Phenotypic and Genotypic Characterization of Some Thermophilic Species of *Bacillus*

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Some thermophilic species of *Bacillus* were characterized using biochemical tests, antibiotic sensitivity, bacteriocin and bacteriophage sensitivity, esterase patterns, DNA hybridization and % G+C content. The three caldo-active strains of bacilli isolated by Heinen (1971) were compared with strains of *B. stearothermophilus*. Eight of the strains examined showed characteristics which enabled them to be placed in the three main taxonomic groups suggested by Walker & Wolf (1971). Two strains showed characteristics of both groups 1 and 3. Examination of genotype data showed taxonomic groupings which differed from those based on phenotypic characterization.

INTRODUCTION

Over the past decade there has been a considerable increase in interest in the thermophilic bacteria and their enzymes (Brock & Freeze, 1969; Atkinson, 1973; Singleton & Amelunxen, 1973; Jung *et al.*, 1974; Williams, 1975; Atkinson *et al.*, 1975). We have examined the taxonomic position and relationships between strains of *Bacillus stearothermophilus*, *B. coagulans*, the caldo-active bacteria isolated by Heinen (1971) and the thermophile isolated by Epstein & Grossowicz (1969). The latter was at first identified as a subspecies of *B. coagulans* but later as a starch-negative strain of *B. stearothermophilus* (Cazzulo *et al.*, 1969, 1970; Atkinson *et al.*, 1975). Three other thermophilic bacilli isolated in this laboratory were also examined.

METHODS

Strains and growth. Strains of *Bacillus stearothermophilus* used were: ATCC 12016; NCA 1503 (NCIB 8924); strain 10, from Dr N. Welker, North Western University, Ill., U.S.A.; strain RS93, isolated from the hot water supply to the fermentation plant at the Microbiological Research Establishment, Porton, Salisbury (Sharp *et al.*, 1979); strain 240, isolated from chalky soil in the Salisbury area; strain 262, isolated from an Icelandic hot water spring; and strain 136, isolated by Epstein & Grossowicz (1969). Cultures of *B. caldodenax*, *B. caldovelox* and *B. caldolyticus* were from Dr W. Heinen, University of Nijmegen, Holland. *Bacillus coagulans* ATCC 8038, *B. coagulans* ATCC 12245, *B. subtilis* 168 (Trp⁻), *B. amyloliquefaciens* H1, *B. megaterium* KM and *Escherichia coli* K12 (Hfr) were also used.

All strains were normally stored as lyophilized cultures and routinely grown on Tryptone Soya Agar (TSBA) (Oxoid CM131). The liquid media used were as described by Sargeant *et al.* (1971).

Bacteriophages. Bacteriophages TP1C and TP84 were from Dr N. Welker. Bacteriophage ATCC 12016b was also used. Bacteriophages JS017, JS020 and JS025 were isolated from compost samples taken from the Salisbury area.

Biochemical tests. Sugars in basal peptone water were inoculated from a 6 h peptone water broth culture derived from an overnight plate culture. Citrate utilization was tested on Simmon's citrate slopes. Gelatin liquefaction, starch hydrolysis, casein hydrolysis, nitrate and nitrite reduction, indole production, acetoin production, growth in 0.02 % NaN₃, catalase production and oxidase reaction were all examined by the

methods of Cowan & Steel (1974). Tolerance to saline was determined by inoculating bottles containing 10 ml nutrient broth no. 2 (Oxoid CM67) supplemented with 1 to 10 % (w/v) NaCl. Sabouraud dextrose agar plates were based on Oxoid agar (CM41). Readings were made at 1, 2 and 3 d.

Antibiotic sensitivity. Cultures were grown in peptone water broth for 6 h. Disposable bioassay plates were prepared with 200 ml Oxoid Sensitest agar (CM409) with agar at 3 % (w/v). The plates were seeded with 3 ml culture, and allowed to dry. Oxoid antibiotic sensitivity discs (15) were then placed on them and the plates were incubated overnight at 60 °C. The zone width from the edge of the disc to the edge of the bacterial growth was then measured.

Temperature range for growth. Cultures were streaked on to plates of TSBA and incubated at a range of temperatures; above 50 °C, plates were incubated in closed polythene bags; above 65 °C, glass Petri dishes were used. The plates were examined daily up to 3 d.

Bacteriocin sensitivity. A crude preparation of thermocin 93 (Sharp *et al.*, 1979) was used. Growth inhibition was observed on agar plates using the well diffusion method.

Bacteriophage sensitivity. Phages were harvested after growth on *B. caldodenax* and titres were adjusted to 10^6 plaque forming units (p.f.u.) ml⁻¹. Dilutions of the bacteriophage were mixed with 0.1 ml of a 6 h culture of the bacteria in 2 ml of Trypticase (BBL) soft agar (0.6 %, w/v) containing 0.015 M-CaCl₂ at 50 °C. After mixing, the soft agar was poured over the surface of TSBA plates and incubated at 60 °C overnight.

Constitutive enzyme production using API-ZYM test strips. The API-ZYM system (API, Invincible Rd, Farnborough) is a semi-quantitative micro-system designed for the study of enzyme activities. Bacteria were grown for 16 h on TSBA plates at 60 °C. Suspensions of cells were prepared in distilled water to give an absorbance of 1.5 at 450 nm, and 60 µl was added to each reaction cupule. The strips were incubated at 60 °C for 10 h before reading the results.

Esterase electrophoresis. Cultures were grown overnight at 60 °C in the medium of Sargeant *et al.* (1971). Cells were harvested by centrifuging at 10000 g for 30 min at 4 °C, washed twice in 0.15 M-NaCl and finally suspended in 0.15 M-NaCl to give 3 g wet wt cells per 15 ml saline. The suspensions were disrupted ultrasonically (20 kHz, 5 × 1 min) on ice and cell debris was removed by centrifugation at 48000 g for 1 h at 4 °C. Cell extracts were concentrated 25-fold in a Minicon B15 concentrator (Amicon) and stored at -20 °C.

Gels were prepared by dissolving 23 g soluble starch in 200 ml electrophoresis buffer (90 mM-Tris, 90 mM-boric acid, 3 mM-EDTA, pH 8.0) and heated with continuous stirring until maximum viscosity was attained. The solution was then poured on to plates (190 × 140 mm) to give a 4 mm layer. Graphite electrodes were inserted 150 mm apart and wells (9 × 1 mm) were cut 6 mm apart. The plates were stored overnight at 4 °C covered with Clingfilm.

Bromophenol blue was added as a marker to each extract prior to electrophoresis. Samples of 100 µl were loaded and electrophoresis was carried out at 120 V for 2.5 h at ambient temperature. Electrophoresis buffer was dropped on to the gel surface intermittently to prevent excess heating or drying out.

The gels were stained as described by Baillie & Norris (1963) and R_F values for the esterase bands relative to the bromophenol blue front were determined.

Preparation and analysis of DNA. [³²P]DNA was prepared from *B. stearothermophilus* NCA 1503 and *B. caldodenax* grown at 60 °C and from *B. subtilis* 168 and *B. coagulans* ATCC 8038 grown at 35 °C in the following medium (g l⁻¹): Tris, 6; glucose, 10; KCl, 5; MgSO₄·7H₂O, 1; NaCl, 4; glutamic acid, 5; Difco Casamino acids, 1; Difco yeast extract, 0.1; L-tryptophan, 0.025; plus ³²PO₄³⁻ [1.2 mCi l⁻¹ (44 MBq l⁻¹)]. The pH was adjusted to 7.4 with HCl. For unlabelled DNA, all strains were grown at 60 °C in the medium of Sargeant *et al.* (1971) but with 1 % (w/v) glucose replacing sucrose as the major carbon source. *Bacillus subtilis* 168 (Trp⁻), *B. amyloliquefaciens* H1, *B. megaterium* KM, *B. coagulans* ATCC 8038 and ATCC 12445 and *E. coli* K12 HfrH were grown at 35 °C in this medium.

Cells washed with 0.15 M-NaCl, 0.01 M-Na₂EDTA, 0.01 M-Tris/HCl (pH 8.0) were treated with lysozyme (0.2 mg ml⁻¹, 22000 units mg⁻¹; Boehringer) in similar buffer, but containing 0.05 M-Na₂EDTA, for 20 min at 25 °C and lysed with sodium dodecyl sulphate (20 g l⁻¹) at 70 °C for 15 min. The lysed material was incubated with Proteinase K (50 µg ml⁻¹, 20 units mg⁻¹; Boehringer) for 16 h at ambient temperature. DNA was isolated as described by Marmur (1961) and further purified as described by Shah *et al.* (1976).

Thermal denaturation of purified DNA was carried out as described by Shah *et al.* (1976) in 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0 (SSC) (Marmur & Doty, 1962).

DNA-DNA hybridization was carried out essentially as described by Denhardt (1966) on Millipore HADP 25 mm diam. filters. Filters preloaded with 25 µg sonicated, heat-denatured (105 °C, 15 min) unlabelled DNA were annealed in 6 × SSC with 0.2 µg sonicated, heat-denatured [³²P]DNA at 65 °C for 16 h under paraffin oil. Filters were then washed with 2 × SSC, dried and counted. DNA from *E. coli* K12 HfrH was used as a standard for thermal denaturation and as a blank in hybridization experiments.

Table 1. Sugar fermentations and biochemical reactions of *Bacillus* species

All results were recorded after 48 h; further incubation did not result in any additional activity.
w denotes weak positive reactions.

	<i>B. stearo- thermophilus</i> NCA 1503	<i>B. stearo- thermophilus</i> ATCC 12016	<i>B. stearo- thermophilus</i> 240	<i>B. stearo- thermophilus</i> 262	<i>B. stearo- thermophilus</i> RS93	<i>B. stearo- thermophilus</i> 136	<i>B. stearo- thermophilus</i> 10	<i>B. cald- otenax</i>	<i>B. cald- ovelox</i>	<i>B. cald- olyticus</i>	<i>B. coagu- lans</i> ATCC 8038	<i>B. coagu- lans</i> ATCC 12245
Sugar fermentations:												
Adonitol	—	—	—	—	+	—	—	—	—	—	—	—
Arabinose	—	+	—	—	—	—	w	—	—	—	—	—
Dextrin	+	—	+	+	—	+	+	+	+	+	+	+
Erythritol	—	—	—	—	—	+	—	—	—	—	—	—
Galactose	w	+	—	—	—	—	w	—	—	—	+	+
Glucose	+	+	+	+	w	+	+	+	+	+	+	+
Glycerol	+	+	+	—	+	w	+	+	+	w	+	w
Glycogen	+	w	+	+	—	—	+	—	—	+	w	—
Inositol	—	—	—	—	w	w	—	—	—	—	—	—
Lactose	—	—	—	—	—	—	w	—	—	—	+	+
Laevulose	+	w	+	—	w	+	+	+	w	w	+	+
Maltose	+	+	+	+	—	+	+	+	+	w	+	+
Mannitol	—	w	—	—	+	+	—	w	+	w	w	—
Mannose	+	+	+	w	w	—	+	+	+	+	+	+
Raffinose	+	—	—	—	—	—	+	w	w	—	—	—
Rhamnose	—	—	—	—	—	w	—	—	—	—	+	—
Salicin	—	w	—	—	—	w	—	—	—	—	+	+
Starch	+	—	+	w	—	—	+	—	—	+	w	w
Sucrose	+	—	+	+	+	+	+	+	+	+	—	+
Sorbitol	—	—	—	—	w	+	—	—	—	—	+	—
Trehalose	+	—	+	—	w	w	+	—	+	+	+	+
Xylose	—	+	—	—	—	—	+	—	—	—	w	w
Biochemical reactions:												
NaCl tolerance (%)	3	1	3	4	5	7	3	3	2	2	2	2
Sodium azide	—	—	w	w	—	—	—	—	—	—	+	+
Sabouraud agar	—	—	—	—	—	—	—	—	—	—	+	+
NO ₃ ⁻ reduction	+	+	+	+	+	—	+	+	+	+	—	+
NO ₂ ⁻ reduction	+	+	+	+	+	—	+	—	—	—	—	—
Catalase production	—	—	+	+	+	+	—	—	—	—	+	+
Oxidase	—	+	+	+	+	+	—	+	+	+	—	—
Gelatin hydrolysis	—	w	—	+	—	w	+	+	+	+	—	—
Casein digestion	w	w	+	+	—	—	+	+	+	+	—	w
Starch digestion*	+	+	R	R	—	—	+	R	R	+	—	—
Growth in anaerobic dextrose broth	+	—	+	+	—	—	+	w	w	w	+	+
Anaerobic reduction of NO ₃ ⁻ to gas	—	+	—	—	—	—	—	—	—	—	—	—

No strain utilized aesculin, dulcitol, inulin or citrate; no strain produced indole or acetoin.

* +, Large zone of hydrolysis; R, restricted zone of hydrolysis.

RESULTS AND DISCUSSION

The main taxonomic characters which distinguished *B. coagulans* ATCC 8038 and ATCC 12245 from the other 10 thermophiles tested agreed with those recorded by Buchanan & Gibbons (1974). The *B. coagulans* strains grew readily on Sabouraud dextrose agar and in sodium azide broth (Table 1). Their maximum and minimum growth temperatures were 61 °C and 25 °C, respectively (Table 5). They also showed a greater overall sensitivity to

Table 2. *Antibiotic sensitivity of thermophilic Bacillus cultures*

Cultures grown for 6 h in peptone broth were used to seed bioassay plates which, after drying, were tested with Oxoid antibiotic sensitivity discs. Plates were incubated at 60 °C for 18 h and then the widths of the inhibitory zones were measured.

Strain	Sensitivity to antibiotic* (zone width, mm)														
	C10	CB10	CH10	CT10	E10	FD10	FR50	K5	N10	OB5	OT10	PN2	P1.5	S10	S25
<i>B. stearothermophilus</i> NCA 1503	5	20	7.5	3.5	11	11	10	4.5	12	17	8	19	19	6	9
<i>B. stearothermophilus</i> ATCC 12016	5	22	9.5	4.5	12	9	8	8.5	10	18	9.5	20	18	7.5	9
<i>B. stearothermophilus</i> 240	5	19	5	4	10.5	9	8	6	10	16	5	17	14	6	8
<i>B. stearothermophilus</i> 262	5	21	5	3.5	9	7	8	4	10	15	6	19	15	5.5	7
<i>B. stearothermophilus</i> RS93	3.5	19	8	2.5	6.5	7.5	5	6	9	14	7.5	13	12	7	10
<i>B. stearothermophilus</i> 136	3	20	5	2	6	5	4	5	11	12	—	15	12	7	6
<i>B. stearothermophilus</i> 10	5	19	9.5	3.5	11	11	6	5	8	18	10	17	16.5	5.5	15
<i>B. caldotenax</i>	6.5	20	7	2.5	10	10	7	7	10	15	8	19.5	17	8	9
<i>B. caldovelox</i>	6.0	18	7	2.5	11	9	7	8	9	15	8	17	15	7.5	9
<i>B. caldolyticus</i>	4.5	19	8	4	11	12	6	8	10	15	10	18	17	7	10
<i>B. coagulans</i> ATCC 8038	12	8.5	17.5	9	18.5	22.5	9	10	15	6.5	19	11	12	12	—
<i>B. coagulans</i> ATCC 12245	12	13	14.5	6.5	16	18.5	10	10.5	12	12	15.5	11.5	14.5	11.5	—

* C10, Chloramphenicol (10 µg); CB10, methicillin (10 µg); CH10, chlortetracycline (10 µg); CT10, colistin sulphate (10 µg); E10, erythromycin (10 µg); FD10, fusidic acid (10 µg); FR50, furazolidone (50 µg); K5, kanamycin (5 µg); N10, neomycin (10 µg); OB5, cloxacillin (5 µg); OT10, oxytetracycline (10 µg); PN2, ampicillin (2 µg); P1.5, penicillin G (1.5 units); S10, streptomycin (10 µg); S25, streptomycin (25 µg).

Table 3. Phage and bacteriocin sensitivity of thermophilic *Bacillus* cultures

Bacteriophage suspensions (10^6 p.f.u. ml⁻¹) and dilutions were plated with 0.1 ml of 6 h bacterial cultures and incubated at 60 °C for 18 h. Thermocin 93 was added to wells cut into TSBA plates seeded with bacterial suspensions; plates were incubated at 60 °C for 18 h and then zone diameters were recorded.

Strain	Efficiency of plating of phage*						Thermocin 93 (zone diam., mm)
	JS017	JS020	JS025	TP1C	TP84	12016b	
<i>B. stearothermophilus</i> NCA 1503	—	—	—	+	++	—	22
<i>B. stearothermophilus</i> ATCC 12016	+++	—	—	—	++++	++++	20
<i>B. stearothermophilus</i> 240	—	—	—	—	+++	++++	18
<i>B. stearothermophilus</i> 262	++	—	—	+	++	+	21
<i>B. stearothermophilus</i> RS93	++++	+++	++++	+	+	—	0
<i>B. stearothermophilus</i> 136	—	—	—	—	—	—	0
<i>B. stearothermophilus</i> 10	++	—	—	+	++	—	24
<i>B. caldotenax</i>	++++	++++	++++	++++	++++	++++	22
<i>B. caldovelox</i>	++++	++++	++++	++++	++++	++++	22
<i>B. caldolyticus</i>	+++	+++	+++	+++	++	++	22
<i>B. coagulans</i> ATCC 8038	—	—	—	—	—	—	0
<i>B. coagulans</i> ATCC 12245	—	—	—	—	—	—	0

* + + + +, Plating efficiency of 1 to 0.1; + + +, plating efficiency of 0.1 to 0.01; + +, plating efficiency of 0.01 to 0.0001; +, indication of low level phage infection; —, no visible indication of phage infection.

Table 4. Constitutive enzyme production by thermophiles detected using the API-ZYM system

Bacteria were grown for 16 h at 60 °C on TSBA. Cells were suspended in distilled water ($A_{450} = 1.5$) and 60 µl was added to each reaction cupule. The strips were incubated at 60 °C for 10 h before reading the results. Enzyme production was assessed qualitatively on a scale 0 to 5, 0 indicating no enzyme production, and 5 high enzyme production.

Strain	Enzyme detected in reaction cupule*																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>B. stearothermophilus</i> NCA 1503	0	4	3	4	3	2	1	1	2	2	2	4	4	2	2	1	0	0	0	0
<i>B. stearothermophilus</i> ATCC 12016	0	2	2	3	2	1	0	0	1	0	2	4	4	3	2	2	1	0	0	0
<i>B. stearothermophilus</i> 240	0	2	2	3	2	2	1	1	1	4	2	2	0	1	1	3	2	1	0	0
<i>B. stearothermophilus</i> 262	0	1	1	1	1	1	0	0	0	0	1	2	0	0	2	2	1	1	0	0
<i>B. stearothermophilus</i> RS93	0	1	1	1	1	0	0	0	0	0	2	3	0	0	0	1	0	0	0	0
<i>B. stearothermophilus</i> 136	0	2	2	2	1	1	2	1	1	3	3	2	0	0	0	1	1	0	0	0
<i>B. stearothermophilus</i> 10	0	1	2	2	1	0	0	0	0	0	3	2	3	1	2	3	1	0	0	0
<i>B. caldotenax</i>	0	5	3	4	3	3	3	2	3	3	4	4	4	1	1	4	3	1	0	0
<i>B. caldovelox</i>	0	5	4	4	3	2	2	1	2	2	4	4	4	2	3	4	4	2	1	0
<i>B. caldolyticus</i>	0	4	3	3	3	2	1	1	2	2	3	3	3	2	3	3	4	1	1	0

* (1) Negative control, (2) alkaline phosphatase, (3) esterase (C4), (4) esterase lipase (C8), (5) lipase (C14), (6) leucine arylamidase, (7) valine arylamidase, (8) cystine arylamidase, (9) trypsin, (10) chymotrypsin, (11) acid phosphatase, (12) phosphoamidase, (13) α-galactosidase, (14) β-galactosidase, (15) β-glucuronidase, (16) α-glucosidase, (17) β-glucosidase, (18) N-acetyl-β-glucosaminidase, (19) α-mannosidase, (20) α-fucosidase.

antibiotics (Table 2) but were not inhibited by thermocin 93 (Table 3). DNA hybridization results showed a low percentage of hybridization with DNA from *B. stearothermophilus* NCA 1503 and *B. caldotenax* and their DNA % G + C contents were lower than for the other strains examined, falling in the range 47 to 48% (Table 7).

The three caldo-active strains of Heinen (1971) and the other seven thermophiles could be placed into the three major groups of Walker & Wolf (1971) (Table 8). In group 1 *B. stearothermophilus* ATCC 12016 complies with their three main criteria; *B. caldotenax* and *B. caldovelox* also showed restricted hydrolytic activity on starch, grew poorly in anaerobic

Table 5. *Growth temperature of thermophilic Bacillus cultures*

Strains were examined for growth on TSBA following overnight incubation, unless indicated otherwise.

Strain	Growth temperature (°C)			Reference
	Maximum	Minimum	Optimum	
<i>B. stearothermophilus</i> NCA 1503	74	41	60 to 65	
<i>B. stearothermophilus</i> ATCC 12016	76	40	60 to 65	
<i>B. stearothermophilus</i> 240	77	41	60 to 70	
<i>B. stearothermophilus</i> 262	78	42	60 to 70	
<i>B. stearothermophilus</i> RS93	68	39	60 to 65	Sharp <i>et al.</i> (1979)
<i>B. stearothermophilus</i> 136	65	41	55 to 58	Epstein & Grossowicz (1969)
<i>B. stearothermophilus</i> 10	75	41	60 to 65	
<i>B. caldodenax</i>	85	—	80	Heinen & Heinen (1972)
<i>B. caldovelox</i>	76	—	60 to 70	Heinen & Heinen (1972)
<i>B. caldolyticus</i>	82	—	72	Heinen & Heinen (1972)
<i>B. coagulans</i> ATCC 8038	61	25 (3 d)	50 to 55	
<i>B. coagulans</i> ATCC 12245	61	25 (3 d)	50 to 55	

Table 6. *Electrophoretic mobility of esterases in cell extracts of thermophilic Bacillus cultures*

Conditions were as described in Methods. R_F values were determined with respect to bromophenol blue.

Strain	R_F value*
<i>B. stearothermophilus</i> NCA 1503	0.20(s)
<i>B. stearothermophilus</i> 10	0.21(s)
<i>B. stearothermophilus</i> RS93	0.30(s)
<i>B. stearothermophilus</i> 136	0.30(s)
<i>B. stearothermophilus</i> 240	0.14(s)
<i>B. caldolyticus</i>	(i) 0.18(s)
	(ii) 0.30(s)
<i>B. stearothermophilus</i> ATCC 12016	(i) 0.13(w)
	(ii) 0.30(s)
	(iii) 0.67(s)
<i>B. stearothermophilus</i> 262	(i) 0.06(w)
	(ii) 0.18(s)
	(iii) 0.37(s)
<i>B. caldodenax</i>	(i) 0.18(s)
	(ii) 0.37(s)
<i>B. caldovelox</i>	(i) 0.18(s)
	(ii) 0.37(s)

* s, Strongly staining band; w, weakly staining band.

glucose broth but did not reduce nitrate to gas under anaerobic conditions. Walker & Wolf (1971) also described three strains which failed to reduce nitrite to N_2 but which otherwise readily fall into group 1. These three strains were placed into sub-group 1b₄ (Walker & Wolf, 1971).

Bacillus stearothermophilus 136 and RS93 appeared to belong to group 2 since neither of them hydrolysed starch. Strains NCA 1503, 10 and 240 belonged to group 3; they displayed good growth in glucose broth anaerobically and strong hydrolytic action on starch. *Bacillus caldolyticus* hydrolysed starch but grew poorly in anaerobic dextrose broth; *B. stearothermophilus* 262 grew well in anaerobic dextrose broth but starch hydrolysis was restricted. These two thermophiles therefore appeared to have characters of both group 1 and group 3 organisms.

To obtain further evidence for the taxonomic relationships between the 10 thermophiles, a comparison was made of their esterase enzymes. Baillie & Walker (1968) showed a close relationship between characterization on biochemical tests and esterase patterns in

Table 7. DNA homology and % G + C among mesophilic and thermophilic *Bacillus* cultures

Nitrocellulose filters (25 mm diam.) were loaded with 25 µg total denatured DNA and reassociation was performed with 0.2 µg sonicated, denatured [³²P]DNA in 6 × SSC at 65 °C for 16 h under paraffin oil. Reassociation values (α) were defined as:

$$\alpha = 100 \times \frac{(\text{Counts bound to heterologous filters}) - (\text{Counts bound to } E. coli \text{ filters})}{(\text{Counts bound to homologous filters}) - (\text{Counts bound to } E. coli \text{ filters})}$$

DNA % G + C was calculated from mol % G + C = 2.44 ($T_m - 69.3$) (Marmur & Doty, 1962) from melting curves determined in SSC.

Total unlabelled DNA from:	mol % G + C	Percentage of reassociation with [³² P]DNA from:			
		<i>B. stearo- thermophilus</i> NCA 1503	<i>B. caldotenax</i>	<i>B. subtilis</i> 168	<i>B. coagulans</i> ATCC 8038
<i>B. stearothermophilus</i> NCA 1503	51.9	100	23	17	11
<i>B. caldotenax</i>	64.8	22	100	9	9
<i>B. subtilis</i> 168 (Trp ⁻)	44.1	19	11	100	10
<i>B. coagulans</i> ATCC 8038	48.3	9	7	10	100
<i>B. stearothermophilus</i> 10	50.9	85	19	16	12
<i>B. stearothermophilus</i> 240	52.3	92	21	14	15
<i>B. stearothermophilus</i> ATCC 12016	54.2	74	25	8	16
<i>B. stearothermophilus</i> RS93	54.5	77	23	9	15
<i>B. caldolyticus</i>	52.3	53	38	11	9
<i>B. stearothermophilus</i> 262	62.2	28	66	7	11
<i>B. caldovelox</i>	65.1	23	88	5	8
<i>B. stearothermophilus</i> 136	47.5	12	11	10	66
<i>B. coagulans</i> ATCC 12245	46.9	18	6	11	88
<i>B. amyloliquefaciens</i> H1	43.2	5	8	19	10
<i>B. megaterium</i> KM	36.7	9	4	8	10

B. stearothermophilus. The results in Table 6 confirm the subdivision into three groups (Table 9) corresponding closely to those of Walker & Wolf (1971); group 1, however, showed some variation. The esterase enzyme pattern of *B. stearothermophilus* 262 closely resembled that of the caldo-active bacteria but had a third, weakly staining band. *Bacillus stearothermophilus* ATCC 12016 produced two strongly staining bands and one weakly staining band, but with R_F values differing from those of other members of group 1.

Determination of the percentage similarity of the strains based on 43 characters (those in Table 1, thermocin 93 sensitivity, and sensitivity to phages TP1C, TP84 and 12016b) showed two groups with > 80% similarity (Table 11). *Bacillus caldotenax* and *B. caldovelox* showed 98% similarity, and *B. caldolyticus* showed 90% similarity with this group. *Bacillus stearothermophilus* NCA 1503 and 10 showed 91% similarity and 80% similarity with *B. stearothermophilus* 240 and with *B. caldolyticus*. *Bacillus stearothermophilus* 262 showed 70 to 80% similarity to both groups but had an 88% similarity to *B. stearothermophilus* 240. *Bacillus stearothermophilus* 136 and RS93 showed 80% similarity with each other but less than 65% with the other groups, and appeared as a third group.

DNA hybridization results and % G + C content (Table 7) supported the presence of three main groups (Table 10). *Bacillus stearothermophilus* 136 was similar to both strains of *B. coagulans*. *Bacillus caldotenax* and *B. caldovelox* had similar % G + C contents and showed a high percentage of DNA hybridization. *Bacillus stearothermophilus* 262 had a % G + C

Table 8. *Classification of strains based on the system of Walker & Wolf (1971)*

Group 1	Group 2	Group 3
<i>B. stearothermophilus</i> ATCC 12016	<i>B. stearothermophilus</i> RS93	<i>B. stearothermophilus</i> NCA 1503
<i>B. caldotenax</i>	<i>B. stearothermophilus</i> 136	<i>B. stearothermophilus</i> 10
<i>B. caldovelox</i>		<i>B. stearothermophilus</i> 240
<i>B. caldolyticus</i>		<i>B. caldolyticus</i>
<i>B. stearothermophilus</i> 262		<i>B. stearothermophilus</i> 262

Table 9. *Classification of strains based on the electrophoretic mobility of their esterase enzymes*

Group 1	Group 2	Group 3
Two or more bands	One band, similar R_F values	One band, similar R_F values
<i>B. caldotenax</i>	<i>B. stearothermophilus</i> 136	<i>B. stearothermophilus</i> NCA 1503
<i>B. caldovelox</i>	<i>B. stearothermophilus</i> RS93	<i>B. stearothermophilus</i> 10
<i>B. caldolyticus</i>		<i>B. stearothermophilus</i> 240
<i>B. stearothermophilus</i> 262*		
<i>B. stearothermophilus</i> ATCC 12016†		

* This strain showed three bands: two were identical with those of the caldo-active bacteria, the third was a weakly stained band.

† This strain showed three bands, two strongly stained and one weakly stained; they did not correspond to the bands produced by the caldo-active bacteria.

Table 10. *Classification of strains based on DNA hybridization data and % G + C content*

Group 1	Group 2	Group 3
<i>B. caldotenax</i>	<i>B. stearothermophilus</i> 136	<i>B. stearothermophilus</i> NCA 1503
<i>B. caldovelox</i>	<i>B. coagulans</i> strains	<i>B. stearothermophilus</i> 10
<i>B. stearothermophilus</i> 262		<i>B. stearothermophilus</i> 240
		<i>B. stearothermophilus</i> ATCC 12016
		<i>B. stearothermophilus</i> RS93
		<i>B. caldolyticus</i>

content similar to those of *B. caldotenax* and *B. caldovelox* with a slightly lower percentage of DNA hybridization. *Bacillus stearothermophilus* strains NCA 1503, 10, 240, ATCC 12016, RS93 and *B. caldolyticus* all had similar % G + C contents and showed relatively high levels of DNA hybridization with strain NCA 1503. The implications of these results must be treated with caution since the extent of reassociation was only examined against [32 P]DNA from four organisms.

Characterization of the strains according to antibiotic sensitivity (Table 2) was not possible since the differences in the sizes of the inhibitory zones were small. *Bacillus stearothermophilus* 136 and strain RS93 showed some similarities, having a slightly greater resistance to several antibiotics, including chloramphenicol, erythromycin, fusidic acid, furazolidone, ampicillin, cloxacillin and penicillin G than the other strains examined.

Characterization by phage typing (Table 3) was of limited value although the caldo-active strains were similar. *Bacillus caldolyticus* showed a slightly lower level of infection, perhaps due to the presence of the restriction enzyme *BclI* (Bingham *et al.*, 1978). Bacteriocin typing may become an important system for distinguishing groups of strains. Thermocin 93 clearly distinguished *B. stearothermophilus* 136 and *B. coagulans* from the other strains.

API-ZYM test strips showed the caldo-active strains to be similar, with relatively high levels of extracellular enzyme production (Table 4). *Bacillus stearothermophilus* 262 showed very limited activity and strains NCA 1503 and 10 did not show any overall close similarity.

Our study indicated that taxonomic grouping based on genotype data and phenotypic characterization did not always coincide. Six of the strains examined remained in the same groups when characterized on several different criteria, i.e. Walker & Wolf's (1971) classi-

Table 11. Classification of strains based on matching coefficient

Matching coefficient (Ss) was calculated from $Ss = (a+d)/(a+b+c+d)$, where *a* is the number of characters positive in both strains, *b* is the number of characters positive in strain 1 and negative in strain 2, *c* is the number of characters negative in strain 1 and positive in strain 2 and *d* is the number of characters negative in both strains. The 43 characters used for the calculation were all the tests from Table 1, with growth in NaCl recorded at 2 % and 5 % and starch hydrolysis recorded as R (restricted), positive or negative, and susceptibilities to phages TP1C, TP84 and 12016b and to thermocin 93 from Table 3.

Strain	NCA 1503	10	240	<i>B. caldolyticus</i>	ATCC 12016	<i>B. caldotenax</i>	<i>B. caldovelox</i>	262	RS93	136
NCA 1503	100 %									
10	91	100 %								
240	88	80	100 %							
<i>B. caldolyticus</i>	84	80	86	100 %						
ATCC 12016	65	70	63	67	100 %					
<i>B. caldotenax</i>	74	72	72	88	68	100 %				
<i>B. caldovelox</i>	76	74	74	91	70	98	100 %			
262	77	70	88	77	65	76	74	100 %		
RS93	63	53	70	65	60	65	67	60	100 %	
136	49	44	56	60	53	60	63	51	80	100 %

Matching coefficient	Group 1	Group 2	Group 3
90–99 %	<i>B. caldotenax</i> <i>B. caldovelox</i>		<i>B. stearothermophilus</i> NCA 1503 <i>B. stearothermophilus</i> 10 <i>B. stearothermophilus</i> 240 <i>B. caldolyticus</i>
80–89 %	<i>B. caldolyticus</i>	<i>B. stearothermophilus</i> 136 <i>B. stearothermophilus</i> RS93	<i>B. stearothermophilus</i> 262 <i>B. stearothermophilus</i>
70–79 %	<i>B. stearothermophilus</i> 262		
60–69 %	<i>B. stearothermophilus</i> ATCC 12016		ATCC 12016

fication, esterase analysis, matching coefficients and genotype data. However, *B. caldolyticus* and *B. stearothermophilus* 262 showed features common to groups 1 and 3. *Bacillus stearothermophilus* ATCC 12016, while closely fitting Walker & Wolf's group 1, showed a % G+C content similar to group 3 but had an overall similarity of less than 70 % with either group. Strain RS93, while appearing phenotypically closely related to strain 136, showed a very different genotype.

Bacillus caldolyticus appeared from our study to be a strain of *B. stearothermophilus*, with a % G+C content falling within the upper group for *B. stearothermophilus*. *Bacillus caldotenax* and *B. caldovelox* were almost identical to each other and probably represent closely related strains of the same species. They have a % G+C content of approximately 65 %, higher than that previously found in *B. stearothermophilus*. Strain 262 also showed this high %G+C content and emerged as a related strain. *Bacillus stearothermophilus* 136, while showing close phenotypic similarities with strain RS93, had a genotype very similar to that of *B. coagulans*.

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