Alkalibacterium olivoapovliticus gen. nov., sp. nov., a new obligately alkaliphilic bacterium isolated from edible-olive wash-waters

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INTRODUCTION

Extremely alkaline environments harbour an interesting diversity of micro-organisms, including archaea and bacteria, with representatives ranging from phototrophic cyanobacteria and purple sulphur bacteria to haloalkaliphilic archaea (Kroll, 1990). Both Gram-positive (spore-forming and non-spore-forming) and Gram-negative alkaliphilic bacteria have been isolated (Kroll, 1990). Alkaliphiles are generally aerobes or facultative anaerobes, but do include some anaerobes such as Clostridium and Methanobacterium spp. Alkaliphiles can also be divided into psychro-alkaliphiles, mesoalkaliphiles, thermoalkaliphiles, haloalkaliphiles and haloalkaliphilic methanogens on
the basis of growth characteristics other than pH. They are found in saline alkaline lakes and ponds, and in Ca²⁺-rich alkaline environments, e.g. spring of Oman and soda deserts (Grant & Tindall, 1986; Jones et al., 1994). Marine environments are dominated by weakly alkalitolerant bacteria, although a few alkaliphilic strains have been reported. The predominant extreme alkaliphiles found in soil are Bacillus species (Kroll, 1990), but only some of these species are also found in highly alkaline saline environments (Weisser & Trüper, 1985; Grant & Tindall, 1986). In addition, alkaliphiles have been isolated from enriched environments such as potato-processing-plant effluents (Collins et al., 1983).

In this paper, we describe the morphological, physiological, chemotaxonomic and phylogenetic characteristics of three novel obligately alkaliphilic strains isolated from wash-waters which are formed as a waste product during the preparation of edible olives (EWW, edible-olive wash-waters), according to the procedures used to give so-called 'Spanish green olives' (Garrido Fernandez, 1982). These wash-waters are extremely alkaline, having a pH between 10.5 and 13.5, with aged samples generally having a lower pH value than that of freshly prepared EWW (probably due to CO₂ dissolution). On the basis of their unique taxonomic properties, we conclude that these novel isolates are members of a new bacterial genus and new species, for which we propose the name Alkalibacterium olivoaptiviticus. Alkalibacterium olivoaptiviticus is proposed as the type species of the new genus and strain WW2-SN4a³ (= DSM 13175ᵀ = NCIMB 137107ᵀ) is proposed as the type strain of this species.

METHODS

Bacterial strains, cultural and physiological conditions. The three obligately alkaliphilic strains were isolated from a sample of fresh EWW (pH 10-9), kindly provided by Professor C. Balis, Harokopio University, Athens, Greece. Primary isolation of bacteria involved dilution plating on a solid medium consisting of diluted EWW (50 %, v/v, aq.) and 2 % (w/v) bacteriological agar (Oxoid). Cultures were maintained and grown routinely using an alkaliphilic medium (GYEC) composed of 0.05 M sodium-t-glutamate (Sigma), 0.5 % (w/v) yeast extract (Oxoid) and a buffer (0.1 M Na₂CO₃/1 mM K₂HPO₄, pH 10.5, or 0.1 M NaHCO₃/1 mM K₂HPO₄, pH 9) containing 0.1 % (w/v) NH₄SO₄ plus 0.1 mM MgSO₄ (Quirk et al., 1991). To investigate growth at pH values between 9.0 and 10.5, the relative proportions of Na₂CO₃ and NaHCO₃ were varied whilst keeping their overall concentration at 0.1 M. To investigate growth at pH values above 10.5, the pH of the medium was adjusted with 5 M NaOH. For medium with a pH value of 8.5 the carbonate buffer was replaced with borate buffer (0.125 M Na₂B₄O₇/0.15 mM HCl). To investigate bacterial metabolism, sodium-t-glutamate was replaced with specific sugars, amino acids or other substrates and the yeast extract concentration was lowered to 0.005 % (w/v) (no bacterial growth was observed in medium containing this concentration of yeast extract alone as carbon source). Salts, metabolic substrates, yeast extract and the buffer were autoclaved separately and mixed aseptically. Solid media were made by adding 2 % (w/v) bacteriological agar.

Tolerance to salt was investigated by using NaCl concentrations (3–20 %, w/v) in a glutamate/yeast extract solid medium. The effect of temperature on growth was determined on plates of glutamate/yeast extract solid medium placed in a gradient incubator over the temperature range 4–39 °C. Anaerobic growth was tested using an anaerobic jar (Merck) with sodium-t-glutamate medium containing 0.2 % (w/v) sodium thioglycollate. The anaerobic bacterium Clostridium pasteurianum (from the Wye Microbiology Laboratories culture collection) and a strictly aerobic Bacillus sp. (Ntougias & Russell, 2000) were used as controls. The presence of catalase and oxidase were tested as described in Cowan & Steel (1965).

Antibiotic sensitivity was investigated by placing antibiotic discs (Oxoid) or a small amount of antibiotic impregnated in a filter-paper disc on plates of glutamate/yeast extract medium. Spore-formation was checked and the Gram test (Hucker modification) was performed as described in Cowan & Steel (1965). The so-called ‘Gram test’ with KOH was performed according to Powers (1995) and the aminopeptidase test as described by Cerny (1976).

Microscopy. Cellular morphology and size were examined by phase-contrast light microscopy of unstained bacteria and by electron microscopy of negatively stained bacteria. For electron microscopy, one drop of an exponentially growing culture in glutamate/yeast extract liquid medium was placed on gold-coated grids, covered with carbon. The bacteria were allowed to grow on the grids in a humid atmosphere for 2 d and then fixed with 2.5 % (v/v) glutaraldehyde, pH 10.5; the grids were washed with carbonate buffer and water successively prior to staining with 2 % (w/v) ammonium molybdate at pH 9.0. The bacteria were viewed using a Hitachi H7000 electron microscope (acceleration voltage, 75 kV).

Lipid analysis. Polar lipids were extracted using the method of Bligh & Dyer as described by Kates (1982). Cellular fatty acid methyl esters obtained by methylation of the total lipid extract (Kates, 1982) were analysed by capillary-GC using a Hewlett Packard 5880A series chromatograph equipped with a flame-ionization detector (injector temperature, 250 °C; detector temperature, 250 °C), fitted with a splitter (ratio 100:1) and a 30 m × 0.25 mm (i.d.) × 0.2 μm film thickness Supelco SP-2380 fused-silica capillary column. Nitrogen was used as the carrier gas with a flow rate of 1 ml min⁻¹. Fatty acid methyl esters of the three obligate alkaliphiles were separated isothermally at 150 °C or by using a temperature program consisting of an initial holding period at 130 °C for 10 min, rising to 250 °C at a ramp rate of 4 °C min⁻¹ and a final period of 10 min at 250 °C. The results were analysed using a Spectra-Physics SP 4270 Computing Integrator with a Hewlett Packard 5880A series GC-terminal. Fatty acids were identified by comparison of their retention times with authentic standards (Supelco, before and after hydrogenation and/or bromination (Kates, 1982). Individual phospholipids and (phospho)glycolipids were separated by one-dimensional TLC using CHCl₃/CH₃OH/H₂O/CH₃COOH (85:15:3:5:10, by vol.) as the solvent system, or by two-dimensional TLC using CHCl₃/CH₃OH/7 M NH₄OH (65:30:5, by vol.) in the first dimension and CHCl₃/CH₃OH/CH₃COOH/H₂O (85:15:10:3:5, by vol.) in the second dimension. Phospholipids and glycolipids were identified by using specific sprays and staining reagents for phosphate (mol-
Alkalibacterium olivoapovliticus gen. nov., sp. nov.

### RESULTS

#### Growth characteristics

The alkaliphilic strains WW2-SN4a, WW2-SN4c and WW2-SN5 grew as small, round, glistening colonies with diameters of 0.8–1 mm. Individual colonies appeared white, although the bulk biomass colour observed after centrifugation was yellow for strains WW2-SN4a and WW2-SN5 and orange for strain WW2-SN4a. The cells were rods (0.5–0.7 × 1.2–2.1 μm for isolate WW2-SN4a and 0.4–0.6 × 1.3–2.9 μm for isolates WW2-SN4c and WW2-SN5) and they occurred singly, in pairs or in small clusters of up to five cells. Endospores were never observed. The bacteria were weakly motile and polar flagella could be observed by electron microscopy of negatively stained cells (Fig. 1).

Growth in GYEC medium at pH 9.0 (see Methods) gave a maximum yield of $4 \times 10^8$–$5 \times 10^9$ cells ml$^{-1}$ for all three strains. The same maximum cell yield in 100% EWW was observed for the two alkaliphilic strains WW2-SN4a and WW2-SN5, whereas twice this yield ($1 \times 10^7$–$5 \times 10^7$) was obtained for strain WW2-SN4c. The isolates stained Gram-negative with conventional Gram staining. However, Duckworth et al. (1996) have proposed that, because alkaliphilic strains do not give clear results with the conventional Gram-staining technique, the characterization of their Gram status must be based on a combination of the results of the Gram test with 3% (w/v) KOH and the aminopeptidase reaction (combined with the type of quinone, vide infra). Strains WW2-SN4a, WW2-SN4c and WW2-SN5 gave a negative reaction with 3% KOH and lacked aminopeptidase activity: both of these

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**DNA base composition.** Genomic DNA was isolated and its G+C content was determined by HPLC of the derived deoxyribonucleosides as described by Tamaoka & Komagata (1984).

**DNA–DNA hybridization.** DNA–DNA hybridization was carried out commercially by DSMZ. DNA was isolated from a broken-cell paste by chromatography on hydroxyapatite using the procedure of Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) with the modification described by Huss et al. (1983) and Escara & Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermostated programmer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992).

**Nucleotide sequence accession number.** Sequences of the bacterial strains used in this study are under the following accession numbers: bacterial sp. WN16, X92169; *Lactobacillus malaromaticus*, M58825; *Carnobacterium piscicola*, X54268; *Carnobacterium gallinarum*, X54269; *Carnobacterium divergens*, M58816; *Carnobacterium alterfudundium*, L08623; *Carnobacterium funditum*, S86170; *Carnococcus atlanticus*, M94844; *Lactosphaera pasteurii*, X87150; *Aerococcus urinae*, M77819; *Aerococcus viridans*, M58797; *Abiotrophia detectiva*, D50541; *Dolosigranulum pigrum*, X70907; *Alloiovococcus otitis*, X59765; and *Clostridium paradoxum*, L06838.

**Organism deposition.** Strains WW2-SN4a, WW2-SN4c and WW2-SN5 have been deposited in the DSMZ as DSM 13175T, DSM 12937 and DSM 12938, respectively, and in the NCIMB as NCIMB 13710T, NCIMB 13711 and NCIMB 13712, respectively.

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The amplified product was analysed using the Perkin Elmer ABI Prism, 310 Genetic Analyser. The sequences of PCR products of each isolate were assembled using the MegAlign, EditSeq and SeqMan software using a Power Macintosh computer. Homology searches against the NCBI database were carried out using the BLAST program (Altschul et al., 1990). Percentage sequence similarity between the organisms from the present study and the selected described species were calculated using the BESTFIT programme of the Wisconsin Genetics Computer Group (GCG) computer package version 9 (1996). Alignment of the sequences was carried out using the ARB Sequence Database Tools (http://www.mikro.biologie.tu-muenchen.de/).

The phylogenetic trees were generated by maximum-likelihood (DNAML and parsimony analyses (DNAPARS) [maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969)] using the PHYLIP version 3.55c suite of programmes (Kuhner & Felsenstein, 1994). Bootstrap values were determined the by SEQBOOT program. TREECON for Windows 95/NT (Version 1.3b) was also used for constructing phylogenetic trees (Van der Peer & De Wachter, 1993). In this programme, evolutionary distances were calculated using the method of Jukes & Cantor (1969) and the topology was inferred using the ‘neighbour-joining’ method (Saitou & Nei, 1987) based on bootstrap analysis of 100 or 1000 trees. *Clostridium paradoxum* was chosen as the outgroup sequence.

The 16S rRNA secondary structure model of the alkaliphilic isolates was based on the secondary structure predictions of *Bacillus subtilis* and *Escherichia coli*, obtained from small-subunit rRNA (Van de Peer et al., 1993) and the Ribosomal Database Project (Maidak et al., 1997), plus the thermodynamic interpretation of its secondary structure using MFOLD version 3.0 (http://mfold1.wustl.edu/mfold/rna/ form1.cgi) for RNA analysis.

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results indicate that the strains have a Gram-positive cell wall structure. They were also catalase- and oxidase-negative. All strains can grow at pH values from 8-5 to 10-8 with an optimum at pH 9-0-9-4 for isolates WW2-SN4aT and WW2-SN5, and pH 9-8-10-2 for isolate WW2-SN4c, therefore indicating that all three strains are obligate alkaliphiles, since no growth was observed at pH values ≤ 8-0 or > 11-0. The value of optimum pH is based on a consideration of growth rate and maximum cell yield. At suboptimal pH values there was rapid cell lysis once stationary phase had been achieved for cultures of WW2-SN4c and WW2-SN5, but not WW2-SN4aT. Isolates WW2-SN4c and WW2-SN5 grew over a temperature range from 4 to 35 °C with a broad optimum from 27 to 32 °C; isolate WW2-SN4aT had the same broad optimum of 27–32 °C and a lower limit of 4 °C, but an upper limit of 37 °C. Therefore, all three strains are psychrotolerant. The three strains grew well under anaerobic conditions and therefore are facultative anaerobes. All three strains could tolerate up to 15% (w/v) NaCl. No significant differences in the growth rate or yield were observed at 0, 3, 5, 10 and 15% NaCl. No growth was observed at 20% NaCl. Thus, all three strains are also halotolerant (Table 1).

**Biochemical characteristics**

No differences in the ability to use a wide range of specific compounds tested as sole carbon and energy sources were observed among the three alkalophilic strains: all had relatively narrow metabolic abilities in this respect. D (+)-Glucose, D (+)-glucose 6-phosphate, D (+)-cellobiose, starch and sucrose were the carbohydrate substrates which were utilized most effectively. All strains were moderately positive (OD050 0-06–0-09) for maltose, mannose and trehalose utilization, weakly positive (OD050 0-04–0-06) for glutamate and malate, and negative for L (+)-arabinose, α-cellulose, D(−)-fructose, D(−)-galactose, lactose, D(+) -melezitose, α-D(+)-melibiose, D(+) -raffinose, L (+)-rhamnose, D(−)-ribose, D(−)-xylose, acetate, ethanol, glycerol, D-mannitol, myo-inositol, sorbitol and succinate utilization. None of the common amino acids supported growth of the three alkaliphilic strains. Some hexoses or disaccharides consisting of hexoses were utilized by the cells, although none of the pentoses tested were metabolized. Growth was also observed in alkali salts of some organic acids and yeast extract could be utilized as sole carbon and energy source.

**Table 1. Morphological and growth characteristics of alkaliphilic strains WW2-SN4aT, WW2-SN4c and WW2-SN5**

<table>
<thead>
<tr>
<th>Morphological and growth characteristics</th>
<th>WW2-SN4aT</th>
<th>WW2-SN4c</th>
<th>WW2-SN5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Colour of biomass</td>
<td>Orange</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Growth yield in EWW (c.f.u. ml⁻¹)</td>
<td>4 × 10⁷</td>
<td>1 × 10⁸</td>
<td>5 × 10⁷</td>
</tr>
<tr>
<td>Specific growth rate μ (h⁻¹)</td>
<td>0-17</td>
<td>0-24</td>
<td>0-17</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>9-0–9-4</td>
<td>9-8–10-2</td>
<td>9-0–9-4</td>
</tr>
<tr>
<td>Optimum NaCl concn (%)</td>
<td>3–5</td>
<td>0–10</td>
<td>3–10</td>
</tr>
<tr>
<td>Optimum growth temperature (°C)</td>
<td>27–32</td>
<td>27–32</td>
<td>27–32</td>
</tr>
</tbody>
</table>
**Table 2. Antibiotic sensitivity of the alkaliphilic strains WW2-SN4a\textsuperscript{T}, WW2-SN4c and WW2-SN5**

All strains were sensitive to ampicillin (10 and 25 µg), carbenicillin, chloramphenicol, kanamycin, penicillin G (2, 5 and 10 IU), streptomycin and trimethoprim (25 µg). All strains were resistant to ceftazidime (30 µg), cephalexin (30 µg), ketoconazole and sulphamethoxazole (25 µg). +, sensitive; –, resistant; ±, weakly sensitive.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>WW2-SN4a\textsuperscript{T}</th>
<th>WW2-SN4c</th>
<th>WW2-SN5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxycillin (25 µg)</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Amoxycillin (2 µg)</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ampicillin (2 µg)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Miconazole</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Neomycin</td>
<td>–</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td>Penicillin G (1 IU)</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulphamethoxazole/trimethoprim (25 µg)</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Trimethoprim (125 µg)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 3. Glycolipid compositions (semi-quantitative) of the alkaliphilic strains WW2-SN4a\textsuperscript{T}, WW2-SN4c and WW2-SN5**

+ + +, Major component; + +, main component; +, component present; TR, trace.

<table>
<thead>
<tr>
<th>Glycolipid ( R_F ) value</th>
<th>WW2-SN4a\textsuperscript{T}</th>
<th>WW2-SN4c</th>
<th>WW2-SN5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>+</td>
<td>TR</td>
<td>+</td>
</tr>
<tr>
<td>0.27</td>
<td>+</td>
<td>TR</td>
<td>+</td>
</tr>
<tr>
<td>0.85</td>
<td>TR</td>
<td>+ + +</td>
<td>TR</td>
</tr>
<tr>
<td>0.97</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

These observations indicate that the strains are heterotrophic bacteria. In contrast to their common ability to metabolize carbon compounds, the three alkaliphiles could be distinguished by their different sensitivities to a range of antibiotics (Table 2). For instance, strain WW2-SN4c was sensitive to amoxycillin, and WW2-SN4a\textsuperscript{T} was less sensitive to amoxicillin, trimethoprim and penicillin G than the other two strains.

**Chemotaxonomic characteristics**

The total lipid fraction of all three alkaliphilic strains contained four glycolipids, having \( R_F \) values of 0.15, 0.27, 0.85 and 0.97 in the TLC solvent system used (see Methods). The glycolipids have not been identified structurally, but their chromatographic behaviour indicates that they are likely to contain either one (\( R_F = 0.85, 0.97 \)) or two or more (\( R_F = 0.15, 0.27 \)) sugars. Specific staining showed that they do not contain a phosphate residue. Semi-quantitative analysis was possible by using an α-naphthol spray to reveal clear differences (based on staining intensity) in the relative proportions of the glycolipids in the three strains (Table 3). The major phospholipids in all three strains are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and phosphatidylserine (PS), plus an unknown phospholipid designated PL-1; there was more PG and DPG but less PL-1 in WW2-SN5 compared with WW2-SN4a\textsuperscript{T} and WW2-SN4c (Table 4). No growth-
The fatty acid profiles of all three strains were reproducible. The 16S rDNA sequences of all three alkaliphiles were 98% of the size of the 16S rDNA of *Bacillus subtilis* (known to contain MQ-7).

### 16S rDNA sequence analysis

The 16S rDNA sequences of isolates WW2-SN4a<sup>T</sup>, WW2-SN4c and WW2-SN5 were almost identical: the sequence for WW2-SN4c (1508 bases) contained one extra base compared to that of WW2-SN4a<sup>T</sup> (1507 bases), while the length of the 16S rDNA sequence (1507 bases) of WW2-SN5 was the same as that of WW2-SN4a<sup>T</sup> but the identity of the last three bases have not been determined. In repeated analyses of independently prepared samples, the single base difference between WW2-SN4a<sup>T</sup> and WW-SN4c was reproducible. The 16S rDNA sequences of all three alkaliphiles were 98% of the size of the 16S rDNA sequence of *E. coli*.
None of the three obligately alkaliphilic bacteria are phylogenetically related to any named organism, the closest match (96.8% homology) being with an unknown Gram-positive alkaliphile WN16 isolated from Lake Nakuru by Duckworth et al. (1996). The next match (93.2% homology) was with Carnobacterium funditum.

Construction of phylogenetic trees using distance matrix methods (Jukes & Cantor, 1969; Fig. 2), character-based methods (maximum-parsimony according to Kluge & Farris, 1969) and the maximum-likelihood method according to Cavalli-Sforza & Edwards (1967) all placed the three obligate alkaliphiles in a phylogenetic position related to Alloiooccus otitis (91.8% similarity) and Dolosigranulum pigrum (91.2% similarity), despite the fact that Carnobacterium funditum (93.2% similarity) was the organism with the highest similarity. Isolate WN16 was the closest related organism and was placed on the same branch. However, the three obligate alkaliphiles isolated from EWW were taxonomically distinct from WN16, which was positioned on a different sub-branch (Fig. 2). The phylogenetic topology of this alkaliphilic branch is conserved, as indicated from the bootstrap analysis of 1000 trees (Fig. 2).

The G+C content of the DNA in all three strains was 39.7±1.0 mol% and there were no significant differences between the strains (P < 0.05). The results of DNA–DNA hybridization for the three strains were as follows: WW2-SN4aT–WW2-SN4c, 88%; WW2-SN4aT–WW2-SN5, 99%; and WW2-SN4c–WW2-SN5, 92%.

Fig. 2. Phylogenetic tree of the three obligately alkaliphilic bacteria isolated from EWW associated with members of the genera Carnobacterium, Aerococcus, Abiotrophia, Alloiooccus and Dolosigranulum. Evolutionary distances were calculated using the method of Jukes & Cantor (1969) and the topology was inferred by using the ‘neighbour-joining’ method (Saitou & Nei, 1987) based on bootstrap analysis of 1000 trees. Scale bar represents 0.02 inferred substitutions per nucleotide position. The 16S rRNA sequence of Clostridium paradoxum was arbitrarily chosen as the outgroup sequence.

16S rRNA secondary structure

The 16S rRNA secondary structure predictions of the three obligate alkaliphiles and their closest related species also showed different structures in the variable regions (Fig. 3). For instance, the secondary structure of the V6 region in 16S rRNA was the same for the three obligate alkaliphiles and isolate WN16, apart from a single substitution of a CG pair with UG. The terminal ring structure of Carnobacterium funditum 16S rRNA was also found in the four alkaliphilic bacteria, although the AC shoulder was structurally different in this terminal loop. Differences in the overall nucleotide composition were observed for all the related strains. Dolosigranulum and Alloiooccus species possess a small ring (ACCGCUU), which is substantially different to the shoulder observed at the same position in the 16S rRNA of Carnobacterium species. The corresponding ring in the V6 region of Dolosigranulum pigrum is smaller by one base compared to that in the other organisms.

DISCUSSION

Based on their 16S rRNA sequences, the three obligately alkaliphilic bacteria present almost identical genotypic characteristics, since their phylogenetic topology and 16S rRNA secondary structure are almost the same. They have the same G+C content in their DNA and the level of hybridization of the DNA between all pairs of the three bacteria is > 88%, a value well above that of 70% which is generally regarded as being the cut-off point for distinguishing different species (Owen & Pitcher, 1985). Therefore, the genotypic characteristics indicate that the three obligate alkaliphiles are different strains of the same species.

In terms of phenotypic characteristics, the three bacteria possess many similarities as well as differences. For instance, no differences are found in substrate utilization, whereas differences in antibiotic sensitivity and bacterial growth rates and yields are observed. In addition, the three obligate alkaliphiles contain the same fatty acid, phospholipid and glycolipid components, although the percentages of the individual components are different. Therefore, according to their phenotypic characteristics also, the three obligate
alkaliphiles appear to be different strains of the same species.

The three obligate alkaliphiles do not match with any known Gram-positive bacterium, although they appear to belong to the Carnobacterium and Aerococcus-like spectrum based on the fact that the most related organisms belong to that radius. Phylogenetic examination places the three strains together with the alkalophilic isolate WN16 from Lake Nakuru (Duckworth et al., 1996) in a cluster which is a new alkalophilic linkage of the low-G+C Gram-positive bacteria. However, isolate WN16 belongs to a different sub-branch. Carnobacterium funditum is the taxonomically described species that is closest to the novel isolates described here, with 93–2% similarity, even though they are placed in the Alloiococcus and Dolosigranulum spectrum. Despite the fact that the three obligate alkaliphiles isolated from EWW and the Alloiococcus and Dolosigranulum genera display 91% similarity, Alloiococcus and Dolosigranulum are on the same branch of the phylogenetic tree (but on a different sub-branch), whereas the three alkaliphilic strains are accommodated on a different branch of the same cluster, indicating that these organisms are members of a novel genus and species.

The three strains and their closest related organisms are non-spore-forming, Gram-positive, low-G+C bacteria. All these bacteria, apart from Alloiococcus otitis, are catalase-negative and form small colonies (1–2 µm) on agar plates (Aguirre & Collins, 1992a). The pattern of utilization of several substrates reveals taxonomic differences between the three obligately alkalophilic strains and their closest relatives. For instance, Carnobacterium funditum, Carnobacterium alterfunditum and Aerococcus urinae can utilize ribose and fructose, in contrast to the three alkaliphiles (Franzmann et al., 1991; Aguirre & Collins, 1992b).

These three isolates tolerate relatively high salt concentrations, compared to carnobacteria which grow at neutrality and up to NaCl concentrations of only 10% (w/v). In addition, three of the six Carnobacterium species are motile (Collins et al., 1987; Franzmann et al., 1991). Their lower optimum growth temperatures distinguish Carnobacterium funditum and Carnobacterium alterfunditum from the three obligately alkalophilic strains (Franzmann et al., 1991). Aerococcus urinae and Lactosphaera pasteurii differ from the three isolates in terms of their motility and shape, their pH and salt tolerances, and their temperature ranges for growth (Aguirre & Collins, 1992b; Janssen et al., 1995). Abiotrophia and Dolosigranulum species are distinguished from the three isolates in terms of motility, pH tolerance, and temperature and salt concentration ranges for growth (Aguirre et al., 1993; Kawamura et al., 1995). Growth at pH 7.0 under strictly aerobic conditions and a catalase-positive reaction constitute the distinctive features of Alloiococcus otitis (Aguirre & Collins, 1992a). The lack of menaquinones, motility, and the temperature, salt and gaseous requirements distinguish the three obligately alkalophilic strains from isolate WN16.

The differences in the variable region V6 of the 16S rRNA gene of the three obligate alkaliphiles compared to the most closely related bacteria also support the fact that these three strains constitute a novel alkalophilic lineage and verify the differences obtained from
the phylogenetic tree, indicating their unique phylogenetic position.

Description of *Alkalibacterium* gen. nov.

*Alkalibacterium* (Al.ka.li.bac’ter.i.um. Ar. n. alkali; Gr. n. bacterium bacterium; N.L. n. *Alkalibacterium* bacterium living under alkaline conditions).

Cells are rods and occur singly, in pairs or in clusters up to five cells. The cells stain Gram-negative but show Gram-positive behaviour in the KOH test and the aminopeptidase test. The cells are weakly motile by polar flagella. Endospores are not formed. Facultatively anaerobic. Catalase- and oxidase-negative. The major phospholipids are PG, DPG, PS and an unknown phospholipid; there are also four unidentified glycolipids. The cellular fatty acids are mainly saturated and unsaturated even-carbon-chain, with \( n \)-hexadecanoic, hexadecen(7)\(^\circ\)ic and octadecen(9)\(^\circ\)ic acid as the major components. No quinones could be detected. The G + C content of the DNA is 39-7 mol%.

The type species is *Alkalibacterium olivoapovliticus* (DSM 13175\(^T\) = NCIMB 13710\(^T\)).

Description of *Alkalibacterium olivoapovliticus* sp. nov.

*Alkalibacterium olivoapovliticus* (o.li.vo.a.pov’li.t.i.cus. L. n. olivo olives; Gr. n. apovlito waste disposal; N.L. gen. n. *olivoapovliticus* from the waste of olives).

Cells are rods and occur singly, in pairs or in small clusters. The cells are 0.4–0.6 x 1.3–2.9 \( \mu \)m, showing Gram-positive cell-wall behaviour and are weakly motile by polar flagella. Endospores are not formed. Colonies are small, round and glistening with diameter of 0.8–1.1 mm, appearing as pale white due to their small size, although the colour of bulk biomass observed after centrifugation is yellow or orange. They are halotolerant, psychrotolerant, obligate alkali; Gr. n. alkali; Gr. n. bacterium bacterium; N.L. n. *Alkalibacterium* bacterium living under alkaline conditions.

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