Chemotaxonomic Study of an Alkalophilic Bacterium, *Exiguobacterium aurantiacum* gen. nov., sp. nov.

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Chemical studies were performed on a Gram-positive alkalophilic bacterium of uncertain taxonomic position. On the basis of the present and earlier studies it is suggested that the alkalophilic bacterium be classified in a new genus *Exiguobacterium*, as *Exiguobacterium aurantiacum* gen. nov., sp. nov.

INTRODUCTION

The isolation of five strains of an alkalophilic bacterium from potato-processing effluent was reported by Gee *et al.* (1980). The bacterium was Gram-positive, non-sporing, facultatively anaerobic and varied in shape from rods to almost coccoid forms. In some respects it resembled certain coryneform bacteria but its true taxonomic position was not determined (Gee *et al.*, 1980).

Recent improvements in the classification of coryneform and related bacteria have been made possible by analyses of cell wall peptidoglycan (Schleifer & Kandler, 1972), DNA base composition (Crombach, 1978) and lipid composition (Minnikin *et al.*, 1978; Collins & Jones, 1981). In the present study these techniques have been used to characterize further the alkalophilic bacterium of Gee *et al.* (1980) in an attempt to clarify its classification.

METHODS

Cultures and cultivation. For cell wall and lipid studies, five strains, BL77/1 to BL77/5, were cultivated in shake flasks of nutrient broth no. 2 (Oxoid) pH 7.8 at 30 °C for 3 d. Cultures were checked for purity, harvested by centrifugation (10000 g), washed with distilled water and freeze-dried.

Peptidoglycan analysis. The peptidoglycan type of two strains (BL77/1 and BL77/2) was determined as described by Schleifer & Kandler (1972). Purified walls were prepared from about 500 mg of dry cells, and the qualitative amino acid composition of the partial and complete wall hydrolysates was determined by paper chromatography. Quantitative determinations were made using an automatic amino acid analyser.

DNA base composition determinations. Strains BL77/1 and BL77/2 were grown to late-exponential phase, harvested by centrifugation and washed twice in distilled water. DNA was extracted and purified as described by Garvie *et al.* (1981). The DNA base composition was determined from its melting temperature in standard saline citrate as described by Garvie *et al.* (1981).

Analysis of polar lipids. Free lipids were extracted from dry organisms (50 mg) as described previously (Minnikin *et al.*, 1979). Polar lipids were examined by two-dimensional thin-layer chromatography using HPTLC Kieselgel 60F₂₅₄ (Merck Art. 5628) plates (10 × 10 cm). Chromatograms were developed in the first dimension with chloroform/methanol/water (65:25:4, by vol.) and in the second dimension with chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Spraying with 10% (w/v) molybdophosphoric acid in ethanol followed by heating at 150 °C for 15 min revealed all the lipids present. Specific spray reagents for lipid phosphate (Dittmer &

Lester, 1964), α -glycols (Shaw, 1968), sugars (α -naphthol and anisaldehyde/H₂SO₄) (Jacin & Mishkin, 1965; Stahl & Kaltenbach, 1961) and free amino groups (ninhydrin) were also used.

Analysis of isoprenoid quinones. Isoprenoid quinones were extracted from dry cells (50 mg) and purified as described by Collins *et al.* (1977). Purified quinones were further examined by reverse-phase partition chromatography as described previously (Collins *et al.* 1980*b*, 1982*a*). Mass spectra of the quinones were recorded on an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70 eV and a temperature range of 180 to 200 °C.

RESULTS AND DISCUSSION

The purified peptidoglycan of strains BL77/1 and BL77/2 contained lysine as the dibasic amino acid. In addition alanine, glycine and glutamic acid were detected in the hydrolysates. Analysis of partial hydrolysates indicated the presence of a group A peptidoglycan (type Lysine-glycine,) (Schleifer & Kandler, 1972). This peptidoglycan type has been reported previously in certain coryneform taxa (e.g. *Brevibacterium acetylicum*) and bifidobacteria (Schleifer & Kandler, 1972; Weiss *et al.*, 1981). The peptidoglycan of strains BL77/1 and BL77/2 also resembles that of staphylococci, except that the latter normally have several glycine residues (i.e. Lysine-glycine₄₋₅; Schleifer & Kandler, 1972).

The DNA base composition of strains BL77/1 and BL77/2 was 53.4 and 53.2 mol % G + C respectively. The present result is lower than that reported (56 mol % G + C) for strain BL77/1 by Gee *et al.* (1980). A mol % G + C range of 53 to 54 is compatible with certain coryneform bacteria (see Table 1) but significantly higher than that reported for members of the genus *Staphylococcus* (Schleifer *et al.*, 1979*a, b*). The mol % G + C range of 53 to 54 is also higher than that of most members of the genus *Bacillus*, although some *Bacillus* spp. possess DNA base ratios of 50 to 62 mol % G + C (Gibson & Gordon, 1974).

Components that co-chromatographed with vitamin K were the only isoprenoid quinones detected in the test strains (BL77/1 to BL77/5). UV absorption spectra of the purified quinones showed absorption maxima at 242, 248, 260, 269 and 325 nm, in accord with published data for menaquinones (Collins & Jones, 1981). The mass spectra of the menaquinone samples showed intense peaks at *m/e* 187 and 225 derived from the naphthoquinone nucleus with peaks in the high mass region (*m/e* 648, 580, 512; major component in bold) attributable to molecular ions (M⁺). The major component corresponded to unsaturated menaquinones with seven isoprene units (abbreviated MK-7) although trace amounts of MK-6 and MK-5 were also present. The recovery of major amounts of MK-7 from the alkalophilic strains differentiates them from members of the 'coryneform group of bacteria' (Collins & Jones, 1981). Major amounts of MK-7 have however been reported to be present in a variety of other Gram-positive taxa (e.g. *Bacillus*, *Kurthia*, *Staphylococcus*) (Collins & Jones, 1981).

Diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) were readily identified in the extracts of the test strains by their chromatographic behaviour and staining characteristics. In addition the test strains possessed minor amounts of an unidentified phospholipid (Fig. 1). No glycolipids were detected. The presence of PE in the test strains is of considerable interest. With the exception of a few mycolic acid-containing taxa (Minnikin *et al.*, 1978) PE is not normally found in coryneform bacteria. Although PE is common amongst *Bacillus* species, the latter also possess glycolipids (Shaw, 1970; Lechevalier, 1977). It is worth noting that *Kurthia zopfii* also possesses the simple polar lipid pattern DPG, PG and PE (Goodfellow *et al.*, 1980).

The results of the present study indicate that the alkalophilic bacterium of Gee *et al.* (1980) is quite distinct from all Gram-positive eubacteria examined to date (Table 1). The alkalophilic bacterium can be distinguished from all members of the 'coryneform group of bacteria' on the basis of cell wall peptidoglycan, mol % G + C, polar lipid and menaquinone composition. Although the alkalophilic bacterium resembles *Kurthia zopfii* in possessing a peptidoglycan based upon lysine, unsaturated menaquinones with seven isoprene units and a polar lipid composition comprising DPG, PG and PE, the former has a relatively low G + C content (about 37 mol %; Keddie, 1981). The alkalophilic bacterium can also be distinguished from the vast majority of *Bacillus* spp. on the basis of its cell wall composition. Members of the genus

Fig. 1. Two-dimensional thin-layer chromatogram of polar lipids from strain BL77/1. Chloroform/methanol/water (65:25:4, by vol.) was used in the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) in the second dimension. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL, unknown phospholipid.

Bacillus generally possess a directly cross-linked peptidoglycan based on *meso*-diaminopimelic acid (Schleifer & Kandler, 1972). Two alkalophilic bacilli, *B. pasteurii* and *B. sphaericus*, have however been reported to possess peptidoglycans based on lysine (Schleifer & Kandler, 1972). The DNA base compositions of *B. pasteurii* (42 mol % G + C) and *B. sphaericus* (37 to 43 mol % G + C) (Gibson & Gordon, 1974) are however incompatible with that of the alkalophilic bacterium (about 53 mol % G + C). Thus on the basis of phenetic (Gee *et al.*, 1980), cell wall peptidoglycan, mol % G + C and lipid data, the alkalophilic bacterium appears to be quite distinct from all other Gram-positive taxa examined to date. We therefore propose that the alkalophilic bacterium of Gee *et al.* (1980) be classified in a new genus, *Exiguobacterium* (L. adj. *exiguus* short, small; Gr. neut. dim. n. *bakterion* a small rod; M.L. neut. n. *Exiguobacterium* small rod), as *Exiguobacterium aurantiacum* sp. nov.

Description of Exiguobacterium gen. nov.

Cells vary in shape from rods ($3.2 \times 1.2 \mu\text{m}$) in the exponential phase to short, almost coccoid forms ($1.4 \times 1.1 \mu\text{m}$) in the stationary phase (Gee *et al.*, 1980). Both rods and coccoid cells are Gram-positive, non-acid fast and motile; endospores are not formed. The organism is facultatively anaerobic, catalase positive and oxidase negative. Acid is produced from glucose, sucrose, galactose and some other sugars. In anaerobic conditions with glucose as substrate, lactate, acetate and formate are major end products, the proportions depending on cultural conditions (Gee *et al.*, 1980). The cell wall contains a group A type peptidoglycan based on lysine. Mycolic acids are not present. Menaquinones are the sole respiratory quinones; the principal quinone is MK-7. The major polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The DNA base compositions of two strains are 53.2 to 55.8 mol % G + C. The type species is *Exiguobacterium aurantiacum*.

Table 1. *Chemotaxonomic features of Exiguobacterium and some other Gram-positive taxa*

The data are from: Schleifer & Kandler (1972), Lechevalier (1977), Minnikin *et al.* (1978), Goodfellow *et al.* (1980), Collins (1981), Collins & Jones (1981), Keddle (1981), Keddle & Jones (1981), Collins *et al.* (1980*a*, 1982*b*) and Döpfer *et al.* (1982).

Taxon	Major wall diamino acid	Mol% G + C	Mycolic acids	Fatty acid types*	Major menaquinone(s)	Polar lipid†
<i>Arthrobacter globiformis</i> group	Lysine	59-66	absent	S, A, I	MK-9(H ₂)	DPG, PG, PI, MGDG, DGDG, DMDG
<i>Arthrobacter simplex</i> group	LL-DAP	72-74	absent	S, A, I, U, T	MK-8(H ₄)	DPG, PG, OH-PG
<i>Bacillus pasteurii</i>	Lysine	37-43	absent	ND	MK-7	ND
<i>Bacillus sphaericus</i>	<i>meso</i> -DAP	60-64	absent	S, A, I	MK-8(H ₂)	DPG, PG, G
<i>Brevibacterium</i>	<i>meso</i> -DAP	60-67	present	S, U, T	MK-8(H ₂), MK-9(H ₂)	DPG, PI, PIDM
<i>Caseobacter</i>	Ornithine	71-73	absent	S, A, I	MK-9(H ₄)	DPG, PI, PGLs
<i>Cellulomonas</i>	<i>meso</i> -DAP	51-59	present	S, U, (T)	MK-8(H ₂), MK-9(H ₂)	DPG, PI, PIDM
<i>Corynebacterium sensu stricto</i>	Ornithine	67-75	absent	S, A, I	MK-9	DPG, PG, Gs
<i>Curtobacterium</i>	Lysine	53-56	absent	ND	MK-7	DPG, PG, PE, PL
<i>Exiguobacterium</i> gen. nov.	Lysine	36-38	absent	S, A, I	MK-7	DPG, PG, PE
<i>Kurtalia</i>	Lysine	69-75	absent	S, A, I	MK-10, MK-11, MK-12	DPG, PG, DMDG
<i>Microbacterium</i>	Lysine	30-39	absent	S, A, I	MK-8, MK-7, MK-6	DPG, PG, DGludG
<i>Staphylococcus</i>						

ND, Not determined.

* S, straight-chain acids; A, *anteiso* acids; I, *iso* acids; U, monounsaturated acids; T, tuberculostearic acid.

† DPG, Diposphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIDM, phosphatidylinositol dimannosides; PE, phosphatidylethanolamine; OH-PG, phosphatidylglycerol containing hydroxylated fatty acids; PGL, unknown phosphoglycolipid(s); G, unknown glycolipid(s); MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; DGludG, diglucosyl diacylglycerol; DMDG, dimannosyl diacylglycerol; PL, unknown phospholipid(s).

Description of Exiguobacterium aurantiacum sp. nov.

Exiguobacterium aurantiacum (au.ran.ti'ac.um. L. neut. n. *aurum* gold; M.L. neut. n. *Aurantium* generic name of the orange; M.L. neut. adj. *aurantiacum* orange-coloured).

Surface colonies on PPYG agar (Gee *et al.*, 1980) are 2 to 3 mm in diameter after 3 d at 25 °C, low convex, orange, opaque, butyrous and easily emulsified. Colonies on heart infusion agar are normally flat and fainter orange. Orange pigment does not diffuse into medium; pigment production does not occur anaerobically. Cells vary in shape from short, almost coccoid forms $1.4 \times 1.1 \mu\text{m}$ in the stationary phase to rods $3.2 \times 1.2 \mu\text{m}$ in the exponential phase (Gee *et al.*, 1980). Longer, distorted rods may be formed during exponential growth at pH > 10. Cells are Gram-positive, non-acid fast, non-sporing. Motile (peritrichous flagella). Growth occurs in aerobic and anaerobic conditions. Optimum temperature for growth (at pH 9.5) about 37 °C; temperature range of growth is from 7 to 43 °C; at 25 °C the pH range for growth is about 6.5 to 11.5, with two maxima, at pH 8.5 and pH 9.5 (Gee *et al.* 1980). Catalase positive, oxidase negative, glucose metabolized fermentatively. Acid produced from glucose, galactose, glycerol, maltose, mannitol and sucrose but not from L-arabinose, dulcitol, lactose, melezitose, raffinose, rhamnose, sorbitol or xylose. Acid sometimes produced from fructose and salicin. In anaerobic conditions with glucose as substrate, lactate, acetate and formate are the major end products, the proportions depending on cultural conditions. Starch, casein and gelatin are hydrolysed but carboxymethylcellulose, dextran, pectin, tributyrin and Tween 80 are not attacked. Nitrate is reduced to nitrite. Growth is inhibited by chloramphenicol (10 µg per disc), erythromycin (10 µg), novobiocin (5 µg), oleandomycin (5 µg), penicillin G (1.0 µg) and tetracycline (10 µg) but not sulphafurazole (100 µg).

The cell wall peptidoglycan is based on lysine (type Lysine-glycine₁). Mycolic acids are not present. The principal isoprenoid quinone is MK-7. The major polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The G + C content of the DNA, determined by estimation of the melting point, is 53.2 to 55.8 mol % (Gee *et al.*, 1980; present work). The type strain is NCIB 11798.

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