Isolation and Peptidoglycan of Gram-negative Hydrocarbon-utilizing Thermophilic Bacteria

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Four Gram-negative, non-sporulating, aerobic, obligate thermophilic bacteria, isolated from non-thermal environments by enrichment with *n*-heptadecane as substrate, utilized *n*-alkanes, carbohydrates and organic acids as sole source of carbon and energy and also grew on complex media. The growth rate of these organisms, when utilizing *n*-heptadecane as substrate, was markedly increased by adding a low concentration (7.5 mg l^{-1}) of yeast extract. They grew optimally between 55 and 65 °C, and at a pH between 6.2 and 7.5. The mol % G + C for all was between 51 and 58. On the basis of the amino acid and amino sugar compositions of their peptidoglycan, these organisms and other Gram-negative thermophilic bacteria can be divided into four distinct groups. Group A includes the newly isolated hydrocarbon-utilizing bacteria which have nearly equimolar amounts of glutamic acid, alanine, diaminopimelic acid and glucosamine. Group B consists of obligate hydrocarbon-utilizing microbes that have lower molar ratios of glutamic acid and diaminopimelic acid, and contain either ornithine or lysine. The previously isolated non-hydrocarbon-utilizing thermophiles (κ -2, *Thermus aquaticus* YT-1, *Thermus* x-1) and a newly isolated organism from a hot spring comprise group C and contain glycine, ornithine, no diaminopimelic acid, and much lower molar ratios of glutamic and muramic acids than in groups A and B. *Thermomicrobium roseum* lacked peptidoglycan and is placed separately in group D.

INTRODUCTION

Thermophilic micro-organisms, both Gram-negative and Gram-positive, have been isolated from thermal and non-thermal environments (Allen, 1953; Brock & Freeze, 1969; Farrell & Campbell, 1969; Ramaley & Hixson, 1970; Saiki *et al.*, 1972; Jackson *et al.*, 1973; Loginova & Egorova, 1974; Oshima & Imahori, 1974; Pask-Hughes & Williams, 1975). Gram-negative thermophilic bacteria that can utilize hydrocarbon substrates as sole source of carbon and energy have been reported and two of these are unable to utilize any substrate except long-chain *n*-alkanes (Phillips & Perry, 1976; Merkel *et al.*, 1978).

Delineation of the Gram-negative thermophilic bacteria on the basis of readily determinable characteristics, e.g. substrate specificity, base pair ratio, is difficult, as the morphological and physiological characteristics of these organisms appear to fall in a rather narrow range. We therefore examined, amongst other properties, the peptidoglycan composition of a number of Gram-negative thermophilic bacteria since this is of considerable value in the classification of bacteria (Schleifer & Kandler, 1972).

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METHODS

Organisms and growth conditions. Enrichment culture techniques were as previously reported (Merkel *et al.*, 1978). The obligate hydrocarbon-utilizing Gram-negative thermophilic bacteria, ys-3 and ys-4 (Merkel *et al.*, 1978) and *Thermomicrobium fosteri* PTA-1 (ATCC 29033) (Phillips & Perry, 1976), have been described. *Thermus aquaticus* YT-1 (ATCC 25104) (Brock & Freeze, 1969), *Thermus* x-1 (Ramaley & Hixson, 1970), κ -2 isolate (ATCC 27599) (Ramaley *et al.*, 1975) and *Thermomicrobium roseum* (ATCC 27502) (Jackson *et al.*, 1973) were cultured as described in the respective references. Strain ys-3R was isolated as a mixed culture with ys-3 (hot spring, Yellowstone National Park) and was grown in the media employed for the *Thermus* species.

General characteristics (newly isolated organisms). Cell morphology was determined with a Leitz phase contrast microscope. Thin sections were prepared for electron microscopy by methods previously described (Kellenberger *et al.*, 1958; Kennedy *et al.*, 1975). Cell dimensions were determined from both phase contrast and scanning electron photomicrographs (Merkel *et al.*, 1978).

The micro-organisms were tested for the ability to utilize various substrates as described by Vestal & Perry (1969) and Merkel *et al.* (1978).

The optimum growth temperature was determined from the generation time at various temperatures with glucose (0.2 %, w/v) or heptadecane (0.1 %, v/v) as substrate. Generation time was determined from cell numbers obtained by plate counts on Plate Count Agar (Difco). Similarly, the optimum pH for growth was determined from generation times in media at selected pH values with glucose or heptadecane as substrate.

Biochemical characteristics were determined with API test strips (Analytab Products, New York, U.S.A.). Heptadecane-grown cells were used as the inoculum in one series of ampoules and cells obtained by growth on Plate Count Agar were used in another series. The strips were incubated at 60 °C in sealed, humidified chambers. Catalase, nitrate reduction, casein hydrolysis, spore stain, cellulose digestion and starch hydrolysis were determined by standard procedures (Conn, 1957). Analysis for dipicolinic acid was by the procedure of Janssen *et al.* (1958).

DNA from the thermophilic bacteria was isolated and purified and its mol % G + C was determined by the methods of Marmur (1961) and Marmur & Doty (1962). Early-stationary phase cells grown on glucose at 60 °C were rendered susceptible to lysozyme by seven cycles of freezing and thawing in a dry ice/acetone bath. Lysozyme (0.1 %, w/v) digestion (30 min, 60 °C) was followed by addition of sodium dodecyl sulphate (SDS) to a final concentration of 1.0 % (w/v). Carotenoid pigments were analysed according to the method of Eimhjellen & Jensen (1964).

Isolation and purification of peptidoglycan. Peptidoglycan was isolated and purified by methods modified from Schleifer & Kandler (1972). Washed cells (2 to 4 g wet wt) suspended in saline containing deoxyribonuclease (1 mg ml⁻¹) and ribonuclease (1 mg ml⁻¹) (both from Sigma) were disrupted in a French pressure cell at 35 MPa. After adding SDS (2.5 %, w/v), the suspension was incubated at 100 °C for 10 min and intact cells were removed by centrifugation (4000 g, 20 min, 24 °C). The supernatant was further centrifuged (27000 g, 20 min, 24 °C) and the pellet was washed by repeated centrifugation to remove the SDS. The pellet was suspended in 0.1 M-KH₂PO₄/KOH buffer (pH 7.8) and treated with trypsin (2 mg ml⁻¹, 37 °C, 2 h). Debris was removed by centrifuging at 4000 g and cell walls were recovered by centrifuging the supernatant at 27000 g. The pellet was washed successively with 0.1 M-phosphate buffer (pH 7.0), 0.9 % (w/v) saline and distilled water.

The purified peptidoglycan was hydrolysed with 6 M-HCl in evacuated, sealed vials (12 h, 105 °C). The HCl was removed *in vacuo* and amino acids were determined on a Beckman model 120 or 118 amino acid analyser with a single column, packed with Aminex A4 (BioRad).

RESULTS

Characteristics of the hydrocarbon-utilizing thermophilic bacteria

Strain LEH-1 was isolated from soil obtained on the Lehigh University campus in Bethlehem, Pa, U.S.A. Strains BI-1, CC-6 and NR-9 (BI, Bogue Inlet; CC, Core Creek; NR, Newport River) were isolated from North Carolina estuarine mud that had a surface temperature during the summer between 35 and 40 °C. All were aerobic, Gram-negative rods. Strains LEH-1, BI-1 and CC-6 had a similar cell shape, with some differences in cell dimensions, and were non-motile.

Thin sections of the four strains (represented by BI-1 in Fig. 1) revealed a multilayered cell envelope, and cells grown on hydrocarbons had transparent inclusions (Fig. 1, I) which were possibly membrane-bound stored hydrocarbon (Scott *et al.*, 1976; Scott & Finnerty,

Fig. 1. Thin section of BI-1 after growth with heptadecane as carbon source: I, inclusion; CM, cytoplasmic membrane; P, peptidoglycan; OM, outer membrane. Bar marker represents 0.25 μm .

1976). Qualitative extraction and gas chromatographic analysis (Kennedy *et al.*, 1975) revealed a considerable quantity of unaltered heptadecane in cells after growth on this substrate.

No dipicolinic acid was detected and there were no endospores evident by microscopic examination.

Biochemical characteristics of the new isolates

Strains LEH-1, CC-6, BI-1 and NR-9 were negative for: oxidase, *o*-nitrophenyl- β -D-galactopyranosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S production, urea utilization, tyrosine deaminase, indole production, acetoin production and cellulose digestion. All were catalase positive and strains LEH-1, BI-1 and CC-6 hydrolysed casein and starch and had an inducible nitrate reductase whereas strain NR-9 did not.

None contained measurable carotenoid pigments nor was pigmentation induced by growth under illumination.

All four strains utilized C_{13} to C_{20} *n*-alkanes as sole carbon source. Of the C_1 to C_{12} alkanes, strain LEH-1 utilized C_{10} to C_{12} and strain CC-6 utilized C_{12} . Strain NR-9 grew on 1-heptadecene, but not on other 1-alkenes. Strains LEH-1, CC-6 and BI-1 could not utilize 1-alkenes. Strain BI-1 utilized C_{14} and C_{17} methyl ketones (all from C_5 to C_{17} tested). None utilized cycloalkanones or cycloalkanes. Acetate and butyrate supported the growth of all strains and only NR-9 did not utilize propionate or succinate. None grew on citrate. All grew in a medium composed of yeast extract, peptone or hydrolysed casein.

The sugars utilized as growth substrate were glucose, ribose, mannitol, galactose, sucrose, mannose, cellobiose, maltose, xylose, trehalose and glycerol. All strains grew poorly on lactose and not at all on rhamnose, sorbose, sorbitol or arabinose. Strains LEH-1 and CC-6 could not utilize fructose as a carbon source while BI-1 and NR-9 could, and all the strains except NR-9 utilized melibiose.

The four strains had optimum growth temperatures between 55 and 65 °C. None grew below 42 °C or above 70 °C. They had an optimum pH for growth between 6.2 and 7.5. The mol % G + C values for LEH-1, BI-1, CC-6 and NR-9 were 58.0, 51.9, 56.8 and 57.8, respectively.

Generation times were consistently shorter with glucose as substrate (0.7 to 1.8 h) than with heptadecane (1.8 to 3.7 h), and the growth rates on heptadecane were increased markedly by the addition of yeast extract (7.5 mg l⁻¹). Tests with amino acids and vitamins indicated that the growth-promoting factors were glutamic and aspartic acids.

Table 1. *Amino acid and amino sugar compositions of the peptidoglycan from thermophilic bacteria assigned to group A*

Organisms were grown to early-stationary phase in TYG broth [0.5 % (w/v) tryptone, 0.25 % (w/v) yeast extract, 0.1 % (w/v) glucose; pH 6.8 to 7.0]. Results are expressed as molar ratios (MR) with respect to glutamic acid (= 1) and as percentages (%) of the total moles of amino acids and amino sugars present.

Component	LEH-1		CC-6		BI-1		NR-9	
	MR	%	MR	%	MR	%	MR	%
Muramic acid	0.8	15.5	0.8	16.4	0.7	14.9	1.1	17.6
Glutamic acid	1.0	19.0	1.0	21.0	1.0	20.8	1.0	15.5
Alanine	1.1	21.2	1.0	20.4	1.0	20.6	1.3	19.8
Diaminopimelic acid	1.0	20.0	1.0	22.1	0.9	19.5	1.1	16.4
Glucosamine	1.3	24.3	0.9	20.0	1.2	24.2	2.0	30.6

Table 2. *Amino acid and amino sugar compositions of the peptidoglycan from thermophilic bacteria assigned to group B*

Organisms were grown to early-stationary phase with 0.1 % (v/v) heptadecane as carbon source. Results are expressed as in Table 1.

Component	<i>Thermomicrobium</i> <i>fosteri</i> PTA-1		YS-3		YS-4	
	MR	%	MR	%	MR	%
Muramic acid	0.9	15.4	1.0	13.2	0.9	12.5
Glutamic acid	1.0	16.9	1.0	13.2	1.0	14.2
Alanine	1.6	27.7	1.8	24.0	2.0	27.9
Diaminopimelic acid	0.8	13.7	0.9	12.4	1.0	14.2
Glucosamine	1.2	20.6	1.6	21.5	1.5	20.6
Galactosamine	0.2	2.9	0.4	5.8	0.2	3.2
Ornithine	tr	tr	ND	ND	0.5	7.3
Lysine	0.2	2.9	0.7	9.9	ND	ND

tr, Trace; ND, none detected.

Table 3. *Amino acid and amino sugar compositions of the peptidoglycan of thermophilic bacteria assigned to group C*

Organisms were grown to early-stationary phase in TY broth (TYG without the glucose), pH 8.0. Results are expressed as in Table 1.

Component	YS-3R		<i>Thermus</i> <i>aquaticus</i> YT-1		<i>Thermus</i> x-1		K-2 isolate	
	MR	%	MR	%	MR	%	MR	%
Muramic acid	1.1	9.3	1.0	9.3	1.1	9.1	1.1	10.2
Glutamic acid	1.0	8.5	1.0	9.3	1.0	8.3	1.0	9.2
Alanine	2.5	20.6	2.4	22.6	2.9	23.7	2.7	24.6
Glycine	1.7	14.3	1.9	18.1	1.7	14.5	1.6	14.3
Glucosamine	2.8	23.9	2.5	23.2	3.0	24.7	2.6	24.2
Galactosamine	1.3	10.8	0.9	8.1	1.1	9.1	0.8	7.3
Ornithine	1.5	12.7	1.0	9.3	1.3	10.5	1.1	10.2

Peptidoglycan compositions of Gram-negative thermophilic bacteria

Strains LEH-1, CC-6, BI-1 and NR-9 had similar peptidoglycan compositions (Table 1). The peptidoglycan compositions of YS-3, YS-4 and *Thermomicrobium fosteri* strain PTA-1 (Table 2) suggest that in these organisms muramic, glutamic and diaminopimelic acids occur in nearly equimolar concentrations. Ornithine (YS-4) and lysine (PTA-1 and YS-3) occur in low concentrations, as does galactosamine in all three strains.

Thermus x-1, *Thermus aquaticus* YT-1 and the K-2 isolate all lack diaminopimelic acid (Table 3). The major diamino acid present was ornithine along with alanine, glycine and glucosamine. There were lower concentrations of muramic acid, glutamic acid and galactosamine.

DISCUSSION

There were differences in the dimensions of the four newly isolated strains: strain LEH-1 was the smallest (0.5×1.5 to $2.0 \mu\text{m}$), CC-6 and BI-1 were equal in size (0.5×2.0 to $3.0 \mu\text{m}$) and NR-9 was the largest (0.5×3.0 to $3.5 \mu\text{m}$). Physiologically, strains LEH-1, CC-6 and BI-1 are much alike. Strain NR-9 lacks two major hydrolytic enzymes (for casein and starch hydrolysis). Differences among the organisms with respect to utilizable substrates were minor; occurring mainly with fructose and shorter chain *n*-alkanes.

Growth stimulation by factors in yeast extract (glutamic and aspartic acids) is similar to earlier results where addition of a low concentration of yeast extract to the medium for a Gram-positive thermophilic bacterium (utilizing C_{14} alkane as substrate) reduced the lag from 96 h to 68 h (Klug & Markovetz, 1967). In contrast, others (Brock & Freeze, 1969; Jackson *et al.*, 1973) reported that yeast extract can retard growth of some Gram-negative thermophilic bacteria.

None of the organisms showed the yellow, pink or red pigmentation that is characteristic of members of the genera *Thermus* and *Thermomicrobium*, although one described species (strain x-1) is also non-pigmented. The peptidoglycan of the organisms examined (Tables 1 to 3) indicated that all had a greater proportion of amino sugars than was usually present in mesophilic Gram-negative bacteria. The increased amounts of murein and amino sugars might stabilize cells at high temperatures as suggested by Novitsky *et al.* (1974), who reported that growth of a facultative thermophilic *Bacillus* sp. at an elevated temperature yielded cells with more murein and less teichoic acid. The concentrations of amino sugar in the Gram-negative thermophiles correlate with those found in the murein of bacterial spores (Lugtenberg *et al.*, 1977; Murrell & Warth, 1965; Warth, 1965) and Freese *et al.* (1970) have suggested that the ability to form heat-resistant spores may be related to the level of amino sugar in the spore peptidoglycan.

We propose that the Gram-negative thermophilic bacteria be divided into four groups. The newly isolated hydrocarbon-utilizing bacteria (LEH-1, CC-6, BI-1 and NR-9) are placed in group A based on physiological characteristics and the peptidoglycan composition in the cell wall; these strains would be in a new genus.

The peptidoglycan composition of the members of group B resembled that of group A but diaminopimelic acid was replaced by either ornithine or lysine. With their higher concentration of alanine and their inability to grow with any carbon source other than long-chain hydrocarbons, PTA-1, YS-3 and YS-4 should be placed in a separate genus.

The peptidoglycan analysis of group C thermophilic bacteria revealed similarities to cell walls in Gram-positive bacteria. Diaminopimelic acid was replaced by ornithine, they contained high concentrations of glycine, and glucosamine occurred in a high concentration nearly equimolar with alanine. Because of similar peptidoglycan compositions and physiological characteristics, all members of group C should be placed in the genus *Thermus*. Pask-Hughes & Williams (1978) analysed the peptidoglycan from 12 strains of the genus *Thermus* and found that ornithine was the dibasic amino acid and that significant amounts of glycine were present.

Table 4. *Characteristics of Gram-negative thermophilic bacteria*

Strain	Peptido- glycan diamino acid	mol % G+C	Morphology	Pigment- ation*	Generation time†	Growth on complex media‡	Growth on hydro- carbons‡	Optimum growth temp. (°C)§	Optimum pH
Group A									
LEH-1	Dap	58.0	Rod, 1.5-2.0 µm	—	2.8 h, 0.7 h	+	+	60, 55	6.4-6.8
CC-6	Dap	56.8	Rod, 2.0-3.0 µm	—	3.6 h, 0.8 h	+	+	60, 60	7.0-7.5
BI-1	Dap	51.9	Rod, 2.0-3.0 µm	—	3.7 h, 1.0 h	+	+	55, 65	6.2-6.5
NR-9	Dap	57.8	Rod, 3.0-3.5 µm	—	1.8 h, 1.8 h	+	+	55, 60	6.5-7.0
Group B									
YS-3	Dap, Lys	ND	Rod, 0.7 µm	—	6 h + ¶	—	+	60	6.5-7.5
YS-4	Dap, Orn	ND	Rod, 1.5 µm	—	6 h + ¶	—	+	60	6.0-7.0
<i>Thermomicrobium fosteri</i> PTA-1	Dap, Lys	68.8	Pleomorphic rod, 1-1.5 µm	Pink	4-4.5 h	—	+	60	7.2-7.5
Group C									
<i>Thermus aquaticus</i> YT-1	Orn	65-67	Rod, 5-10 µm	Orange	50 min	+	—	70	7.5-7.8
<i>Thermus</i> x-1	Orn	64	Rod, 5-10 µm	Cream	50-60 min	+	—	70	7.5-7.6
K-2	Orn	65	Rod, 5-8 µm	Pink	50 min	+	—	60	7.5-7.6
<i>Thermus thermophilus</i> ¶	ND	69	Rod, 3 µm	Orange	20 min	+	—	65-72	7.0
Group D									
<i>Thermomicrobium roseum</i>	None	64	Pleomorphic rod, 3-6 µm	Pink	5.5 h	+	—	70-75	8.2-8.5

ND, Not determined.

* —, No detectable pigment.

† For group A strains, generation times were measured with *n*-heptadecane and glucose, respectively, as substrates.

‡ +, Growth; —, no growth.

§ For group A strains, optimum growth temperatures were measured with *n*-heptadecane and glucose, respectively, as substrates.

¶ Slow growth rates and inability to grow on other substrates made it difficult to determine these generation times precisely.

¶ Data from Oshima & Imahori (1974).

Table 4 summarizes some major characteristics of the Gram-negative thermophilic bacteria organized according to our proposed classification scheme.

In a separate communication, we will report on the cell wall of *Thermomicrobium roseum*. This organism has a cell wall totally different from that of *Thermomicrobium fosteri* PTA-1. We thus propose that strain PTA-1 be removed from this genus, since *Thermomicrobium roseum* was the first to be characterized.

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