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Thermoactive extracellular proteases of *Geobacillus caldoproteolyticus*, sp. nov., from sewage sludge

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Abstract A proteolytic thermophilic bacterial strain, designated as strain SF03, was isolated from sewage sludge in Singapore. Strain SF03 is a strictly aerobic, Gram stain-positive, catalase-positive, oxidase-positive, and endospore-forming rod. It grows at temperatures ranging from 35 to 65°C, pH ranging from 6.0 to 9.0, and salinities ranging from 0 to 2.5%. Phylogenetic analyses revealed that strain SF03 was most similar to *Saccharococcus thermophilus*, *Geobacillus caldoxylosilyticus*, and *G. thermoglucosidasius*, with 16S rRNA gene sequence identities of 97.6, 97.5 and 97.2%, respectively. Based on taxonomic and 16S rRNA analyses, strain SF03 was named *G. caldoproteolyticus* sp. nov. Production of extracellular protease from strain SF03 was observed on a basal peptone medium supplemented with different carbon and nitrogen sources. Protease production was repressed by glucose, lactose, and casamino acids but was enhanced by sucrose and NH_4Cl. The cell growth and protease production were significantly improved when strain SF03 was cultivated on a 10% skim-milk culture medium, suggesting that the presence of protein induced the synthesis of protease. The protease produced by strain SF03 remained active over a pH range of 6.0–11.0 and a temperature range of 40–90°C, with an optimal pH of 8.0–9.0 and an optimal temperature of 70–80°C, respectively. The protease was stable over the temperature range of 40–70°C and retained 57 and 38% of its activity at 80 and 90°C, respectively, after 1 h.

Keywords Characterization · Extracellular · *Geobacillus caldoproteolyticus* · Thermoactive protease · Thermophilic

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Introduction

Proteases constitute one of the most important groups of enzymes produced commercially. They are widely applied in detergent, protein, brewing, meat, photographic, leather, dairy, and waste treatment industries, accounting for at least one quarter of the total worldwide sale of enzymes (Godfrey and West 1996; Anwar and Saleemuddin 1998).

Microorganisms are the largest source for proteases. Microorganisms produce both intracellular and extracellular proteases. Intracellular proteases play an important role in various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones, and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz 1988). Meanwhile, these extracellular proteases have also been exploited commercially for various industrial applications.

Thermoactive proteases are advantageous in many industrial applications, because higher processing temperatures can be employed with accelerated reaction rates, increased solubility of nongaseous reactants, and reduced incidence of microbial contamination from mesophilic organisms. Although there is no firm evidence to suggest that thermoactive proteases are necessarily derived from thermophilic microorganisms, there is a greater chance of encountering thermoactive proteases from thermophilic microorganisms. So far, a number of

thermoactive proteases from thermophilic microorganisms have been reported, including those from thermophilic bacterial species within the genera *Bacillus*, *Thermus* (Matsuzawa et al. 1983; Peek et al. 1992), and *Thermoactinomyces* (Tsuchiya et al. 1992; Lee et al. 1996) and those from thermophilic archaea species within the genera *Desulfurococcus* (Cowan et al. 1987), *Thermococcus* (Antranikian and Klingeberg 1991a), *Staphylothermus* (Antranikian and Klingeberg 1991b), and *Pyrococcus* (Eggen et al. 1990; Morikawa et al. 1994). Out of these, thermophilic members of the genus *Bacillus*, some of which have recently been transferred to a new genus *Geobacillus* (Nazina et al. 2001; Fortina 2001), have remained major sources of thermoactive proteases. Examples for such *Bacillus* and *Geobacillus* species include *Geobacillus stearothermophilus* (Dhandapani and Vijayaragavan 1994; Rahman et al. 1994), "*Bacillus caldolyticus*" (Heinen and Heinen 1972), and "*B. thermoproteolyticus*" (Voordouw and Roche 1975). In view of the remarkable industrial and commercial value of thermoactive proteases, the search for new microbial sources for thermoactive proteases is of continued value.

In the present study, we report on the isolation and characterization of a novel thermophilic bacterium with the ability to secrete a highly thermoactive protease. Furthermore, we also report on the factors affecting the protease production and some properties of the enzyme activity of industrial interest.

Materials and methods

Isolation and storage

An anaerobically treated and dewatered sewage sludge sample was collected from the Seletar Water Reclamation Plant, Singapore. A portion (1 g) of sewage sludge (wet weight) was aseptically mixed with 10 ml 1× PBS buffer, serially diluted, and plated onto nutrient agar (Difco Laboratories, USA) plates, tryptic soy agar (Difco Laboratories) plates and plate-count agar (Oxoid, UK) plates. The plates were incubated at 60°C for 2 days under aerobic conditions. Individual bacterial colonies were then transferred onto skim-milk agar plates (skim-milk powder, 10%; K_2HPO_4, 2.9 mM; $MgCl_2$, 5 mM; Bacto agar, 1.5%; pH 7.0) to detect their proteolytic activity. After 2 days of incubation at 60°C, halo-forming colonies were selected as protease-producing strains and further purified by repeated streaking onto fresh skim-milk agar plates. Strain SF03 was obtained after several such transfers. Purity was confirmed by microscopic examination, and purified cultures were preserved in tryptic soy broth (Difco Laboratories) with 25% sterilized glycerol at −80°C.

Morphological and phenotypic characterizations

Cell morphology was examined with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (Leica Stereoscan 420 scanning electron microscope, Cambridge Instruments). Cell size was determined by light microscopy of living cell preparations from a culture grown on tryptic soy agar for 16 h. For scanning electron microscopy preparation, cells were fixed for 4 h in 2% (v/v) glutaraldehyde, washed three times with 0.10 M sodium cacodylate buffer, and dehydrated with *T*-butyl alcohol of increasing concentration [50, 70, 85, 95, 100%, (v/v)]. Dehydrated cells were filtered through a 0.2-μm polycarbonate filter (Millipore, USA), dried with a freeze dryer (Bal-Tec CPC 030), sputter-coated with gold at 20 mA in a high-vacuum (2.8×10^{-6} Torr) and low-temperature (−170°C) cryochamber for 90 s, and then viewed with the scanning electron microscope at 20 kV.

Cell growth was monitored by measuring the OD_{600} on liquid media. The effect of temperature on the cell growth was tested using Luria-Bertani broth (Bacto peptone, 1%; yeast extract, 0.5%; NaCl, 0.5%) at pH 7.0. The effect of pH on the cell growth was determined using Luria-Bertani broth at 60°C. Media were adjusted to the initial pH (ranging from 5.0–10.0, with 0.5 increments) at room temperature with HCl or NaOH and then filter-sterilized. The effect of salinity on the cell growth was tested using Luria-Bertani broth containing different concentrations of NaCl (ranging from 0–3%, with 0.5% increments) at pH 7.0 and 60°C. Anaerobic growth was tested by incubation of the culture in 50-ml rubber-sealed screw-cap tubes containing Luria-Bertani broth medium at 60°C. Anaerobic techniques were followed in the medium preparation and aseptic handling of the culture with the aid of an anaerobic chamber (Model Bactron I, SHELLAB, USA). For all the growth tests, growth was considered to have occurred when the observed OD_{600} value exceeded twice the initial value.

Enzyme profiles and carbon substrate utilization characteristics were determined using the API ZYM and API 20E assays according to the manufacturer's instructions (BioMérieux, France) at the testing temperature of 60°C. API ZYM and API 20E strips were sealed in a zip bag to prevent the strips from drying out in the course of incubation. Meanwhile, growth tests using the mineral source medium (Bushnell-Haas broth medium, Difco Laboratories, USA) supplemented with 0.5% (w/v) of different carbohydrate substrates as sole carbon source were performed to validate the API 20E results. Acid production from carbohydrates was determined by measuring the change in pH after 72 h growth. Gram staining was performed using a Gram-stain kit (Difco Laboratories, USA) according to the manufacturer's instructions.

16S rRNA gene sequencing and phylogenetic analyses

The nearly full-length 16S rRNA gene was amplified by PCR with forward primer Eubac27F and reverse primer Universal 1492R1 (Lane 1991). The PCR products were

purified with a QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. A 16S rRNA gene sequence of 1,506 nucleotide bases was obtained in both directions using the ABI model 310A DNA sequencer (Applied Biosystems–PerkinElmer) and the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit (Applied Biosystems, PerkinElmer). Sequence alignments were performed manually with the software BioEdit (Hall 1999). The 16S rRNA secondary structure of strain SF03 was constructed manually with templates from the Ribosomal Database Project (Maidak et al. 2000) to aid in the identification of homologous sequence positions. The final data set included 1,332 bp after discarding positions of uncertain alignment between strain SF03 and the reference sequences. The PHYLIP software package (Felsenstein 1989) was used to construct distance trees (neighbor joining and Fitch) and maximum likelihood trees (DNAML). Evolutionary distance bootstrap values were determined using the SEQBOOT, DNADIST, FITCH, and CONSENSE programs in PHYLIP.

Extracellular protease assay

Cell-free culture supernatants were assayed for extracellular protease activity, using a modification of the method described by Smiber and Krieg (1994). Azocasein (5 mg/ml) dissolved in Tris-HCl (125 mM, pH 8.0) containing 0.04% NaN_3 was used as the substrate for protease reaction. The cell-free culture supernatant was prepared by centrifugation of cell suspension (13,200 rpm, 2 min), followed by filtration of the supernatant through a 0.2-μm cellulose–acetate filter. Filtered supernatant (300 μl) was incubated with 300 μl of azocasein solution at 60°C (unless otherwise indicated). A preliminary study had established the enzyme reaction time of 60 min in which first-order reaction kinetics was observed under the described conditions. The reaction was terminated by addition of 600 μl trichloroacetic acid (10% w/v). The mixture was allowed to stand for 10 min on ice, followed by centrifugation at 13,200 rpm at 4°C for 6 min to remove unreacted azocasein. The supernatant containing azopeptides (1,100 μl) was then neutralized by adding 100 μl 1.8 N NaOH. The absorbance of the azopeptide supernatant was measured at 442 nm (A_{442}), using a Shimadzu UV-1201 V spectrophotometer. Controls were prepared by the same procedure, except that the trichloroacetic acid was added before the enzyme. One unit of protease activity was defined as the amount that yielded an increase in A_{442} of 0.01 in 1 h. Each determination was performed in triplicate.

Effect of culture medium on cell growth and extracellular protease production

The effect of culture medium on the cell growth and extracellular protease production of strain SF03 was investigated using a basal medium (Bacto peptone, 1.5%; K_2HPO_4, 2.9 mM; $MgCl_2$, 5 mM—hereinafter referred as BM) supplemented with different additional carbon sources [glucose, sucrose, lactose (0.5%)] or nitrogen sources (casamino acids, 0.5%; NH_4Cl, 50 mM) in 250-ml Erlenmeyer flasks. At the same time, the kinetics of cell growth and extracellular protease production of strain SF03 on a 10% skim-milk medium (skim-milk powder, 10%; K_2HPO_4, 2.9 mM; $MgCl_2$, 5 mM; pH 7.0) were also studied. Cell growth was monitored by OD_{600} measurement or by a plate-count method, using tryptic soy agar plates incubated at 60°C for 24 h. Our preliminary study has shown a good correlation between the OD_{600} measurement and the plate-count measurement when OD_{600} value was not higher than 0.8 unit, except for the case of the skim-milk medium (data not shown). Protease activity was assayed as described above at pH 8.0 and 60°C. *Bacillus licheniformis* ATCC 12759 (BBL, QualiSwab, Becton Dickinson Microbiology Systems) was used as a reference in protease-production test. Culture supernatants of *B. licheniformis* ATCC 12759, grown on the BM or the skim-milk medium at 37°C for 24 h, were assayed for protease activity using the method described above at pH 8.0 and 37°C.

Nucleotide sequence accession numbers

The sequence for strain SF03 has been deposited in the GenBank database under accession number AY327448. The GenBank accession numbers of the other sequences used in the phylogenetic analyses are as follows: "*Bacillus caldolyticus*", Z26924; *Geobacillus thermoleovorans*, Z26923; *B. vulcani*, AJ293805; *G. stearothermophilus*, AJ294817; *G. uzenensis*, AF276305; *G. thermocatenulatus*, Z26926; *G. thermodenitrificans*, Z26928; *G. subterraneus*, AF276307; *G. thermoglucosidasius*, AB021197; *G. caldoxylosilyticus*, AJ489326; *Saccharococcus thermophilus*, X70430; *Anoxybacillus flavothermus*, AF001964; *B. thermoalkalophilus*, Z26931; *B. pallidus*, Z26930; *B. thermoamylovorans*, L27478; *B. coagulans*, D16267; *B. smithii*, Z26935; *B. infernus*, U20384; and *Staphylococcus gallinarum*; D83366.

Results

Isolation of strain SF03

Ten thermophilic bacteria with the ability to produce extracellular proteases were isolated from the sewage sludge sample. Out of them, strain SF03 exhibited the highest extracellular protease activity. Strain SF03 could produce 56.0 U/ml of extracellular protease when it was grown on the skim-milk medium. The remaining isolates, which were closely related to *Geobacillus stearothermophilus* by partial 16S rRNA gene sequencing, produced the extracellular proteases ranging from 2.7 to

18.3 U/ml under the same conditions. Therefore, strain SF03 was selected for further characterization.

Colony and cell morphology

After 1 day of incubation on tryptic soy agar at 60°C, strain SF03 formed round, smooth, mucoid, and colorless colonies with diameters of 5–7 mm. Cells were rod-shaped, Gram stain-positive, motile (in the exponential growth phase), and endospore forming. Cells were between 3.0 and 6.0 μm in length and between 0.5 and 0.8 μm in diameter when grown on tryptic soy agar plates at 60°C for 16 h (Fig. 1). Spores were ellipsoidal and located terminally. The typical *Bacillus* life cycle (Sneath 1986), in which vegetative cells could form endospores, which then could grow vegetatively again, was observed for strain SF03 on Luria-Bertani broth medium at 60°C.

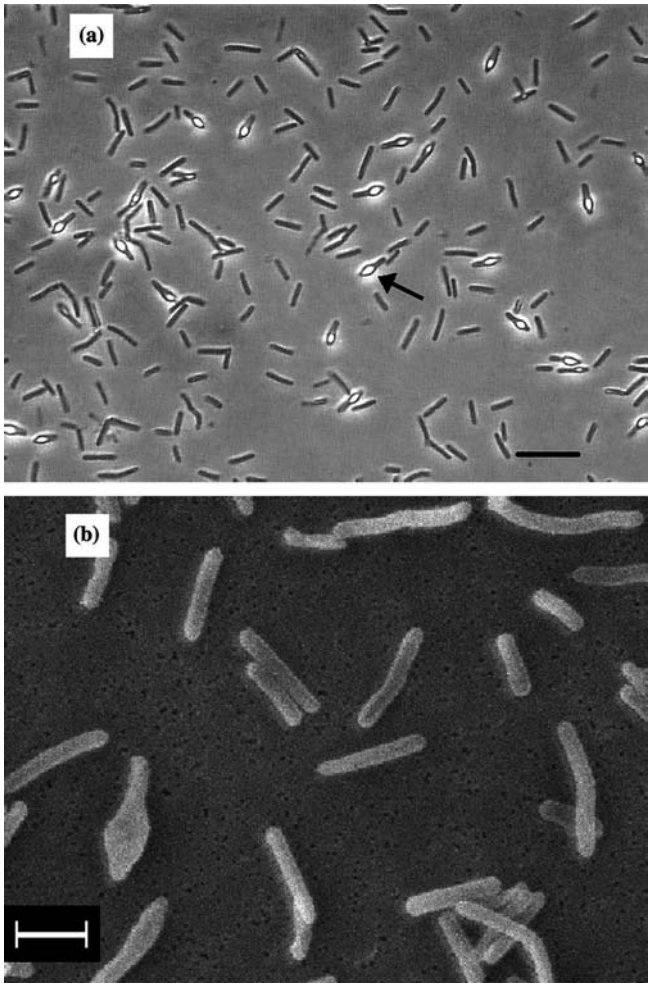


Fig. 1 **a** Phase contrast micrograph (*bar* 10 μm); and **b** scanning electron micrograph (*bar* 2 μm) of strain SF03 grown at 60°C for 16 h on tryptic soy agar. *Arrow* Endospores were located terminally

Physiological and biochemical properties

Strain SF03 was a moderate thermophile that could grow at temperatures ranging from 35–65°C, with an optimum growth temperature of 60°C. Strain SF03 was able to grow over the pH range of 6.0–9.0 and the salinities range of 0–2.5%, with an optimum pH of 6.5 and an optimum salinity of 0–0.5%, respectively. At values outside the mentioned ranges, no growth was observed. Strain SF03 was a strictly aerobic organism, and anaerobic growth was not detected.

The following enzymatic reactions were positive for strain SF03 at 60°C: catalase, oxidase, alkaline phosphatase, acid phosphatase, esterase, esterase lipase, arginine dihydrolase, ornithine decarboxylase, and naphthol-AS-BI-phosphohydolase. The following enzymatic reactions were weak for strain SF03 at 60°C: lipase, leucine acrylamidase, α-glucosidase, and α-chymotrypsin. The following enzymatic reactions were negative for strain SF03 at 60°C: valine acrylamidase, cystine acrylamidase, lysine decarboxylase, tryptophane deaminase, trypsin, urease, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Strain SF03 was able to utilize glucose, mannitol, sucrose, glycerol, starch, casein, gelatin, and citrate. Acid production from glucose and glycerol was observed. The Voges–Proskauer test was positive for the production of acetoin. The substrates that were not utilized by strain SF03 included inositol, sorbitol, rhamnose, melibiose, amygdalin, and arabinose. Utilization of lactose was weak. Tests of H_2S production, indole production, and nitrate reduction were negative. Table 1 summarizes part of the physiological and biochemical properties of strain SF03 together with its closest relatives.

16S rRNA gene sequence analysis

A nearly complete 16S rRNA gene sequence of 1,506 nucleotide bases was determined for strain SF03. Distance and maximum likelihood analyses were performed based on 1,332 unambiguous nucleotide bases. It was found that the topologies near strain SF03 estimated from the distance analyses (neighbor joining and Fitch) and the maximum likelihood analysis were essentially consistent. In all cases, strain SF03 was placed as a peripheral member of the thermophilic *Bacillus* group 5 (Ash et al. 1991). A phylogenetic tree obtained using the maximum likelihood algorithm is shown in Fig. 2. The relationship between strain SF03 and the *Bacillus* group 5 was supported by a high bootstrap value of 85%. The closest phylogenetic neighbors of strain SF03 were *Saccharococcus thermophilus* (97.6% sequence identity) and *Geobacillus caldoxylosilyticus* (97.5% sequence identity), followed by *G. thermoglucosidasius* (97.2% sequence identity). Lower sequence identities were found to other members of *Bacillus* group 5, ranging from 95.4– 96.1%.

Table 1 Phenotypic characteristics of strain SF03 and some related thermophilic species. Data were obtained from this study or from Nystrand (1984) (*Saccharococcus thermophilus*), Ahmad et al. (2000) and Fortina et al. (2001) (*Geobacillus caldoxylosilyticus*) and Suzuki et al. (1983) (*G. thermoglucosidasius*). All isolate and reference strains were positive for Gram staining, catalase, and utilization of glucose and sucrose. They were all negative for the indole-production test. + Growth/activity observed, - no growth/activity observed, *w* weak growth/activity, *ND* not determined, *v* variable within the group

Character	Strain SF03	*S. thermophilus*	*G. caldoxylosilyticus*	*G. thermoglucosidasius*
Cell morphology	Rods	Cocci/irregular clusters	Rods	Rods
Spore formation	+	–	+	+
Growth in the presence of 3% NaCl	–	ND	–	–
Starch hydrolysis	+	–	+	+
Casein hydrolysis	+	ND	+	+
Citrate	+	–	+	–
Anaerobic growth	–	–	w	–
Reduction				
NO_3^-–NO_2^-	–	v	+	+
NO_2^-–gas	–	–	v	–
Urease	–	–	–	+
Utilization				
Glycerol	+	+	+	–
Lactose	w	–	+	–
Arabinose	–	+	+	–
Rhamnose	–	+	v	+
Sorbitol	–	+	+	–

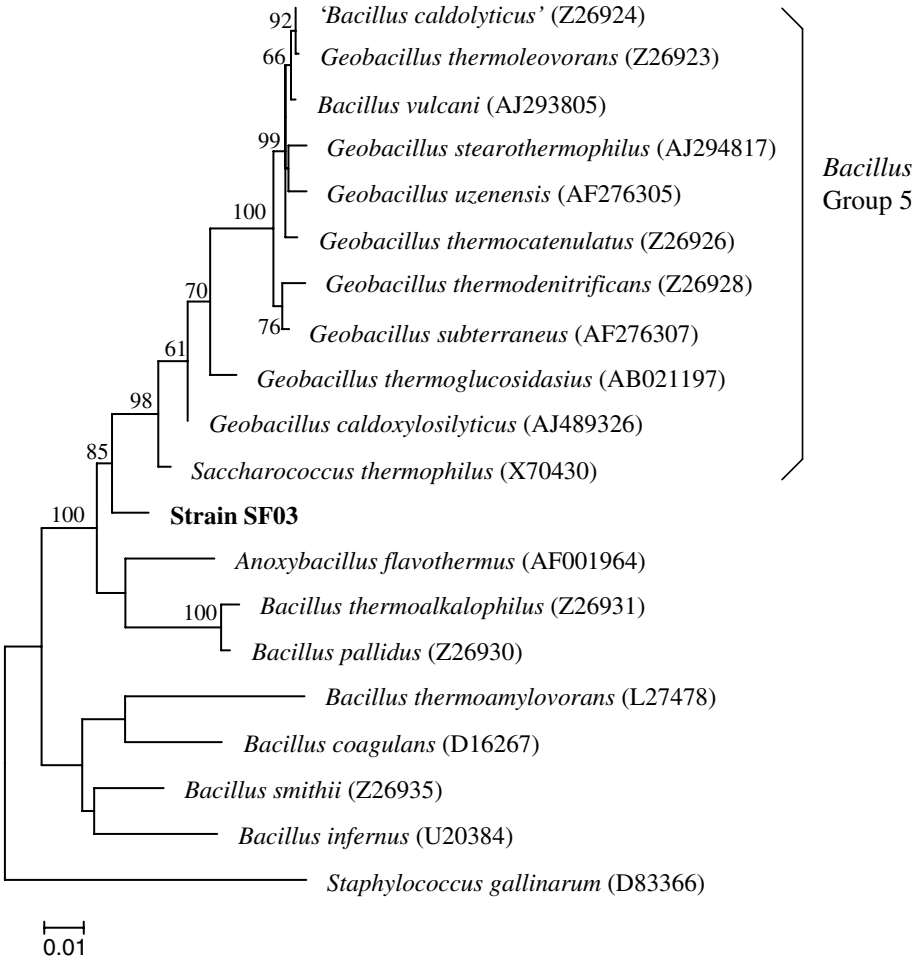


Fig. 2 16S rRNA phylogenetic tree for strain SF03 calculated by the maximum likelihood methods. The *numbers* at the branch nodes are bootstrap values based on 100 resamplings. Only bootstrap values greater than 60% are shown. *Scale bar* Nucleotide divergence of 1%

Effect of culture medium on the growth and extracellular protease production

Strain SF03 grew on the BM, in which peptone was used as a sole carbon and nitrogen source (Fig. 3). The specific growth rate on the BM was 1.48 h^{-1} (Table 2). Supplementation with sugars to the BM accelerated the cell growth, as evidenced by the reduced lag phase. However, the specific growth rate was significantly decreased. When NH_4Cl was supplemented, both cell yield and specific growth rate increased considerably. Protease production by strain SF03 was detected on the BM. The specific protease production was repressed by the supplementation with glucose and lactose but was enhanced by the supplementation with sucrose. Casamino acids also repressed the protease production, whereas NH_4Cl enhanced the protease production remarkably.

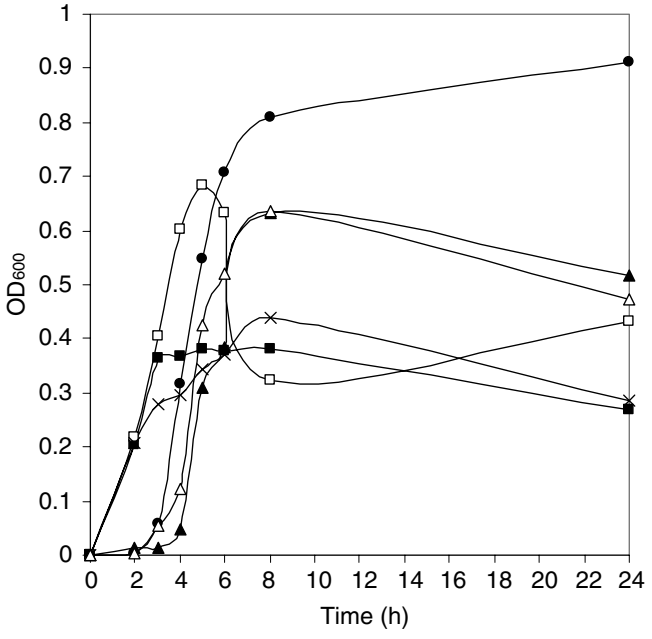


Fig. 3 Growth curves of strain SF03 on the basal medium [(BM) *white triangles*]; the BM supplemented with glucose (*black squares*); the BM supplemented with lactose (*white squares*); the BM supplemented with sucrose (*black x's*); the BM supplemented with NH_4Cl (*black circles*); and the BM supplemented with casamino acids (*black triangles*) at 60°C. Inoculum was prepared using an overnight culture grown on the BM

The cell growth and protease production were significantly enhanced when strain SF03 was cultivated on a 10% skim-milk culture medium (Table 2). The cell concentration on the skim-milk medium reached a value of 1.22×10^8 CFU/ml at 24 h of growth, which was one to two orders higher than those on the other culture media under investigation. The specific protease production on the skim-milk medium was more than twice that on the BM. Protease activity was first detected after approximately 5 h of incubation. It reached a maximum level at 9 h of incubation and leveled off in the following period. In comparison with growth, the protease activity appeared in the late exponential growth phase and reached the maximum level in the stationary growth phase (Fig. 4).

By comparison, *Bacillus licheniformis* ATCC 12759 produced a higher total protease activity but a lower specific protease activity than strain SF03 on the BM (Table 2). However, for the skim-milk medium cultivation, both the total and the specific protease yields by *B. licheniformis* ATCC 12759 were significantly lower than those by strain SF03.

pH, temperature and thermostability profile of protease

The protease activity of strain SF03 was evaluated at various pH values and various temperatures. The protease activity remained high over the entire pH range

Table 2 Effect of culture medium on the cell growth and the protease production of strain SF03 and the reference strain *Bacillus lichenifiormis* ATCC 12759

	Specific growth rate (h^{-1})[a]	Cell yield (CFU/ml)	Protease (U/ml)	Specific protease production (U/ml)/(CFU[10^8]/ml)	Relative protease activity (%)[b]
Strain SF03[c]					
BM[d]	1.48	1.55×10^6	0.3	21.51	100
BM + glucose	0.59	8.81×10^5	ND	0	0
BM + lactose	0.51	1.61×10^6	0.7	13.80	64
BM + sucrose	0.30	9.29×10^5	0.3	35.9	167
BM + NH_4Cl	2.08	2.14×10^7	6.8	31.67	147
BM + casamino acids	1.52	1.68×10^6	0.1	6.61	31
Skim-milk medium	2.23	1.22×10^8	56.0	45.9	213
B. licheniformis ATCC 12759[e]					
BM	–	1.86×10^8	6.7	3.6	–
Skim-milk medium	–	2.28×10^8	6.5	2.9	–

[a]Specific growth rate was determined during the exponential growth phase

[b]Relative protease activity in each medium expressed in relation to the activity in the BM (100%)

[c]Cells were grown at 60°C for 24 h. Protease activity was assayed at pH 8.0 and 60°C, as described in "Materials and methods"

[d]*BM* Basal medium

[e]Cells were grown at 37°C for 24 h. Protease activity was assayed at pH 8.0 and 37°C, as described in "Materials and methods"

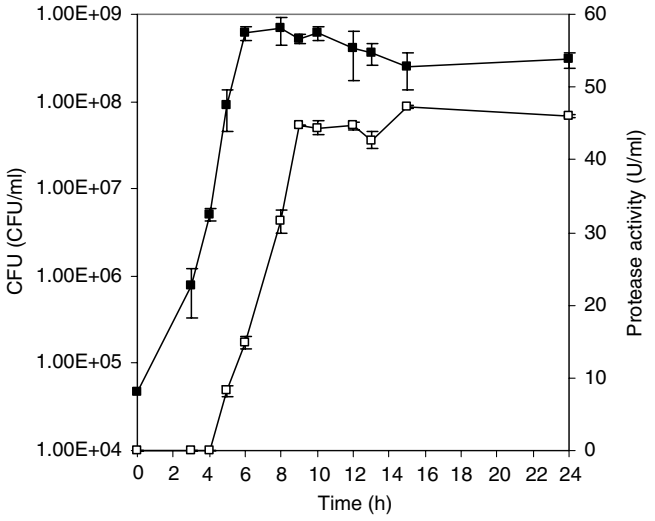


Fig. 4 Time courses of cell growth (*black squares*) and protease production (*white squares*) of strain SF03 on the skim-milk medium at 60°C. The data are averages of three determinations. The standard deviations are represented by *error bars*

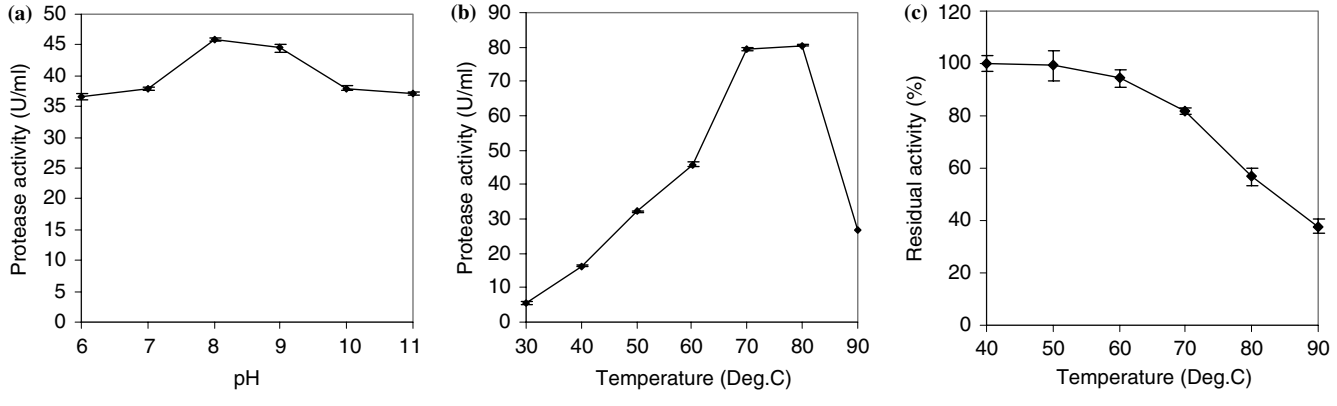
under investigation, i.e., pH 6.0–11.0 (Fig. 5a). The optimal pH was 8.0–9.0. The protease was also active over a wide range of temperatures varying from 40–90°C (Fig. 5b). Very little protease activity was detected at 30°C. The optimal temperature was 70–80°C. At 90°C, the protease retained an approximately one third of its maximal activity.

The thermostability of the protease was examined by measuring the residual activities after incubation of the crude extracellular enzyme solution at various temperatures between 40 and 90°C for 60 min (Fig. 5c). The protease retained more than 80% of the initial activity at temperatures up to 70°C, and 57% of the initial activity at 80°C. About 60% of protease activity was lost at 90°C.

Fig. 5 pH (**a**), temperature (**b**), and thermostability (**c**) profile of protease. Protease samples were obtained from the stationary growth phase cultures (24 h) on the skim-milk medium. For pH profile, phosphate buffer (100 mM) was used over the pH range of 6.0–7.0, while Tris-HCl buffer (125 mM) was used over the pH range of 8.0–11.0. Enzymatic reaction temperature was maintained at 60°C; for temperature profile, enzymatic reaction pH was maintained at pH 8.0 using Tris-HCl buffer (125 mM); for thermostability profile, protease was preincubated for 60 min at various temperatures between 40 and 90°C. Heating of the enzyme was stopped by cooling on ice. Residual activities were measured using the method described in "Materials and methods" at 60°C in 125 mM Tris-HCl buffer (pH 8.0). The enzymatic activity of the unheated enzyme was taken as 100%. All the data are averages of three determinations. The standard deviations are represented by *error bars*

Discussion

In the screening program to obtain thermoactive extracellular protease producers, we have isolated a thermophilic bacterial strain, SF03, from sewage sludge in Singapore. Phylogenetic analyses placed strain SF03 as a peripheral member of the thermophilic *Bacillus* rRNA group 5. *Bacillus* group 5 is a phenotypically and phylogenetically coherent group of thermophilic bacilli, displaying very high similarity among its 16S rRNA sequences (Ash et al. 1991; Rainey et al. 1994). This group comprises the established species of thermophilic bacilli (*B. stearothermophilus*, *B. thermocatenulatus*, *B. thermoleovorans*, *B. kaustophilus*, *B. thermoglucosidasius*, *B. thermodenitrificans*, and *B. thermantarcticus*), the species that have not been validly published ("*B. caldolyticus*", "*B. caldotenax*", and "*B. caldovelox*") and a nonsporulating species, *S. thermophilus* (Ash et al. 1991; Rainey et al. 1994; Sunna et al. 1997; Manachini et al. 2000). Recently, based on phylogenetic and fatty acid analyses, the validly described *Bacillus* species of group 5 have been transferred to a new genus, *Geobacillus*, which was created with two new species, *G. subterraneus* and *G. uzenensis* (Nazina et al. 2001). Subsequently, another species in the genus *Geobacillus*, namely *G. caldoxylosilyticus*, was proposed (Ahmad 2000; Fortina 2001). Our phylogenetic analyses revealed that SF03 shared a common ancestry with the members of the genus *Geobacillus* and other members of *Bacillus* group 5, as well as an even closer relationship with *S. thermophilus* (97.6% sequence identity), *G. caldoxylosilyticus* (97.5% sequence identity), and *G. thermoglucosidasius* (97.2% sequence identity). *S. thermophilus* is a



morphologically distinct species as compared to other members of *Bacillus* group 5. It is a nonsporulating coccus. Phylogenetic study of *S. thermophilus* (Nystrand 1984; Rainey and Stackebrandt 1993) has suggested that it is closely related to *Bacillus* species and can be considered as an offshoot in *Bacillus* group 5. Despite the slightly closer phylogenetic relatedness to *S. thermophilus*, strain SF03 exhibited the morphological and biochemical features typical of other species of the *Geobacillus* genus, e.g., sporulating rod-shaped morphology and thermophilic growth. However, strain SF03 also exhibited unique taxonomic features that can be clearly distinguished from its closest *Geobacillus* relatives (Table 1). Unlike *G. caldoxylosilyticus*, strain SF03 could not denitrify and grow under anaerobic conditions and had a narrower spectrum of substrate utilization. When compared with *G. thermoglucosidasius*, strain SF03 is also phenotypically different based on urease production and the utilization of citrate, glycerol, and rhamnose. Because strain SF03 has at least two differentiating phenotypic characteristics and a divergent 16S rDNA sequence, it can be considered a new species (Stackebrandt and Goebel 1994; Ursing et al. 1995). We therefore propose the creation of a new species, *G. caldoproteolyticus* sp. nov. for strain SF03.

In the study of the extracellular protease production from strain SF03, it was found that the presence of glucose completely inhibited the protease synthesis. This is probably due to the catabolic repression mechanism that has also been reported for *Pseudomonas maltophilia* (Boethling 1975), *Staphylococcus aureus* (Yoshikawa 1974), and *Yersinia ruckeri* (Secades and Guijarro 1999), suggesting that in the absence of glucose, the protease plays a role in supplying peptides or amino acids as the carbon or energy source in addition to being a nitrogen source. Consequently, protease synthesis could be repressed when the energy status of the cells is high in the presence of glucose. Supplementation of casamino acids to the BM also repressed the protease production, probably because of the end-product inhibition. Supplementation of NH_4Cl, however, enhanced the protease production, which is in agreement with the earlier report on a *B. licheniformis* strain (Ferrero et al. 1996). When cultivated on a 10% skim-milk culture medium, SF03 exhibited a significantly increased protease activity. This suggests that the presence of milk components, most likely the proteins such as caseinogen, could induce the synthesis of extracellular protease. A similar induction effect caused by the protein substrates, e.g., casein and mucus, has also been observed on a *B. licheniformis* strain (Ferrero et al. 1996) and a *Vibrio anguillarum* strain (Denkin and Nelson 1999).

G. stearothermophilus and *B. licheniformis* are two important microbial sources for commercial protease production (Anwar and Saleemuddin 1998; Kumar and Takagi 1999). In the present study, it is interesting to find that strain SF03 yielded a higher level of extracellular protease when compared to the *G. stearothermophilus*-like isolates and *B. licheniformis* ATCC 12759. The protease produced by strain SF03 was active over a broad temperature range, with approximately one third of its maximal activity retained at 90°C. The optimal temperature was 70–80°C; however, stability was favored at lower temperatures (40–70°C). At 90°C, a lower stability of the protease was observed. It merits mentioning here that the lower stability of the protease at 90°C might lead to an underestimation of the protease activity due to the possible inactivation of the protease in the assay. The protease was also active over a broad pH range of 6.0–11.0, though such a broad pH response seems to be quite unusual. As the enzyme samples under study were merely crude enzyme solutions, it is likely that more than one protease might be present and therefore result in the unusually broad pH response. To better understand this, isolation and purification of the protease(s) are necessary for further detailed characterization.

Given the high thermoactivity and thermostability of the extracellular protease, in addition to the high protease yield, the newly isolated thermophilic strain SF03 could be a good candidate as a thermoactive protease producer for various biotechnological applications. For instance, it could be used as a protein degrader to bioaugment waste treatment processes, e.g., composting processes. Alternatively, its extracellular protease could also be purified and used when high protease activities at high temperatures are desired, e.g., for the detergents for high-temperature uses.

Description of *Geobacillus caldoproteolyticus* sp. nov.

- *Geobacillus caldoproteolyticus* (cal.do.pro.teo.ly'ti.cus. L. adj. *caldus* hot; M.L. neut. N. *proteo* protein; M.L. adj. *lyticus* dissolving, degrading; *caldoproteolyticus* hot and protein degrading).
- Strictly aerobic, Gram stain-positive rods. 3.0–6.0×0.5–0.8 μm. Terminal endospore ellipsoidal. Colonies round, smooth, mucoid, and colorless.
- Catalase and oxidase positive.
- Growth at temperatures between 35 and 65°C, pH between 6.0 and 9.0 and salinities between 0 and 2.5%.
- Able to utilize glucose, mannitol, sucrose, glycerol, starch, casein, gelatin, and citrate. Poor growth on lactose.
- Acid production from glucose and glycerol.
- Nitrate reduction test negative. Voges–Proskauer test positive. H_2S-production and indole-production tests negative.
- The type strain, *G. caldoproteolyticus* SF03, was isolated from sewage sludge from Seletar Water Reclamation Plant in Singapore. It has been deposited in the American Type Culture Collection (ATCC) under number ATCC BAA-818 and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under number 15730.

References

Ahmad S, Scopes RK, Rees GN, Patel BKC (2000) *Saccharococcus caldoxylosilyticus* sp. nov., an obligately thermophilic, xylose-utilizing, endospore-forming bacterium. Int J Syst Evol Microbiol 50:517–523

Antranikian G, Klingeberg M (1991a) Thermostable protease from *Thermococcus*. PCT Patent Appl WO 9119792

Antranikian G, Klingeberg M (1991b) Thermostable protease from *Staphylothermus*. PCT Patent Appl WO 9119791

Anwar A, Saleemuddin M (1998) Alkaline proteases: a review. Bioresource Technol 64:175–183

Ash C, Farrow JAE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett Appl Microbiol 13:202–206

Boethling RS (1975) Regulation of extracellular protease secretion in *Pseudomonas maltophilia*. J Bacteriol 123:954–961

Cowan DA, Smolenski KA, Daniel RM, Morgan HW (1987) An extremely thermostable extracellular proteinase from a strain of the archaebacterium *Desulfurococcus* growing at 88°C. Biochem J 247:121–133

Denkin SM, Nelson DR (1999) Induction of protease activity in *Vibrio anguillarum* by gastrointestinal mucus. Appl Environ Microbiol 65:3555–3560

Dhandapani R, Vijayaragavan R (1994) Production of a thermophilic extracellular alkaline protease by *Bacillus stearothermophilus* AP-4. World J Microbiol Biotechnol 10:33–35

Eggen R, Geerling A, Watts J, de Vos WM (1990) Characterization of pyrolysin, a hyperthermoactive serine protease from the archaeobacterium *Pyrococcus furiosus*. FEMS Microbiol Lett 71:17–20

Felsenstein J (1989) PHYLIP (PHYlogenetic Inference Package). Cladistics 5:164–166

Ferrero MA, Castro GR, Abate CM, Baigorí MD, Siñeriz F (1996) Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: isolation, production and characterization. Appl Microbiol Biotechnol 45:327–332

Fortina MG, Mora D, Schumann P, Parini C, Manachini PL, Stackebrandt E (2001) Reclassification of *Saccharococcus caldoxylosilyticus* as *Geobacillus caldoxylosilyticus* (Ahmad et al. 2000) comb. nov. Int J Syst Evol Microbiol 51:2063–2071

Godfrey T and West S (1996) Introduction to industrial enzymology. In: Godfrey T, West S (eds) Industrial enzymology, 2nd edn. Macmillan, London, pp 1–8

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Heinen UJ, Heinen W (1972) Characteristics and properties of a caldoactive bacterium producing extracellular enzymes and two related strains. Arch Mikrobiol 82:1–23

Kalisz HM (1988) Microbial proteinases. Adv Biochem Eng Biotechnol 36:1–65

Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561–594

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175

Lee JK, Kim YO, Kim HK, Park YS, Oh TK (1996) Purification and characterization of a thermostable alkaline protease from *Thermoactinomyces* sp. E79 and the DNA sequence of the encoding gene. Biosci Biotechnol Biochem 60:840–846

Maidak BL, Cole JR, Lilburn TG, Parker CT Jr, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen GJ, Pramanik S, Schmidt TM, Tiedje JM (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res 28:173–174

Manachini PL, Mora D, Nicastro G, Parini C, Stackebrandt E, Pukall R, Fortina MG (2000). *Bacillus thermodenitrificans* sp. nov., nom. rev. Int J Syst Evol Microbiol 50:1331–1337

Matsuzawa H, Hamaoki M, Ohta T (1983) Production of thermophilic extracellular proteases (aqualysins I and II) by *Thermus aquaticus* YT-1. Agric Biol Chem 47:25–28

Morikawa M, Izawa Y, Rashid N, Hoaki T, Imanaka T (1994) Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl Environ Microbiol 60:4559–4566

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV (2001) Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. Int J Syst Evol Microbiol 51:433–446

Nystrand R (1984) *Saccharococcus thermophilus* gen. nov., sp. nov., isolated from beet sugar extraction. Syst Appl Microbiol 5:204–219

Peek K, Daniel RM, Monk C, Parker L, Coolbear T (1992) Purification and characterization of a thermostable proteinase isolated from *Thermus* sp. strain Rt41A. Eur J Biochem 207:1035–1044

Rahman RNZA, Razak CN, Ampon K, Basri M, Yunus WMZW, Salleh AB (1994) Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. Appl Microbiol Biotechnol 40:822–827

Rainey FA, Stackebrandt E (1993) Phylogenetic evidence for the relationship of *Saccharococcus thermophilus* to *Bacillus stearothermophilus*. Syst Appl Microbiol 16:224–226

Rainey FA, Fritze D, Stackebrandt E (1994). The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. FEMS Microbiol Lett 115:205–211

Secades P, Guijarro JA (1999) Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. Appl Environ Microbiol 65: 3969–3975

Smiber RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, pp 607–654

Sneath PHA (1986) Endospore-forming Gram-positive rods and cocci. In: Sneath PHA, Mair NS, Sharp ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1104–1139

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Sunna A, Tokajian S, Burghardt J, Rainey F, Antranikian G, Hashwa F (1997) Identification of *Bacillus kaustophilus*, *Bacillus thermocatenulatus* and *Bacillus* strain HSP as members of *Bacillus thermoleovorans*. Syst Appl Microbiol 20:232–237

Suzuki Y, Kishigami T, Inoue K, Mizoguchi Y, Eto N, Takagi M, Abe S (1983) *Bacillus thermoglucosidasius* sp. nov., a new species of obligately thermophilic bacilli. Syst Appl Microbiol 4:487–495

Tsuchiya K, Nakamura Y, Sakashita H, Kimura T (1992) Purification and characterization of thermostable alkaline protease from alkalophilic *Thermoactinomyces* sp HS682. Biosci Biotechnol Biochem 56:246–250

Ursing JB, Rosselló-Mora RA, García-Valdés E, Lalucat J (1995) Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. Int J Syst Bacteriol 45:604

Voordouw G, Roche RS (1975) The role of bound calcium ions in thermostable, proteolytic enzymes. II. Studies on thermolysin, the thermostable protease from *Bacillus thermoproteolyticus*. Biochemistry 14:4667–4673

Yoshikawa M, Matsuda F, Naka M, Murofushi E, Tsunematsu. J (1974) Pleiotropic alterations of activities of several toxins and enzymes in mutants of *Staphylococcus aureus*. J Bacteriol 119:117–122