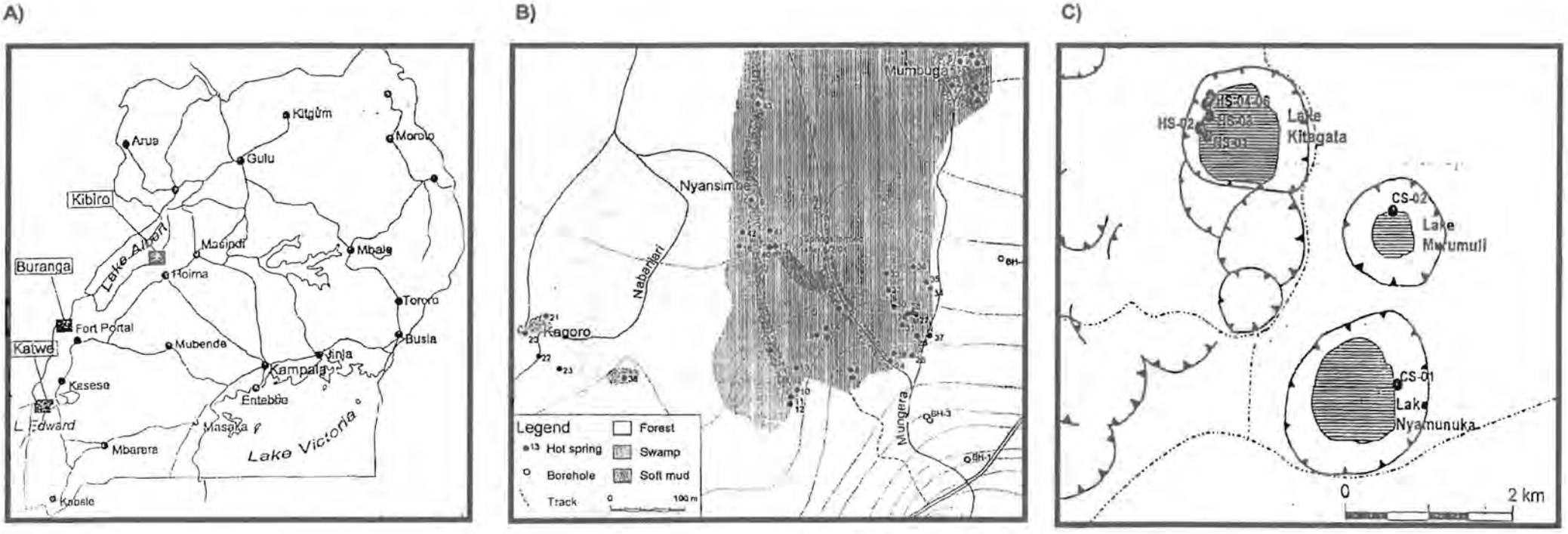

Fig. 1.2 A map of Uganda indicating the three major geothermal areas of Katwe, Buranga and Kibiro is shown in A, while survey maps indicating the distribution of hot springs in the Katwe and Buranga areas are shown in B and C, respectively.

1.3. MICROORGANISMS IN HOT ENVIRONMENTS

Hot environments have been successfully colonised predominantly by prokaryotes (Clive, 1990; Cowan, 1992; Stanier *et al.*, 1995; Ali *et al.*, 1998; Brown and Lupas, 1998; Summit and Baross, 1998; Lengeler *et al.*, 1999; Reysenbach and Cady 2001), and to a lesser extent by eukaryotes such as fungi (Maheshwani *et al.*, 2000) and algae (Sartoni and De Biasi, 1999). Thermophilic bacteria belong to two major domains; Bacteria, which include, among others, members of genera such as *Bacillus, Thermus, Clostridium, Thiobacillus, Thermotoga* and *Streptomyces* (Bergquist and Morgan, 1992; Kim *et al.*, 1998; Xu *et al.*, 1998; Saul *et al.*, 1999), and Archaea, which include, among others, members of genera such as *Methanococcus*, *Thermopterus*, *Sulfolobus*, *Pyrodictium*, *Thermoplasma* and *Pyrococcus* (Bergquist and Morgan, 1992; Adams and Kelly, 1998; Chen and Roberts, 1999).

Whereas thermophilic algae have been isolated from shallow hydrothermal vents and other warm marine environments (Sartoni and De Biasi, 1999), thermophilic fungi have been isolated mostly from heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter. Notably, only 30 species from the approximately 50 000 recorded fungal species are thermophilic with optimal growth temperatures above 40 or 45°C (Maheshwani *et al.*, 2000). They comprise a heterogeneous physiological group and include *Mycelia sterilia*, various species of the *Phycomycetes* and *Ascomycetes* genera, as well as members of the Fungi imperfecti (Maheshwani *et al.*, 2000).

1.4. IMPORTANCE OF THERMOPHILIC MICROORGANISMS

Thermophilic organisms are promising sources of thermostable enzymes. Since these enzymes tend to remain stable in the presence of denaturing agents such as detergents and organic solvents, they are being exploited in a number of biotechnological bioprocesses (Sonnleitner, 1983; Jaenicke *et al.*, 1996). Thermostable enzymes have been used as constituents of laundry detergents (Nagami and Tanaka, 1986; Tran *et al.*, 1991; Mala *et al.*, 1998) and in the bioconversion of proteinaceous waste into biomass (Anwar and Saleemuddin, 1998), as well as in the degradation of xenobiotic compounds (Ali *et al.*, 1998). They are also being applied in different industries such as the leather tanning, waste management (Hameed *et al.*, 1996; Mehrotra *et al.*, 1999), dairy and food processing (Harwood 1992), as well as pharmaceutical industries (Cowan, 1992).

Due to the inherent stability of thermophilic proteins (Talbot and Sygusch, 1990), they are also used as models to elucidate the factors governing protein thermostability (Karshikoff and Landenstein, 1998; Kannan and Vishveshwara, 2000). The results from such investigations may in future aid in the manipulation of existing enzymes from mesophilic organisms to enhance their thermostability. Recombinant gene technology has already revolutionalized the enzyme and protein industry by enabling the successful isolation, cloning and expression of a number of genes from thermophilic origin in mesophilic host organisms (primarily *Bacillus subtilis* and *Escherichia coli)*. This, in turn, has allowed for the large-scale production of thermostable enzymes, which would be difficult to achieve in the original hosts, as few laboratory methods have been developed to grow Archaea economically.

1.5. AIM OF THIS STUDY

Thermophilic bacteria had been preveously isolated from soil samples collected from Buranga hot springs in western Uganda. The isolates (38) had previously been screened for production of protease enzymes and two isolates that displayed the highest proteolytic activity were selected for use in this study with the specific aim of isolating and characterising genes encoding novel enzymes. The characterisation of protease enzyme(s) and a 4-hydroxyphenylacetic acid hydroxylase is reported in this investigation.

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CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

Classic methods for the isolation and identification of microorganisms require their cultivation in pure culture. Often, only a minor fraction of bacteria present in an environmental sample can actually be grown under standard culturing conditions, and the selectivity of the cultivation techniques hardly reflects the true extent of the microbial community composition or diversity (Amann *et al.*, 1995). When it comes to isolating thermophilic bacteria, few laboratory methods have been developed whereby Archaea can be isolated or cultured. Many thermophilic microorganisms, as a result, have thus not been recovered from the environment (Adams and Kelly, 1998; Rondon *et al.*, 1999). Consequently, the true extent of microbial diversity in general and of thermophiles in particular, probably far exceeds the current estimates. With the advances being made in isolation and culturing techniques, culturing of some fastidious organisms is becoming increasingly more possible. Furthermore, advances in molecular biology methods (DNA- and RNA-based methods) and in the development of improved DNA extraction and amplification techniques applicable to soil and sediment samples (Tsai and Olson, 1991; Steffan *et al.*, 1998) have enabled the identification and phylogenetic placement of bacteria that have thus far eluded cultivation (Head *et al.*, 1998; Rondon *et al.*, 1999; Rondon *et al.*, 2000). These methods have opened new avenues for studying bacterial diversity and also for the isolation of potentially novel products from bacteria inhabiting many different and extreme environments.

2.2. PROKARYOTIC SYSTEMATICS

All life forms have been assigned to one of three taxonomic domains, namely: Eukarya, Bacteria and Archaea (Woese *et al.*, 1990). Although thermophilic members belonging to the domain Eukarya have been described (e.g. thermophilic fungi), most studies have been directed at the many genera and species of the two prokaryotic domains, i.e. Archaea and Bacteria (Peinemann-Simon *et al.*, 1995; Summit and Baross, 1998; Reysenbach and Cady, 2001; Kannan *et al.*, 2001; Hemmi *et al.*, 2002).

Members of the archaeal domain are regarded as ancient and primitive. Their membrane lipids characteristically consist of predominantly isoprenoid glycerol diethers or diglycerol tetraethers, and their ribosomes contain an archaeal type rRNA (Woese *et al.*, 1990). The

domain Archaea is subdivided into two phylogenetically distinct groups. The first group, Methanogens, comprises genera such as *Archaeoglobus* (extreme halophiles and sulphate-reducing species), *Thermoplasma* and the *Thermococcus-Pyrococcus*. The second group, Thermoacidophiles, comprises extremely thermophilic, sulphate-dependent archaebacteria (Yang *et al.*, 1985). The niches archaebacteria are entirely thermophilic and they constitute a relatively homogeneous physiological group.

Compared to Archaea, members of the domain Bacteria are regarded as advanced. Their definitive features include membrane lipids consisting of predominantly diacylglycerol diesters, and ribosomes containing an (eu)bacterial type of rRNA (Woese *et al.*, 1990). Within the domain Bacteria, various thermophilic and hyperthermophilic species have been isolated and described (Wery *et al.*, 2001). They include: (a) aerobic heterotrophs, many of which have been assigned to the genera *Thermus* and *Bacillus*, (b) microaerophilic autotrophs (*Aquifex* spp.), (c) anaerobic autotrophs (*Desulfurobacterium* spp.), and (d) anaerobic heterotrophs (*Clostridium* spp. and *Thermotoga* spp.) (Wery *et al.*, 2001).

2.2.1. Methodological approaches to prokaryotic systematics

2.2.1.1. Phenotypic traits used in prokaryotic systematics

Phenotypic methods form the foundation of numerical taxonomy and comprise all methods that are not directed toward characterisation of the DNA or RNA of the microorganism (Vandamme *et al.*, 1996). They include methods to characterise: (a) morphological features such as shape, endospore formation, flagella, inclusion bodies, Gram stain and colony colour, dimension and form (Chung *et al.*, 2000; Hattori *et al.*, 2000; Nazina *et al.*, 2001; Alain *et al.*, 2002; Seyfried *et al.*, 2002), (b) physiological features such as growth at different temperatures, pH, salt concentrations, atmospheric conditions (aerobiosis and anaerobiosis), ability to metabolise different carbon sources, resistance to antibiotics, and production of different metabolic products and enzymes (Fiala *et al.*, 1986; Huber *et al.*, 1992; Sokolova *et al.*, 2001; Seyfried *et al.*, 2002; Zavarzina *et al.*, 2002), and (c) biochemical features such as analysis of the cell wall composition (Kim *et al.*, 1999), polar lipids and fatty acid composition (Nunes *et al.*, 1992a; 1992b; Chung *et al.*, 2000; Nazina *et al.*, 2001), isoprenoid (respiratory) quinones (Yamamoto *et al.*, 1998; Hattori *et al.*, 2000), whole-cell protein (Karnauchow *et al.*, 1992; Agnew *et al.*, 1995), antigenic analysis (Suzuki *et al.*, 1983; Karnauchow *et al.*, 1992), isoenzyme analysis (Xu *et al.*, 1998), electron acceptors (Burggraf

et al., 1990; Wery *et al.*, 2001; Alain *et al.*, 2002; Seyfried *et al.*, 2002; Zavarzina *et al.*, 2002) and polyamines and histones (Vandamme *et al.*, 1996).

Many of these individual traits are insufficient as parameters for determining genetic relatedness, but when integrated, they may provide coherent descriptive information enabling the recognition of taxa (Martiensson *et al.*, 1996). Furthermore, features such as colour, colony morphology and form, whole-cell proteins and endospore formation (in some cases) are at times influenced by the culture conditions. Standardisation of methodologies is therefore a *sine qua none* in bacterial systematics.

2.2.1.2. Genotypic methods

Molecular methods such as DNA base composition, DNA-DNA hybridisation and ribosomal RNA-based nucleic acid sequence homology analyses have been, among others, useful in defining bacterial species, in phylogenetic placement of newly characterised bacterial strains (Vandamme *et al.*, 1996), and in the restructuring of existing taxons (Rőssler *et al.*, 1991; Ash *et al.*, 1991; Rainey *et a*l., 1994). Figure 2.1 summarises current methods used in bacterial systematics and their respective levels of resolution.

2.2.1.2.1. DNA base composition

Determination of DNA base composition, which is expressed as mole percent of guanine plus cytosine (mol % G+C), is considered part of the standard description of bacterial species (Vandamme *et al.*, 1996). Strains that are assigned to a single microbial genus tend to have closely similar or identical mol % G+C values (Stanier *et al.*, 1995). The mol % G+C varies greatly between genera, with values ranging from 24-25% for certain clostridia and mycoplasmas (Brian and Fergus, 1986; Lengeler *et al.*, 1999) to 76% for strains of certain *Actinomycetes* spp. (Lengeler *et al.*, 1999). While the mol % G+C-content of strains of a phylogenetically coherent species usually does not differ by more than *ca.* 3-5% (Brian and Fergus, 1986; Vandamme *et al.*, 1996), values between species of the genus may differ more. A range of more than 15% mol % G+C between species of the genus is usually taken as an indication of phylogenetic heterogeneity of the genus (Lengeler *et al.*, 1999). The mol % G+C of members of the genus *Bacillus*, for example, ranges between 32 to 69%, indicating that it is a very heterogeneous genus (Goodfellow and O'Donnel, 1994). However, the genus

Bacillus has since been subdivided, though the newly established genera have not yet been widely accepted. Therefore, *Bacillus* in this thesis should be taken to include all *Bacillaceae*.

2.2.1.2.2. DNA-DNA hybridisation

DNA-DNA hybridisation is widely used to delineate species (Vandamme *et al.*, 1996) and to resolve the finer taxonomic relationships between closely related organisms (Kim *et al.*, 1999). It is generally agreed that species should encompass strains, which show approximately 70% or more DNA-DNA relatedness under suitable experimental conditions (Kim *et al.*, 1999). A number of DNA hybridisation protocols have been developed and are being used in different laboratories. However, depending on the method used, different results may be obtained between laboratories (Vandamme *et al.*, 1996). Nevertheless, it remains a powerful method in the delineation of bacterial species.

2.2.1.2.3. Ribosomal RNA (rRNA)-based methods

Phylogenetic methods, which are based on the sequencing and cataloguing of rRNAs (5S, 16S, and 23S), have played a major role in modern systematics and phylogenetic studies. Comparative analysis of rRNA by means of T1 ribonuclease oligonucleotide catalogues led to the definition of a third primary kingdom, that of the archaebacteria, in addition to those of the eubacteria and eukaryotes (Jarsch and Bőck, 1985). Nevertheless, this analysis has since been replaced by methods involving comparison of full-length 16S rRNA sequences in order to resolve branching points between phylogenetically very distant groups. The analyses of full-length 16S rRNA sequences furthermore allow comparison of secondary structural features, in addition to primary structures (Jarsch and Bőck, 1985; Yang *et al.*, 1985). Both archaeal and bacterial domains have been phylogenetically described using rRNA-based methods. The strength of the rRNA sequencing approach has been illustrated in its application to the phylogenetic placement of unculturable microorganisms (Head *et al.*, 1998; Rondon *et al.*, 1999; Rondon *et al.*, 2000; Nogales *et al.*, 2001).

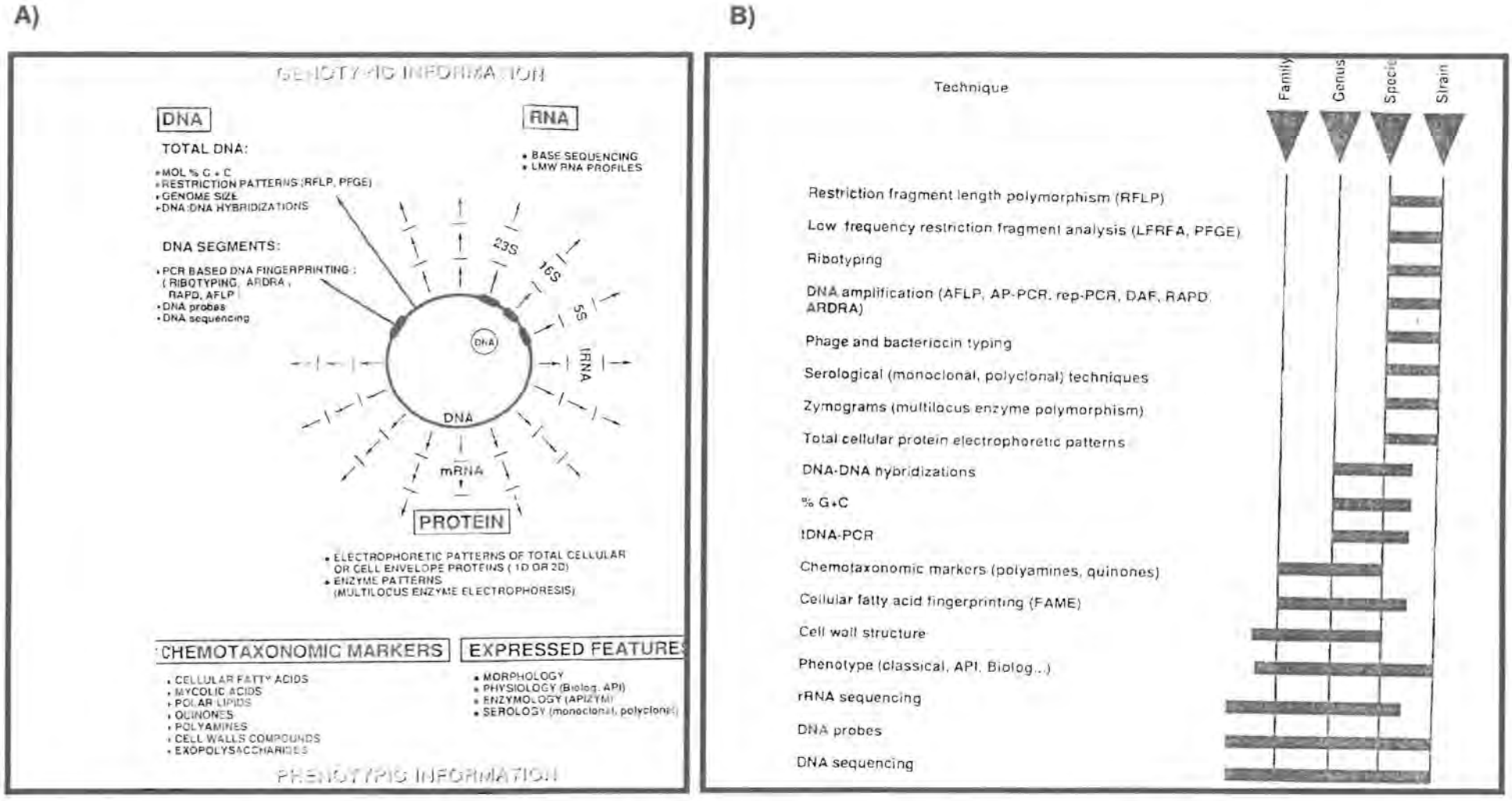

Fig. 2.1 Schematic overview of various cellular components and currently used techniques in systematics (A), as well as the taxonomic resolution of the respective techniques (B). (*Adapted from Vandamme et al., 1996*)

(Abbreviations: RFLP, restriction fragment length polymorphism; PFGE, pulse-field gel electrophoresis; AFLP, amplified fragment length polymorphism; AP-PCR, arbitrarily primed PCR; rep-PCR, repetitive extragenic palindromic PCR; RAPD, randomly amplified polymorphic DNA; ARDRA, amplified rDNA restriction analysis; 1D, one-dimensional SDS-polyacrylamide gel electrophoresis; 2D, two-dimensional SDS-polyacrylamide gel electrophoresis; FAME, fatty acid methyl esters analyses).

2.3. DISTRIBUTION OF THERMOPHILES IN THE ENVIRONMENT

2.3.1. Archaea

Archaebacteria have been isolated from very hot and acidic environments. It is thus not surprising that most of the thermophilic Archaea are both hyperthermophilic and anaerobic. Hydrothermal fluids resulting from sea floor fissures in axial valleys have frequently served as sources of viable anaerobic microorganisms with hyperthermophilic growth range. Moreover, most of the isolates from the sub-sea floors do not tolerate atmospheric concentrations of O_2 at temperatures that permit growth (Reysenbach and Cady, 2001).

Thermoacidophilic archaeabacteria, e.g. *Sulfolobus solfataricus* (Hemmi *et al.*, 2002) and *Thermococcus kodakareansis* KOD1 (Kannan *et al.*, 2001), have been isolated from acid marine solfataric environments. *Methanopyrus*, a rod-shaped methanogen, is endemic to marine vents (Reysenbach and Cady, 2001), while *Thermococcus siculi* sp. nov. has been isolated from a deep-sea thermal vent (1 394 m deep) at the mid-Okinawa trough (Grote *et al.*, 1999). These strains grow well at temperatures between 50 to 100°C with growth temperature optima ranging from 85 to 90°C (Kannan *et al.*, 2001). Thermophilic piezophiles, encompassing genera such as *Methanococcus*, *Palaeococcus*, *Pyrococcus* and *Thermococcus* (Abe and Horikoshi, 2001), have been isolated from high-pressure environments such as deep-sea trenches. Other archaeal genera, such as *Archaeoglobus*, *Pyrodictunum* and *Pyrococcus*, have been isolated from terrestrial and marine solfataric springs, as well as sub-marine and deep-sea hydrothermal vents around the world (Grote *et al.*, 1999).

Oil field reservoirs and surrounding environments are also sources of novel thermophilic archaeabacteria. For example, both *Methanobacterium thermoaggregans* and *M. thermoalcalphilum* have been isolated from oil field water and from sea water-flooded oil wells in California (Nilsen and Torsvik, 1996). Also, *Methanococcus thermolithotrophicus*, isolated from oil field reservoir water, is capable of growing at temperatures between 17 to 62°C, with an optimum growth temperature at 60°C (Nilsen and Torsvik, 1996).

2.3.2. Bacteria

Members of the domain Bacteria (eubacteria) have been isolated from diverse marine and terrestrial environments, which in the case of most spore-formers also include environments non-permissible for their growth and survival (Peinemann-Simon *et al.*, 1995; Summit and

Baross, 1998). Furthermore, many species of thermophilic eubacteria have been isolated from acidic (acidophiles/acidotolerant), alkaline (alkaliphiles/alkalitolerant), high salt (halophiles/halotolerant), and high-pressure (1000 bar) environments (Abe and Horikoshi, 2000).

2.3.2.1. Hyperthermophilic eubacteria

Hyperthermophilic eubacteria, such as *Thermotoga maritima*, *Aquifex* spp., *Staphylothermus* spp. and *Thermus* spp., have been isolated from terrestrial hot springs (Archenbach-Richter *et al.*, 1987; Nelson *et al.*, 1999), turf mats (Yamamoto *et al.*, 1998), deep-sea and shallow marine hydrothermal vents, as well as deep-sea hydrothermal chimneys (Gugliandolo and Maugeri, 1997; Reysenbach and Cady, 2000). Hyperthermophilic eubacteria, with the exception of the genus *Thermus*, are anaerobic and are closely related to hyperthermophilic archaeabacteria. The genus *Thermus* comprises Gram-negative, extremely thermophilic (hyperthermophilic) aerobes and is ubiquitous to many terrestrial hot springs (Gudni *et al.*, 1985; Peek *et al.*, 1992; Friedrich *et al.*, 2002). Representatives of this genus have a wide geographical distribution and have been isolated from hot springs in New-Zealand (Hudson *et al.*, 1987), Iceland (Gudni *et al.*, 1985) and from the Octopus hot spring in the Yellowstone National Park (USA) (Nold and Word, 1995).

2.3.2.2. Thermophilic eubacteria

Whereas most hyperthermophilic archaea and eubacteria are predominantly anaerobic, thermophilic eubacteria are represented almost equally among anaerobes and aerobes. Numerous genera, from a diverse array of both terrestrial and marine environments, have been described.

Anaerobic thermophiles, encompassing genera such as *Clostridium*, *Desulfotomaculum*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Caloramator*, *Thermoacetogenium* and *Anoxybacillus* have been isolated from both geothermal and non-geothermal environments, and comprise both sporulating and non-sporulating species (Patel *et al.*, 1986). For example, *Fervidobacterium nodosum* has only been isolated from geothermal environments (Patel *et al.*, 1985a), while *Clostridium thermocellum* has been isolated from non-geothermal environments. The sporulating *Clostridium thermosulfurogenes*, as well as non-sporulating *Thermoanaerobium brockii*, *Thermoanaerobacter ethanolicus* and *Thermobacteriodes*

acetoethylicus have all been isolated from geothermal environments that are rich in algal and bacterial biomass (Patel *et al.*, 1985a).

Additionally, thermophilic anaerobes, such as *Carboxydobranhium pacificum* (Sokolova *et al.*, 2001), *Caminicella sporogenes* (Alain *et al.*, 2002), *Caloranaerobacter azorensis* (Wery *et al.*, 2001), *Thermovenabulum ferrioganovorum* and *Caloramator veterbensis* (Zavarzina *et al.*, 2002; Seyfried *et al.*, 2002), *Petrotoga olearia* and *P. sibirica* (Haridon *et al.*, 2002) and *Thermacetogenium phaeum* (Hattori *et al.*, 2000), have been isolated from environments such as sub-marine and deep-sea hydrothermal vents, terrestrial hydrothermal springs, deep continental oil reservoirs and from a Kraft pulp-waste water methanogenic reactor.

Aerobic thermophilic eubacteria encompass both sporulating and non-sporulating genera. The genus *Rhodothermus* comprises obligately aerobic, non-sporulating, Gram-negative rod-shaped members (Alfredsson *et al.*, 1988). *Rhodothermus* strains have been isolated from shallow marine hot springs in Isafjardarjup Bay in Iceland and from Ribeira Quente, on the island of Miguel Azores (Alfredsson *et al.*, 1988; Nunes *et al.*, 1992a; 1992b). The sporulating genera include members of the *Bacillaceae*, *Thermoactinomyces* and *Streptomyces* (Stanier *et al.*, 1996).

Genera of the *Bacillaceae* have a very wide distribution, which ranges from air, hot springs, deep-sea hydrothermal vents, solfataras, geothermally heated soils, man-made thermal systems (e.g. hot water pipelines, heat exchangers), composting vegetation, and soil (Martiensson *et al.*, 1995; Maugeri *et al.*, 2001; Baker *et al.*, 2001). Members of these genera have been extensively studied, because of their relatively easier growth requirements. Their diverse distribution is attributed to their formation of heat-resistant endospores, which are spread to different environments where they remain viable for long periods (Baker *et al.*, 2001). Although most thermophilic *Bacilli* are thermophiles (Nazina *et al.*, 2001; Maugeri *et al.*, 2001), having optimal growth temperature ranging between ≥ 45 to $\leq 70^{\circ}C$, a few hyperthermophilic *Bacillus* spp. have been isolated from both natural hot springs and deep-sea hydrothermal vents (Marteinsson *et al.*, 1996; Baker *et al.*, 2001).

Streptomyces species, like *Bacillaceae* and *Thermoactinomyces*, are aerobic and form spores. The ability to form spores may account partly for their wide distribution in terrestrial habitats. Unlike *Bacillus*, however, *Streptomyces* spores are heat-labile. The lability of their spores

may, in part, account for them being distributed in less hostile environments. This is evidenced by their abundance in soil (Stanier *et al.*, 1995; Kim *et al.*, 1998; Kim *et al.*, 1999), lake sediments (Xu *et al.*, 1998) and, to a lesser extent, hot springs (Xu *et al.*, 1998). Members of this genus are moderately thermophilic, growing at temperatures between 25 to 55°C (Kim *et al.*, 1998; Kim *et al.*, 1999). However, Xu *et al.* (1998) described a new species, *Streptomyces thermogriseus,* which exhibited an upper growth limit ranging between 65 to 68°C. This is the highest growth limit so far recorded for a member of the *Streptomyces* genus.

Although a comprehensive analysis of thermophilic bacterial diversity is beyond the scope of this literature review, it is nevertheless very clear that thermophiles, like their mesophilic and psychrophilic counterparts, are widely distributed in the environment. As culturing methods become available, knowledge regarding their distribution and diversity will likewise increase.

2.4. PHYSIOLOGICAL ADAPTATIONS OF THERMOPHILES

The diverse environments of high temperatures ranging from ≥45 to ≤115°C (Huber *et al.*, 2000; Maugeri *et al.*, 2001), pH ranging from pH 2 to pH ≥10 (Darland and Brock, 1971; Horikoshi, 1999), and high salt environments (Santos and da Costa, 2002) have exposed thermophiles and hyperthermophiles to different physiological challenges, namely: (a) the requirement to meet the energy needs of the organism under both aerobic and anaerobic conditions, (b) the requirement to maintain an intracellular homeostatic state and (c) stabilisation of proteins. Consequently, these organisms have, in turn, acquired different physiological adaptations necessary to meet these challenges.

2.4.1. Respiratory / Energy needs

Chemolithotrophic thermophiles obtain energy from the oxidation of inorganic compounds and carbon dioxide (CO_2) serves as the sole carbon source (Reysenbach and Cady, 2001). A variety of inorganic compounds are used as electron donors and acceptors (Huber *et al.*, 2000). Under aerobic conditions, molecular hydrogen is converted to water, while elemental sulphur, sulphides or thiosulphates are oxidised to sulphuric acid in energy-yielding reactions. The above energy-yielding reactions have been identified in hyperthermophilic

genera such as *Aquifex*, *Sulfolobus*, *Metallosphaera* and *Acidianus* (Huber *et al.*, 1992; Huber *et al.*, 2000). Anaerobically, elemental sulphur, sulphides or thiosulphates, nitrates, nitrites and CO_2 may serve as suitable electron acceptors for growth, while molecular hydrogen may act as the electron donor in these energy-yielding reactions (Karnauchow *et al.*, 1992; Miroshnichenko *et al.*, 1998; Huber *et al.*, 2000). In some organisms, e.g. *Ferrioglobus placidus* (Huber *et al.*, 2000), ferrous ions serve as electron donors for nitrate reduction, while in *Thermovenabulum ferriorganovorum*, ferric ions act as electron acceptors (Zavarzina *et al.*, 2002).

Autotrophic thermophiles, on the other hand, obtain cellular carbon and energy through oxidative reduction of CO_2 (Reysenbach and Cady, 2001) via the tricarboxylic acid cycle or reductive Acetyl-CoA cycle, and the recently described 3-hydroxypropionate cycle (Huber *et al.*, 2000).

Heterotrophic thermophiles and hyperthermophiles satisfy their energy requirements through the oxidation of organic matter either aerobically or anaerobically. Many obligate aerobes described among genera such as *Bacillus*, *Thermus* and *Rhodothermus* oxidise organic carbon to CO_2 and H_2O to yield energy. Anaerobic species, such as *Clostridium* spp. (Rani and Nand, 2000), *Clostridium thermolacticum* and *Moorella thermoautotrophica* (Talabardon *et al.*, 2000), *T. phaeum* (Hattori *et al.*, 2000), *C. pacificum* (Sokolova *et al.*, 2001), *C. azorensis* (Wery *et al.*, 2001), *T. toyohensis* (Mori *et al.*, 2002) and *C. sporogenes* (Alain *et al.*, 2002), exhibit fermentative/chemoorganoheterotrophic respiration. While obligate fermentative strains do not require external electron acceptors (Alain *et al.*, 2002), facultative strains do. Electron acceptors, including thiosulphates, elemental sulphur, sulphates and ferrous or ferric ions, are reduced in a number of coupled oxidation reactions with a concomitant yield of energy (Wery *et al.*, 2001; Hattori *et al.*, 2000; Mori *et al.*, 2002). Since enzymes catalyse the above reactions and other metabolic processes, it requires that the cell's enzymes and other proteins be thermostable.

2.4.2. Stabilisation of proteins at high temperatures

Proteins from both thermophilic and hyperthermophilic bacteria are inherently thermostable. This has raised researcher's interest towards understanding of the factors that govern protein thermostability (Karshikoff and Landenstein, 1998; Kannan and Vishveshwara, 2000).

Comparative analytical studies of the primary structures of isofunctional enzymes from thermophilic, mesophilic and psychrophilic bacteria, for example, have revealed no substantial differences between them (Feller *et al.*, 1997; Lengeler *et al.*, 1999; Nèmeth *et al.*, 2002). Furthermore, analysis of 18 nonredundant protein families, containing pairs of high resolution protein structures from both thermophilic and mesophilic organisms, have indicated that both mesophile-thermophile homologous pairs were similar as regards their hydrophobicities, compactness, oligomeric states, main chain-main chain and main chain-side chain hydrogen bonds (Kumar *et al.*, 2000a; 2000b). Although, there appears to be a general increase in these factors among thermophilic members in a family of isofunctional proteins (Vieille and Zeikus, 1996), it has not been possible to develop general rules governing protein stabilisation (Jaenicke, 2000).

It has been established that a combination of cumulative small stabilising and destabilising factors operate either singularly or in synergy to stabilise thermophilic proteins. For example, few amino acid substitutions occur in regions occupied by thermolabile residues. Residue substitutions such as Lys to Arg, Ser to Ala and Gly to Ala, coupled with the general increase in the Arg to Arg+Lys ratio with increasing temperature, have been observed (Russel and Taylor, 1995; Feller *et al.*, 1997; Chung *et al.*, 2000). Such substitutions may contribute to the stabilisation of the tertiary structure of the enzyme through increased internal hydrophobicity, which is accompanied by a decrease in flexibility (Russel and Taylor, 1995). Furthermore, amino acids such as Arg and Tyr may be useful both in short-range local interactions and in long-range interactions. This, in some instances, results in an increase in the number of ion-pairs and ion-pair networks, while in other instances salt bridges are formed (Chang *et al.*, 1999; Kumar *et al.*, 2000a). Additionally, helix-destabilising residues such as Pro, His and Cys are avoided, while Arg and Ala, the helix-stabilising residues, are favoured (Kumar *et al.*, 2000b; Vieille and Zeikus, 1996).

While factors such as hydrophobicity, compactness, oligomeric states, main-chain and side-chain hydrogen bonds do not show consistent trends between mesophilic and thermophilic proteins, an increase in the number of salt bridges and side chain-side chain hydrogen bonds are commonly found in the majority of thermophilic proteins (Waldburger *et al.*, 1995; Kumar *et al.*, 2000a; 2000b). The stabilising nature of salt bridges lies in their networks and the co-operative nature of electrostatic interactions. The co-operativity of the network can resist unfolding, thereby opposing disorder and thus provide a mechanism to counteract

melting/unfolding (Kumar *et al.*, 2000b). This is through an increase in the electrostatic interactions that accompanies the structural plasticity in protein folds. The role of ion networks was also demonstrated in experiments with alcohol dehydrogenase from *Bacillus stearothermophilus* (Fiorentino *et al.*, 1998) whereby the elimination of ion-pair networks, through disruption of specific salt bridges in α-helices, decreased its thermostability. The main chain-main chain, main chain-side chain and side chain-side chain hydrogen bonds have been reported to increase among monomers of most thermophilic proteins and at their interface (Tanner *et al.*, 1996; Kumar *et al.*, 2000b). It has been suggested that hydrogen bonds may be favoured over salt bridges or neutral-neutral hydrogen bonds, because of the lower entropy and enthalpy costs for burying a charged-neutral hydrogen bond, which is lower than that associated with burying a salt bridge or a neutral-neutral hydrogen bond (Vieille and Zeikus, 1995; Kumar *et al.*, 2000a).

Thermophilic proteins also improve their thermostability through the reduction of possible conformations in the unfolded protein state by replacing amino acid residues with Gly and introducing Pro residues (Vieille and Zeikus, 1995), or by introducing covalent linkages such as disulfide bonds (Zhou *et al.*, 1993; Clarke and Fersht, 1993; DeDecker *et al.*, 1996; Li *et al.*, 1998; Németh *et al.*, 2002). As non-Gly residues present in proteins with a left-handed helical conformation often cause protein instability, replacement of such residues with Gly leads to a reduction in the conformation strain with an accompanying increase in stability. For example, Glu_{15} in the *Bacillus subtilis* DNA-binding protein HU and Lys_{95} in *E. coli* RNaseHI (both situated in turn regions) are substituted with Gly residues in their thermophilic counterparts (Vieille and Zeikus, 1995). When the contribution of the substitution was tested in *E. coli* DNA-binding protein HU by site-directed mutagenesis, the $Glu_{15} \rightarrow$ Gly mutation increased the stability of the protein, and it was found to account for 70% of the stability difference between the mesozymes and thermozymes. Disulfide bonds reduce the conformational strain through a decrease in the entropy of the unfolded state of the protein, while increasing their enthalpy state (Clarke and Fersht, 1993; Zhou *et al.*, 1993; Li *et al.*, 1998; Németh *et al.*, 2002). Most disulfide crosslinks occur in the flexible regions (terminal regions) rather than in the rigid regions (α-helices) and may therefore bring the terminal regions in close proximity to each other, and in effect improve on protein packing.

Aromatic clusters or enlarged aromatic networks (Kannan and Vishveshwara, 2000), deletion or shortening of loops (Vieille and Zeikus, 1996; Kumar *et al.*, 2000b), elimination/avoidance of potential sites for deamidation and isoaspartate formation (Chang *et al.*, 1999), and specific chaperones (Russel and Taylor, 1995) are all considered additional contributory factors to thermostability, and operate at a structural level to further strengthen the tertiary structure of the proteins (in the case of aromatic clusters and enlarged aromatic networks). In halophiles, protein stability and function are maintained by increased ion-binding and glutamic acid-content, both allowing the protein inventory to compete for water at high salt concentrations (Jaenicke, 2000).

2.4.3. Maintenance of a homeostatic intracellular environment

Survival at high temperatures requires maintenance of a homeostatic intracellular environment. This, in turn, requires that the membrane remains both stable and fluid to allow movement across, into and out of the cell, in addition to maintenance of a stable intracellular pH and ionic state. In order to achieve the former, saturated lipids are favoured in the membrane (Stanier *et al.*, 1996). An increase in saturated lipids with increasing temperature eliminates the leakage of ions, as an increase in the packaging of saturated lipids results in increased hydrophobic interactions. Thermophilic archaeabacteria, on the other hand, have solved the problem of membrane stability by producing ether lipids, which have a high melting temperature, and thus aid in maintaining the membrane integrity at high temperatures (Fiala *et al.*, 1998; Ko *et al.*, 1996; Pancost and Damsté, 2003).

Among acidophiles and alkalophiles, the internal cytoplasmic pH is maintained around neutral (Horikoshi, 1999; Jaenicke, 2000). This is achieved in part through an increase in the content of ionisable amino acids on proteins facing the outside extremes of pH and through transporter systems within the cell membrane. Furthermore, the ABC (ATP-binding cassette), the Na^+-dependent antiporter system and the solute-binding-protein-dependent transporter systems (Jaenicke, 2000; Driessen *et al.*, 2000), contribute to maintaining the internal homeostatic state through regulation of the rate of proton pumping at elevated temperatures to counter leakage (Van der Oost *et al.*, 1996).

2.5. THERMOSTABLE ENZYMES

Enzymes of industrial importance are broadly grouped into three major categories, namely: carbohydrate-degrading enzymes (amylases, cellulases, xylanases, chitinases, etc.), lipid-degrading enzymes (lipases and phopholipases) and protein-degrading enzymes (proteinases and proteases).

2.5.1. Carbohydrate-degrading enzymes

Carbohydrate-hydrolysing enzymes, commonly known as glycosidases or carbohydrases (Uhlig, 1998), hydrolyse glycosidic bonds between two or more carbohydrates (such as starch, cellulose, xylan, chitin, pectin) or between carbohydrate and non-carbohydrate moieties (Uhlig, 1998; Eichler, 2001). Thermostable glycosidases, e.g. chitinases, chitosanases and cellulase 103, have been isolated from thermophilic bacteria and are being applied in the medical and agricultural sectors, detergent formulations, and in the textile and pharmaceutical industries (Uhlig, 1998; Lengeler *et al.*, 1999; Yoon *et al.*, 2000; Schiraldi and De Rosa, 2002).

2.5.2. Lipid-degrading enzymes

Thermostable microbial lipases and esterases have also been isolated from thermophilic bacterial species, such as *Bacillus thermoleovorans* ID-1 and *Bacillus stearothermophilus* JY144, and are finding increasing industrial application in the synthesis of chiral compounds in non-aqueous solvents and in stereospecific enantioselectivity of enantiomers (Lee *et al.*, 2001; Kim *et al.*, 2002). Furthermore, thermostable lipases have been found to exhibit enhanced activity in the presence of organic solvents such as dimethylsulfoxide (DMSO), ethanol and β-mercaptoethanol (Eichler, 2001).

2.5.3. Proteases

Proteases are the most important industrial enzymes, accounting for about 60% of the total worldwide sales (Harwood, 1992; Mala *et al.*, 1998; Anwar and Saleemuddin, 1998; Mehrotra *et al.*, 1999). The wide range of applications of proteases, coupled with the need for enzymes that can operate under harsh conditions and in the presence of organic solvents and denaturing agents, has made thermophiles a promising source of such enzymes (Jaenicke *et al.*, 1996).

2.5.3.1. Classification of proteases

Proteases are classified based on (a) evolutionary history, (b) type of reaction catalysed, and (c) the nature of the active site (Mala *et al.*, 1998). The evolutionary history of proteases is hard to follow due to the lacunae in the data presented and appears, to some extent, contradictory. Depending on the type of reaction that is catalysed, proteases are classified as either exopeptidases (aminopeptidases and carboxypeptidases), which cleave peptide bonds proximal to the amino or carboxyl termini of the substrate, respectively, or endopeptidases, which cleave peptide bonds distant from both amino and carboxyl termini of the substrate. Based on the nature of the active site, proteases are classified as (a) serine proteases, i.e. a serine amino acid residue present in their active site, (b) aspartic proteases (acid proteases), which depend on the presence of an aspartic amino acid residue in the active site for their catalytic activity, (c) cysteine/thio-proteases, which depend on a catalytic dyad consisting of cysteine and histidine, and (d) metalloproteases, which require a divalent metal ion for their activity (Mala *et al.*, 1998).

2.5.3.2. Bacterial proteases of commercial importance and their synthesis in heterologous hosts

The development of bacterial enzymes for industrial and other biotechnological applications requires that the reaction kinetic properties, substrate specificities, catalytic activity, stability at different temperatures and pH, as well as stability in the presence of reducing agents and metal ions be characterised. The results from such studies have helped in the elucidation of catalytic mechanisms, which, in turn, has been used to guide bioengineering of the enzyme(s) for industrial/biotechnological applications. Furthermore, many bacterial species have been screened for enzyme production and the appropriate genes isolated, cloned and expressed in hosts such as *E. coli*, *B. subtilis* and *B. licheniformis* (in some cases) (Harwood, 1992).

The proteases of commercial importance are mainly neutral and alkaline proteases (Van Den Burg *et al.*, 1991; Kobayashi *et al.*, 1995). These proteases have been isolated from various mesophilic and thermophilic bacteria, with *Bacillus* spp. occupying the central position (Table 2.1). *Bacillus* spp. have an inherent ability to secrete a number of hydrolytic enzymes (e.g. carbohydrate-degrading and proteolytic enzymes) and other proteins (e.g. small peptides), into the extracellular milieu (Van der Laan *et al.*, 1991; Bolhuis *et al.*, 1999). This characteristic of *Bacillus* spp. has been exploited in the production of industrially important

enzymes such as alkaline proteases and neutral proteases (Harwood, 1992), and heterologous proteins such as human α-amylase (Bolhuis *et al.*, 1999), interferons (Henner, 1986) and streptolysin O (Yamada *et al.*, 1995).

Currently, *E. coli* and *B. subtilis* are the most widely used hosts for cloning and expression of recombinant proteins (Wu *et al.*, 1991; Van der Laan *et al.*, 1991; Pang *et al.*, 1991; Peek *et al.*, 1993; Bolhuis *et al.*, 1999; Chavagnat *et al.*, 1999). This is due to their well-characterised physiology and genetics, and a number cloning and/or expression vectors have been developed over the years for use in these expression hosts. Despite the success of the two organisms, there are some limitations to their general application. For example, *E. coli* is not an ideal system for expression of secreted proteins. Most of the proteins are not exported to the extracellular environment, but rather accumulate in the periplasmic space or remain attached to the outer or inner membranes (Mezes *et al.*, 1983; Ikemura *et al.*, 1987; Tan *et al.*, 2002). Recovery of such proteins involves cell disruption and aggregation of cell debris by flocculants, which leads to increased costs of downstream processing of the recombinant protein.

Bacillus subtilis, on the other hand, exports proteins efficiently to the extracellular milieu, but its high extracellular protease activity coupled with limited chaperone and signal peptidase (SipS and SipT) activities affect the efficiency of processing, export and stability of heterologous proteins, especially of eukaryotic origin (Bolhuis *et al.*, 1999). In some instances, the high extracellular protease activity renders many of the recombinant proteins functionless due to proteolysis. This disadvantage has been addressed by developing mutant *Bacillus* strains deficient in extracellular protease activity. *Bacillus subtilis* strain WB600, for example, is a mutant strain deficient for six of the seven proteases produced by *Bacillus subtilis* wild-type strains (Wu *et al.*, 1991). Another potentially useful host for the production of heterologous proteins is *B. brevis*. Like *B. subtilis*, it also secretes proteins directly into the culture medium where they accumulate to high levels in a relatively pure state (Yamada *et al.*, 1981; Yamagata *et al.*, 1985; Udaka and Yamagata 1993). *B. brevis*, however, has very low protease activity and a thin cell wall, which makes the export of recombinant proteins efficient, and the secreted proteins remain stable since they are not significantly degraded. Despite these advantages, its genetics is largely uncharacterised. Consequently, few vectors have been constructed for use in the cloning and expression of heterologous recombinant proteins in *B. brevis*.

2.5.3.3. Export of extracellular proteins

Many secreted proteins are exported as precursors (pre-proproteins). They consist of a signal peptide (pre-sequence) that is responsible for directing protein secretion across the membrane, and a peptide extension between the signal peptide and the mature protein that consists of varying number of amino acids residues, depending on the protein secreted (pro-sequence). The pro-sequence is essential for guiding appropriate folding of the secreted protein to yield an enzymatically active conformation (Wong and Doi, 1986; Ikemura *et al.*, 1987; Zhu *et al.*, 1989; Navarre and Schneewind, 1999; Tjalsma *et al.*, 2000). It is cleaved through an intra-molecular self-processing mechanism to produce a mature protein (Ikemura and Inouye, 1988; Zhu *et al.*, 1989). With the exception of the staphylococcal nuclease A gene (Takahara *et al.*, 1985; Peek *et al.*, 1993), successful expression, in their active conformation, of proteases originating from *Bacillus* spp. by hosts such as *E. coli* and *B. subtilis* has only been achieved by cloning the whole pro-proteinase gene (Takahara *et al.*, 1985; Peek *et al.*, 1993). Therefore, failure to identify foreign genes by screening for function in either *E. coli* or *B. subtilis* may often be a result of failure of processing the gene into a mature protein, because the prosequence was lost during random restriction digestion whilst preparing a genomic DNA library (Dalbøge, 1997).

2.5.3.4. Biotechnological applications of bacterial proteases

Enzymes, as opposed to chemical reagents, are increasingly being used as biocatalysts as a means of not only offsetting the impact of high production costs involved in using chemical catalysts but also to protect the environment from toxic recalcitrant industrial wastes. An obvious source of enzymes is microorganisms, i.e. viruses, bacteria and fungi, as they produce many enzymes that display a range of activities (Mala *et al.*, 1998).

Interest in the application of enzymes from hyperthermophiles for biotechnologically-related processes has rapidly accelerated (Eichler, 2001), since it is often advantageous to perform such processes at high temperatures. This is partly due to the fact that an increase in temperature may lead to a decrease in medium viscosity and an increase in the diffusion coefficient at elevated temperatures, thus resulting in higher reaction rates. Such considerations are relevant to a variety of processes including those involving hydrophobic compounds that normally display low solubilities. Initially, studies on hyperthermophiles were hampered by a lack of specialised growth vessels and protocols. However, as protocols

for the large-scale growth of such organisms are becoming available (Eichler, 2001), coupled with advances in recombinant DNA technology, the discovery of novel hyperthemophilic enzymes is greatly being enhanced.

2.5.3.4.1. Industrial uses

Currently, a number of enzymes, amongst them, alkaline and neutral proteases, are being used in different industrial processes. Table 2.1 shows various thermostable microbial proteases that have been studied with the aim of applying them in different industrial and biotechnological processes. For example, neutral proteases, e.g. thermolysin produced by *Bacillus thermoproteolyticus,* and alkaline proteases, e.g. subtilisins such as subtilisin Carlsberg produced by *B. licheniformis*, are constituents of most laundry detergents (Nagami and Tanaka, 1986; Tran *et al.*, 1991; Mala *et al.*, 1998). Takami *et al.* (1992a; 1992b) and Friedrich and Antranikian (1996) reported thermostable alkaline serine protease from *Bacillus* spp. No. AH-101 and from *Fervidobacterium pennavorans*, respectively, with keratinolytic activity. The properties of the two enzymes, i.e. their high thermostability, activity at alkaline pH (between pH 11-13) and resistance to denaturants, suggests that they may be suitable candidates for application in the leather tanning and poultry farming (waste management) industries. Another keratinolytic alkaline serine protease, produced by *Streptomyces albidoflavus*, which may also be applicable to the leather tanning industry, has been reported by Bressollier *et al.* (1999). The enzyme is active optimally at pH values ranging from 6 to 9.5 and at temperatures ranging from 40 to 70°C. Furthermore, proteolytic enzymes are being used in the dairy industry in the manufacture of cheese (Harwood, 1992), as well as in the bioconversion of proteinaceous waste into useful biomass (Anwar and Saleemuddin, 1998; Mala *et al.*, 1998).

2.5.3.4.2. Catabolism of aromatic compounds and xenobiotics

Degradation of aromatic compounds including phenolics, such as phenol, cresol, benzoate, catechol (Dong *et al.*, 1992; Duffner and Müller, 1998; Ali *et al.*, 1998; Duffner *et al.*, 2000), and substituted aromatics, such as 4-hydroxyphenylacetic acid (4-HPA) and dihydroxyphenylacetic acid (DHPA), has been widely studied among mesophiles and several degradation pathways have been elucidated. Mesophilic enzymes, with a few exceptions, have often been found very unstable, which has, in part, contributed to the failure of purifying a number of phenol-degrading enzymes. Thus far, successful purification of 4-

hydroxyphenylacetate 3-hydroxylase from *Pseudomonas putida* and *Pseudomonas putida* U, to homogeneity, has been reported (Raju *et al.*, 1988; Fernandez-Medarde and Luengo, 1997). The physiological role of these enzymes is to convert phenolic compounds into metabolic intermediates and the subsequent generation of energy through their catabolism. A number of phenol degradation pathways and their constituent enzymes have also been described among thermophilic *Bacillus* (Dong *et al.*, 1992; Duffner *et al.*, 2000). However, few genes have been cloned and sequenced (Dong *et al.*, 1992; Duffner *et al.*, 2000). Since it has been recognised that thermostable enzymes from thermophiles tend to be resistant to chemical denaturation (Gurujeyalakshmi and Oriel, 1989), thermophilic degradation conditions may be more advantageous in terms of higher metabolic rates, bio-availability of many organic pollutants, and increased enzyme stability (Duffner and Müller, 1998).

Table 2.1. Thermostable protease enzymes and their potential industrial relevance

Species	Protease type	Optimal pH	Optimal Temp. (°C)	Application	Reference
Bacillus sp. NKS-21	Alkaline serine protease	10-11	50-55	NS	Tsuchida *et al.* (1986)
B. stearothermophilus	Neutral protease	ND	ND	NS	Kubo and Imanaka (1988)
Bacillus sp. No. AH-101	Alkaline serine protease	12-13	80	Laundry detergent	Takami *et al.* (1989)
B. stearothermophilus	Neutral protease	ND	ND	NS	Nishiya and Imanaka *et al.* (1990)
B. caldolyticus	Neutral protease	ND	ND	NS	Van den Burg *et al.* (1991)
Bacillus sp. Ak.1	Alkaline serine protease	7.5	60-70	Laundry detergent	Peek *et al.* (1993)
Bacillus sp. KSM-K16	Alkaline serine protease	12.3	55	Laundry detergent	Hakamada *et al.* (1994)
Bacillus sp. EA1	Neutral protease	ND	ND	NS	Saul *et al.* (1996)
F. pennavorans	Alkaline serine protease (keratinolytic activity)	10.0	80	Keratin waste management	Friedrich and Antranikian (1996)
B. licheniformis	Alkaline serine protease	12.0	60	Laundry detergent	Ferrero *et al.* (1996)
Pimelobacter sp. Z-483	Alkaline serine protease	9.0	50	NS	Oyama *et al.* (1997)
S. albidoflavus	Alkaline serine protease	6-9.5	40-70	Leather industry	Bressollier *et al.* (1999)
Bacillus sp. BT1	Neutral protease	ND	ND	NS	Vecerek and Venema (2000)
T. kodakaerensis KOD1	Alkaline serine protease	9.5	80	NS	Kannan *et al.* (2001)
B. pumilus MK6-5	Alkaline serine protease	11.5	55-60	Ultrafitration membrane cleaning	Kumar (2002)

ND, not done; NS, no suggestion

2.6. REFERENCES

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CHAPTER THREE*

THERMOPHILIC PROTEASE-PRODUCING *Geobacillus* FROM BURANGA HOT SPRINGS IN WESTERN UGANDA

ABSTRACT

Two thermophilic aerobic bacterial strains, PA-9 and PA-5, were isolated from hot spring environments in western Uganda. The cells were rods, approximately 9.7 to 12 μm in length. Whereas isolate PA-9 grew at temperatures between 38 and 68°C (optimum, 62°C), isolate PA-5 grew at temperatures between 37 and 72°C (optimum, 60°C). Both isolates grew optimally at pH 7.5 - 8.5. The 16S rRNA gene sequencing of isolates PA-9 and PA-5 indicated that they belong to the genus *Bacillus*. During growth on casein, the isolates produced enzymes with caseinolytic activity. Zymogram analysis of the crude enzyme extracts revealed the presence of two extracellular enzymes for isolate PA-5, and at least eight for isolate PA-9. The optimum temperature and pH for casein-degrading activity were 70°C, pH 6.5 for isolate PA-9, but caseinolytic activity could also be observed at 2°C. In the case of isolate PA-5, optimal activity was observed over a temperature and pH range of 50 - 70°C and pH 5 - 10, respectively.

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3.1. INTRODUCTION

The use of enzymes as diagnostic tools, as analytical reagents and as industrial catalysts is an important and rapidly expanding technology (Cowan, 1992). This expansion is driven by the requirements of some industries for high levels of specificity in the biosynthesis of high value products (as in the pharmaceutical industries) and by the success of some enzyme products as speciality reagents, e.g. the DNA polymerases in the polymerase chain reaction.

Research on thermo- and extremophiles, as promising sources for highly stable enzymes, has remained an active research subject (Davis, 1998). Thermophiles represent an obvious source of thermostable enzymes, being reasonable to assume that such character will confer their proteins a high thermal stability (Adams and Kelly, 1998). Enzymes isolated from these microorganisms are not only thermostable and active at high temperature, but are also often resistant to and active in the presence of organic solvents and detergents (Jaenicke *et al.*, 1996). Despite being sources of novel biochemical pathways, enzymes and bioproducts, the technological use of thermophiles still faces several challenges since knowledge on physiology, genetics and diversity of such organisms is generally poor (Prieur, 1992). Thermophiles or the genes derived from them are, however, still the preferred source for thermostable enzymes (Adams and Kelly, 1998).

By now, a high number of thermostable enzymes from thermophiles have been reported; most of them belonging to eubacterial and archaebacterial kingdoms. Hyperthermophilic archaebacteria and eubacteria, such as *Pyrococcus furiosus* and *Thermotoga* spp., have yielded several biotechnologically relevant enzymes (Adams and Kelly, 1998). However, *Bacillus* spp. have remained major sources of enzymes of industrial and commercial value and a number of thermophilic *Bacillus* spp. have been reported to produce useful enzymes (Mala *et al.*, 1998).

In this part of the study, two thermophilic protease-secreting, Gram-positive, spore forming aerobes were isolated from the Buranga hot springs in western Uganda and were characterised morphologically and physiologically. The preliminary characterisation of extracellular proteases with caseinolytic activity produced by these isolates is furthermore reported.

3.2. MATERIALS AND METHODS

3.2.1. Isolation of protease-secreting thermophiles

Soil and water samples collected from the Buranga hot springs in western Uganda, were serially diluted, plated onto milk agar plates (pH 8.5), and incubated at 55°C for 48 h. Individual colonies that produced clear zones were selected and purified via subculturing, and two isolates producing the largest hydrolysis zones were selected and designated as PA-5 and PA-9, respectively.

3.2.2. Culture conditions

The isolates were grown at 55°C for 48 h in modified Castenholz medium D (Castenholz, 1969) containing 5 g peptone, 2 g yeast extract, 2 g glucose, 100 ml Solution A, 1 ml Solution B and 10 ml Solution C per litre. Solution A contained (g/l) $CaCl_2$, 0.6; $MgSO_4.7H_2O$, 1; NaCl, 0.8; $NaNO_3$, 3; KNO_3, 1; Na_2HPO_4, 0.11; $FeCl_3$, 0.03 and $NH_4CH_3COO^-$, 0.36. Solution B contained (g/l) $MnCl_2$, 0.513; $ZnSO_4.7H_2O$, 0.25; H_3BO_3, 0.25; $(NH_4)_2MoO_4$, 0.0125; $CoCl_2$, 0.023 and $CuSO_4$, 0.0125. Solution C contained 10 mg/ml each of nicotinamide, riboflavin and *p*-aminobenzoic acid.

3.2.3. Morphological properties and growth limits

Differences in cellular morphology were examined by microscopic analysis of Gram-stained cells (Zeiss Axioskop, Germany), and images were captured using a COHU monochrome CCD camera (RS-170, Cohn Inc., San Diego, CA, USA) at a magnification of ×1000. The optimal pH for growth of each isolate was determined in 50 ml of the medium buffered with 10 mM benzoic acid at pH 4, 10 mM pyridine at pH 5, 10 mM phosphate buffer at pH 5, 10 mM Tris-HCl at pH 7 to 9, 10 mM $Na_2HPO_4.2H_2O$ at pH 10, and 10 mM methylamine-HCl at pH 11. The optimum growth temperature of each isolate was determined after inoculation of 15 ml of the medium, pre-equilibrated to temperatures ranging from 37 to 72°C. The tubes were incubated in a temperature gradient incubator (Scientific Industries, Mineola, USA) and the bacterial growth was followed by measuring optical density at 600 nm every 30 min for 10 h. At each of the investigated temperatures, the growth rate (μ) was calculated for the period of their exponential increase.

3.2.4. Phylogenetic analysis by 16S rDNA sequence analysis

Genomic DNA of both isolates PA-5 and PA-9 was isolated using the method described by Marmur and Doty (1962) with the following modifications. Cells from overnight cultures of the two isolates were collected by centrifugation and the pellet suspended in 2 ml of 50 mM TE buffer (pH 8.0), containing lysozyme (5 mg/ml). Following incubation at 37°C for 1 h, an equal volume 10% (w/v) SDS was added and the cell suspensions were incubated at 60°C for 20 min. The DNA was subsequently extracted with phenol/chloroform/isoamylalcohol (25:24:1) after which the chromosomal DNA was precipitated from the aqueous phase through the addition of 2 volumes 95% ethanol and sodium acetate (pH 5.2) to a final concentration of 0.3 M. The genomic DNA was recovered by spooling, washed in 70% ethanol and suspended in ddH_2O.

The 16S rDNA gene of each isolate was subsequently amplified by using the universal primers fD1 and rP2 (Weisburg *et al.*, 1991). The purified PCR product was directly sequenced with an ABI Prism model 377 DNA sequencer and the resulting sequence was edited to a total length of 1 347 nucleotides. The phylogenetic relationship of isolates PA-5 and PA-9 was determined by comparing the sequencing data with sequences of related *Bacillus* and *Geobacillus* spp. (GenBank database of the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/GenBank/) by using the neighbour-joining method of Saitou and Nei (1987) in combination with the bootstrap method (Felsenstein, 1985).

3.2.5. Preparation of crude extracellular protease extracts

The isolates were cultured at 55°C in medium containing 0.5% (w/v) meat extract and soybean-casein digest, and 1% (w/v) of either glucose or glycerol. Following incubation, cells were harvested by centrifugation (13 000 rpm, 5 min) and the supernatants were dialysed against 5 mM Tris buffer (pH 8) containing 2 mM $CaCl_2$ at 4°C overnight. The dialysates were concentrated by lyophilization and resuspended in the same buffer.

3.2.6. Proteolytic activity assays

For cup plate assays, 100 µl of crude enzyme extract was added to wells punched into casein or milk agar plates. After incubation at 55°C for 12 h, zones of hydrolysis around the wells were measured as an indicator of proteolytic activity. Proteolytic activity was also assayed by using azocasein (Sigma, St. Louis, MO, USA) as a substrate according to the method of

Smibert and Krieg (1994). The protein concentrations were determined according to the method of Bradford (1976) by using the Pierce assay reagent (Pierce, Rockford, Ill., USA) and bovine serum albumin as the standard.

3.2.7. Influence of pH and temperature on protease activity

The influence of the pH on caseinolytic activity was determined by using the azocasein protocol described above, except for the use of different reaction buffers (benzoic acid, pyridine, phosphate buffer, Tris-HCl, $Na_2HPO_4.2H_2O$, methylamine-HCl) to obtain values from pH 4 to pH 11. All the buffers were pH-adjusted for the incubation temperature and all of the assays were performed at 55°C. To determine the influence of temperature on the caseinolytic activity, samples were incubated for 10 min at temperatures of 2, 20, 37, 40, 50, 60, 70, 80, 90, and 94°C, respectively, in 10 mM Tris-HCl buffer (pH 7.5).

3.2.8. Electrophoresis and zymograms

The method used for SDS-PAGE analysis was essentially the method described by Laemmli, (1970). For zymogram analysis, 2% (w/v) caseinate co-polymerised with the gels was used. Samples were loaded into the gel without prior heating, and electrophoresis was performed at 4°C at 25 V/gel. Following electrophoresis, gels were washed successively, first with 2.5% (v/v) TritonX-100 in water and then with 50 mM Tris-HCl (pH 8) buffer containing 2.5% (v/v) TritonX-100, each for 10 min at room temperature. The gels were equilibrated for 10 min in 50 mM Tris-HCl buffer (pH 7.5) and then incubated for 5 min at 55°C. Finally, gels were stained with 2% Coomassie brilliant blue and destained in deionized water, to reveal zones of substrate hydrolysis. For electrophoretic analysis of low-molecular-weight proteins, a Tricine gel was used according to the method described by Judd (1996).

3.2.9. Nucleotide sequence accession numbers

The 16S rRNA sequences obtained in this study have been deposited in GenBank under accession no. AF267508 and AF267509 for isolates PA-5 and PA-9, respectively.

3.3. RESULTS AND DISCUSSION

Microbial studies of hot spring communities may provide an unique and wide-ranging source of novel microorganisms, many of which may show significant variance from known terrestrial phylotypes. These organisms, each containing a catalogue of enzymes and other bioproducts, could provide a highly valuable resource for biotechnological developments and applications. In this study, protease-secreting thermophilic bacteria were isolated from Buranga hot springs in western Uganda. The temperature and pH of the hot springs ranged between 45°C and 101°C and from pH 6.8 to 8.5, respectively. Two isolates that demonstrated high protease activity on milk and casein agar plates, designated as PA-5 and PA-9, respectively, were selected and characterised.

3.3.1. Morphology

Both isolates PA-9 and PA-5 are aerobic, sporulating, Gram-positive, rod-shaped bacteria. The cell morphology of isolate PA-9 differed from that of PA-5 grown under similar conditions (Fig. 3.1). The cells of isolate PA-9 were long straight rods (12 µm in length and 3 µm in width), whereas cells of isolate PA-5 were slightly shorter (10 µm in length by 3 µm in width). There was a general tendency to increase in size with time. After 24 h of stationary broth culture, isolate PA-9 grew as single cells and as filament-like chains at least 53 µm long. In contrast, isolate PA-5 grew as single cells, and short chains (19 µm long) were observed only after 48 h of incubation.

3.3.2. Effect of temperature and pH on growth

The growth rates of isolates PA-9 and PA-5 were determined within the temperature range of 36 to 72°C. For isolate PA-5, the optimum growth rate was at 60°C (Fig. 3.2a), while isolate PA-9 had the highest maximum growth rate at 62°C (Fig. 3.2b). At the temperatures for maximum growth rates, the generation time was approximately 35 and 21 min for isolate PA-5 and PA-9, respectively. Isolate PA-5 was unable to grow at 72°C or below 38°C, and isolate PA-9 was unable to grow above 68°C or below 36°C. Regarding pH growth limitation, isolates PA-9 and PA-5 were grown in modified Castenholz medium that had a range of pH values between 4 and 12. A pH range around neutrality (7.5 - 8.5) favoured optimal growth of the two isolates. The isolates did not grow at a pH lower than pH 4. However, reduced growth at pH 11 and pH 12 was noted for isolates PA-5 and PA-9, respectively.

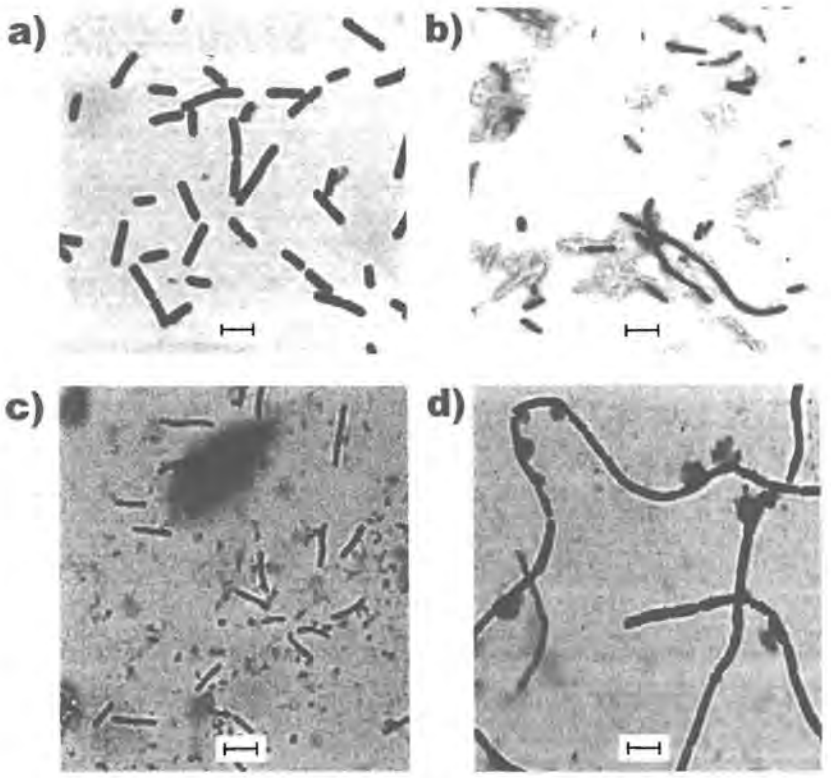

Fig. 3.1 Micrographs showing typical cell morphologies of isolates PA-5 after 10 h (a) and 48 h (b) of incubation, and of isolate PA-9 after 8 h (c) and 75 h (d) of incubation. The bar corresponds to 2 µm and applies to all micrographs.

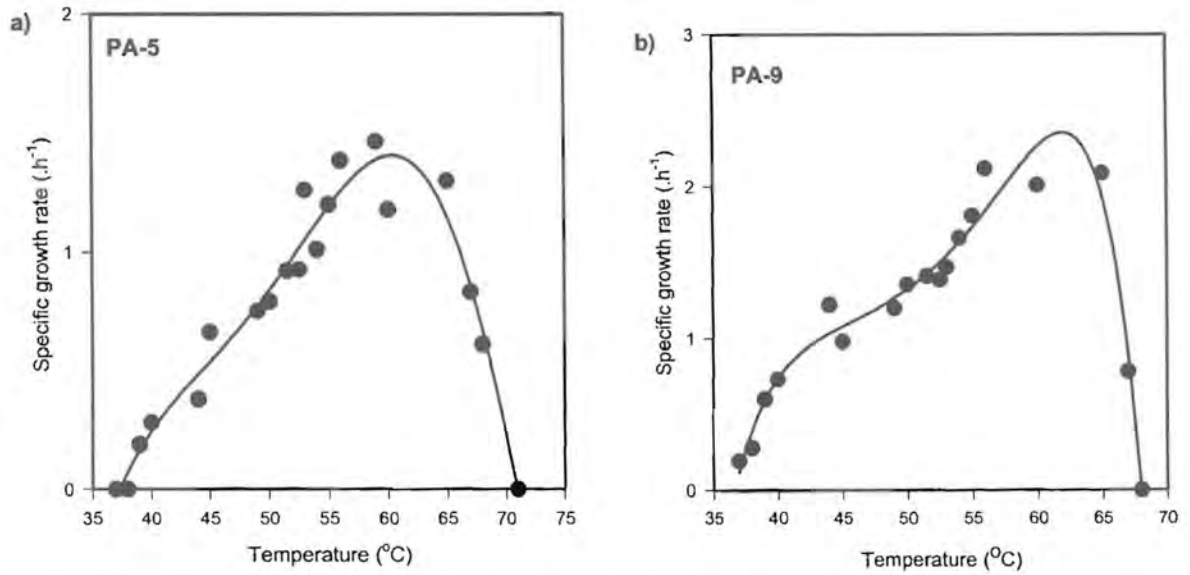

Fig. 3.2 Influence of temperature on maximal specific growth rates of isolates PA-5 (a) and PA-9 (b) cultivated in modified Castenholz medium D. All values are the average of two separate experiments.

3.3.3. Phylogenetic analysis

Based on their morphologies, PA-5 and PA-9 were tentatively identified as *Bacillus* species. To confirm their identity, PCR amplification and sequencing of the 16S rRNA gene of these isolates allowed us to determine approximately 96% of the complete sequence. A phylogenetic tree (Fig. 3.3) was constructed by the neighbor-joining method and indicated that both isolates PA-5 and PA-9 were part of the cluster within the thermophilic group of the genus *Bacillus*. All of these species, like the presently obtained isolates, grow at high temperatures and neutral pH (Rainey *et al.*, 1994). Among the described species, the closest relatives of isolate PA-5 were *B. thermoleovorans* B23 and DSM5366, while those for isolate PA-9 were *Geobacillus uzenensis* strain X and *B. kaustophilus* NCIMB8547. It has recently been proposed that the thermophilic *Bacillus* growing at neutral pH be transferred to a newly proposed genus, *Geobacillus*, with *Geobacillus stearothermophilus* (formally *B. stearothermophilus*) as the type species (Nazina *et al.*, 2001). The 16S rDNA sequence analysis indicated that isolates PA-5 and PA-9 were phylogenetically related to a low degree (96%) when compared with the relatedness between other known thermophilic *Bacilli*. A more definitive classification in the future, however, should take additional characteristics, especially DNA-DNA hybridisation data, into account (Stackebrandt and Goebel, 1994).

3.3.4. Caseinolytic characteristics of the different *Bacillus* isolates

Isolates PA-5 and PA-9 were cultured at 55°C in broth containing either glycerol or glucose as the sole carbon source. Analysis of extracellular protease extracts by plate cup assays at various time intervals indicated that maximal levels of extracellular enzymatic activity could be obtained after 3 days for isolate PA-9, and after 6 days for isolate PA-5. In the case of isolate PA-9, similar levels of protease production was observed when either glucose or glycerol was added to the medium. In contrast, for isolate PA-5, production of proteases was significantly favoured by glycerol in the medium (data not shown). Thus, glucose and glycerol was used as the sole carbon source for isolate PA-9 and PA-5, respectively, in all subsequent experiments.

To investigate the activity of the extracellular proteases, their ability to hydrolyze azocasein at pH 4 to 11 was investigated spectrophotometrically. For isolate PA-9, high levels of protease activity were detected from pH 5 to 9, and with two optima, one at pH 6.5 and a

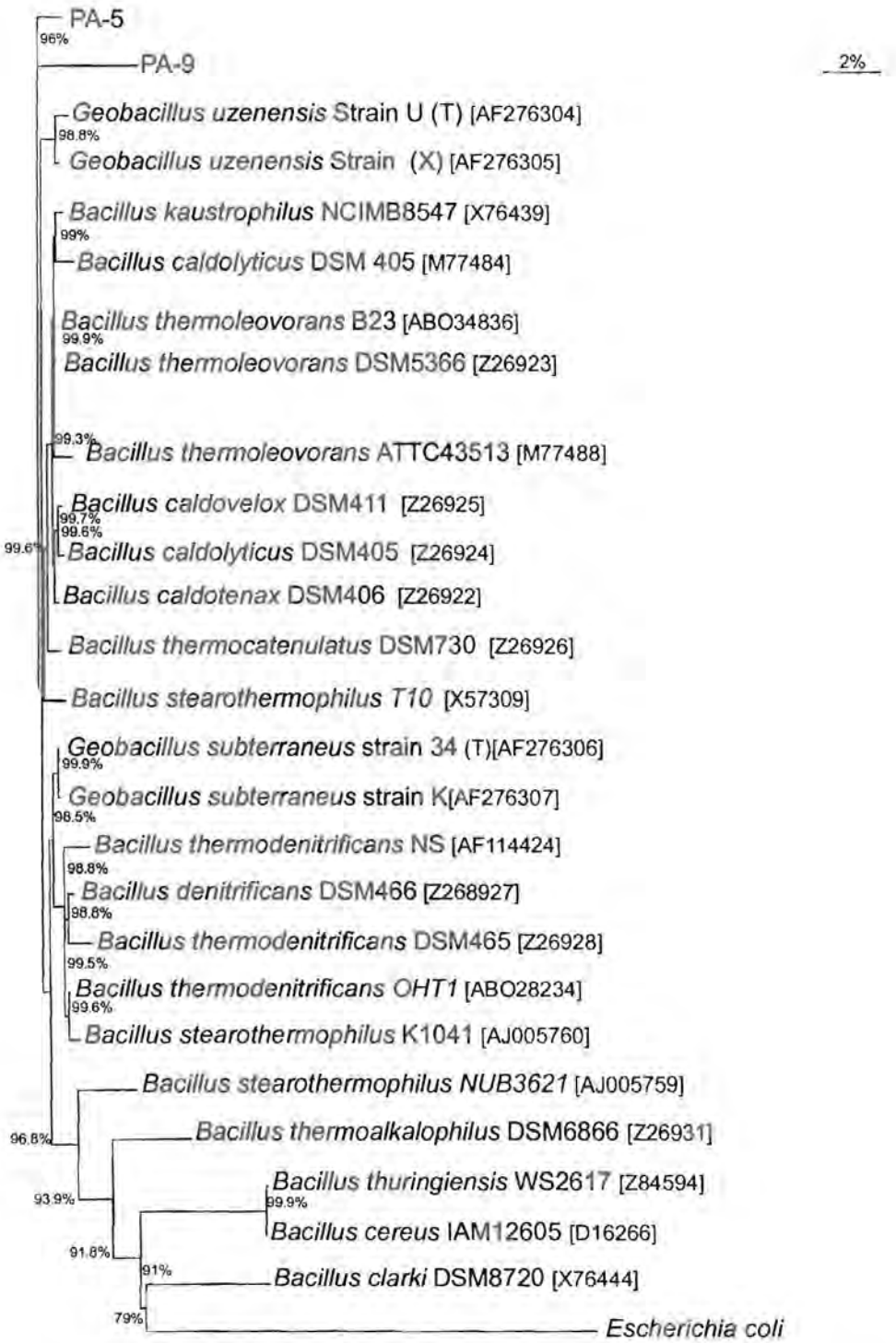

Fig. 3.3 Neighbor-joining tree showing the phylogenetic affiliation of isolates PA-9 and PA-5 to a selected number of members of *Bacillus* spp., of which the GenBank Accession no. are indicated in brackets. Bootstrap values greater than 50% are indicated. The scale bar represents 2% of the estimated substitutions per nucleotide position.

second at pH 8.0 (Fig. 3.4a). The protease extract of isolate PA-5 displayed optimal activity between pH 5 and 10, albeit low when compared to that of isolate PA-9.

The optimum temperature for caseinolytic activity of the crude extracellular enzyme extracts prepared from isolates PA-5 and PA-9 was investigated at various temperatures from 2 to 94°C at pH 7.5. For isolate PA-9, the optimum reaction temperature of the enzyme solution was 70°C (Fig. 3.4b). The enzymes remained active over a wide range of temperatures varying from 37 to 80°C. In the case of isolate PA-5 (Fig. 3.4b), the optimal temperature of the enzyme solution was between 50 and 70°C. It is noteworthy that the extracellular enzyme preparation of PA-9 displayed significant activity at 2°C. Furthermore, the enzyme preparations prepared from both isolates were active at 94°C.

3.3.5. Zymogram analysis of secreted proteases

The extracellular enzyme solutions prepared from isolates PA-5 and PA-9 had different caseinolytic activities as measured by the azocasein method. Zymogram analysis was therefore carried out to compare the different proteases secreted by the respective isolates. Like many reported *Bacillus* species (Priest, 1977), isolates PA-5 and PA-9 also produced multiple extracellular proteases.

The zymogram obtained for isolate PA-5 (Fig. 3.5), indicated the presence of two bands with protease activity. A larger zone of hydrolysis was observed for the 25-kDa band compared to the 24-kDa band. For isolate PA-9, eight proteins with protease activity could be identified in the extracellular enzyme extract and were found to have molecular masses of approximately 97, 72, 50, 27, 22, 17 and 12 kDa (resolved as two bands by Tricine gel electrophoresis) (Fig. 3.6b). Whether each of the observed proteases in fact represent distinct protease species or whether some of these may represent unprocessed forms of other proteases must await primary amino acid sequence comparisons. Following incubation at 55°C for 10 min, the two protein bands of approximately 97 and 72 kDa, respectively, displayed larger zones of hydrolysis and thus appear to be heat-activated proteases (Fig. 3.6a, lane 2). The 12-kDa protease appeared to be responsible for the observed protease activity at low temperature (2°C) (Fig. 6a, lane 1). The use of a Tricine gel revealed that the small molecular weight protease band of 12 kDa could be resolved as two separate bands (Fig. 3.6b), each displaying caseinolytic activity.

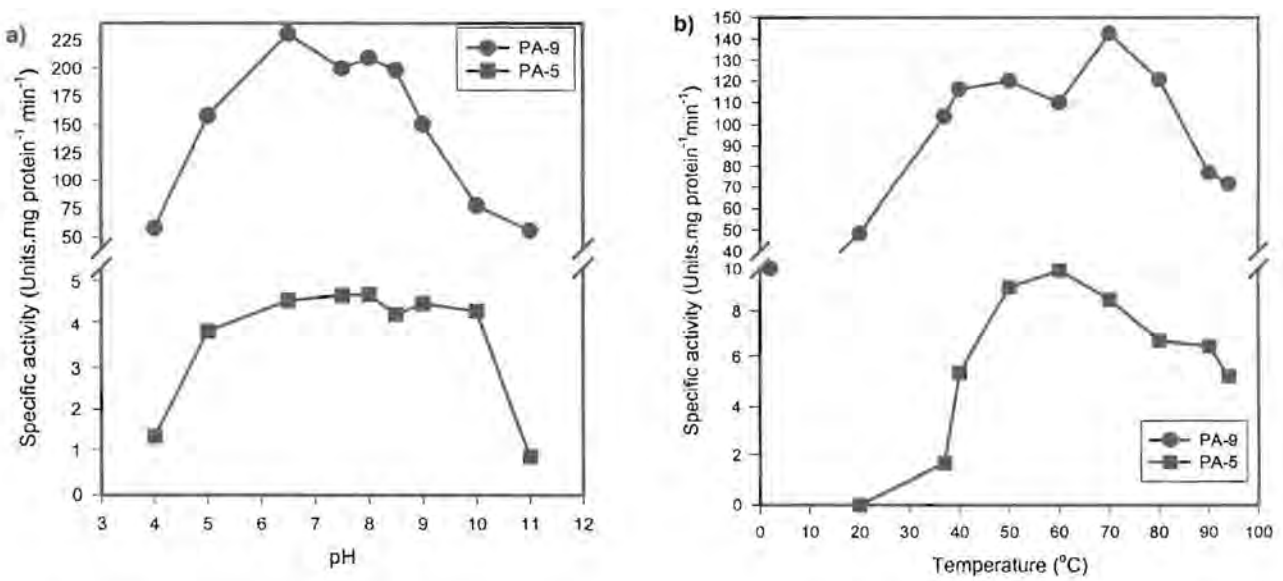

Fig. 3.4 Influence of (a) pH and (b) temperature on the activity of crude extracellular enzyme preparations prepared from the culture supernatants of isolates PA-5 and PA-9. For the pH profile, activity was measured at 55°C in 10 mM Tris buffer adjusted to the correct pH. For the temperature profile, activity was measured in 10 mM Tris buffer (pH 7.5) at different temperatures. Values are the means of results of duplicate experiments.

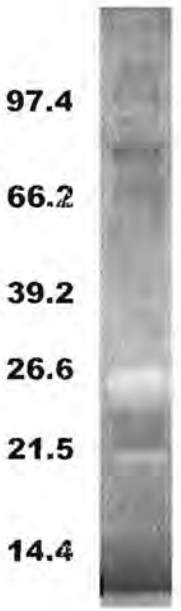

Fig. 3.5 Analysis of the presence of caseinolytic proteases in cell-free supernatants of isolate PA-5. Aliquots of culture supernatants from cells grown for 6 days were used to analyze the presence of caseinolytic proteases by zymograms (lane 1). The sizes of the molecular mass standards (in kilodaltons) are indicated to the left.

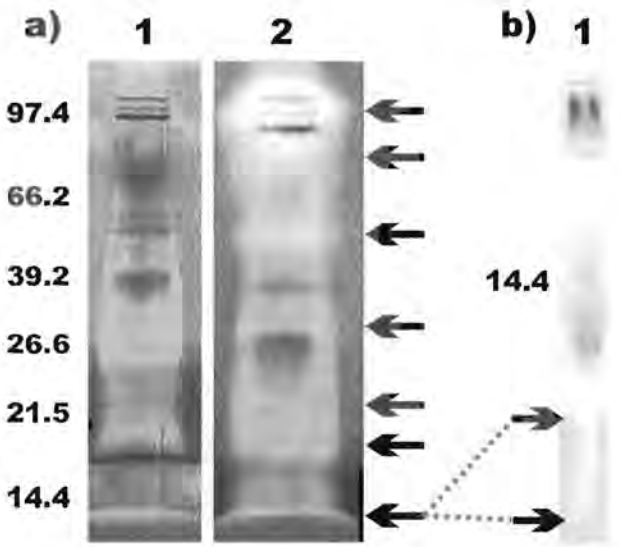

Fig. 3.6 Analysis of the presence of caseinolytic proteases in cell-free supernatants of isolate PA-9. (a) Aliquots of culture supernatants from cells grown for 3 days were used to analyze the presence of caseinolytic proteases by zymograms without (lane 1) and with incubation at 55°C for 5 min (lane 2). (b) Zymogram of a Tricine gel indicating two low-molecular-weight proteases. The positions of the proteases are indicated by arrows, and the sizes of the molecular mass standards (in kilodaltons) are indicated to the left.

The observation that proteases produced by isolate PA-9 displaying activity at 2°C may be of significance. The genes of cold-active proteases have been cloned from two psychrotrophic *Bacillus* strains, strains TA39 (Narinx *et al.*, 1997) and TA41 (Davail *et al.*, 1992). Both of these proteases are members of the subtilisin subfamily. Such enzymes have also been isolated from Gram-negative psychrotrophs such as *Shewanella* strain Ac10, as well as a few mesophiles (Kulakova *et al.*, 1999). The two small proteases produced by the thermophilic *Bacillus* PA-9 therefore constitute a novel finding with potential for the development of recombinant cold-active proteases. There is a growing biotechnological interest in cold-adapted enzymes as they are particularly suited to low temperature biotransformations where substrates and/or co-factors are thermolabile, where low temperature operation is considered as a means of reducing microbial growth, or simply to save on heating expenses (Margesin and Schinner, 1994).

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