**Vagococcus humatus** sp. nov., isolated from soil beneath a decomposing pig carcass

Aravind Sundararaman,† Sathiyaraj Srinivasan† and Sang-Seob Lee*

### Abstract

A Gram-stain-positive, non-motile, coccus-shaped bacterium, designated strain C25\(^T\), was isolated from the soil beneath a decomposing pig carcass in Korea and was characterized using a polyphasic taxonomic approach. Comparative 16S rRNA gene sequence analysis showed that strain C25\(^T\) belongs to the genus *Vagococcus* in the family *Enterococcaceae* of the *Lactobacillales*. 16S rRNA gene sequence analysis showed that strain C25\(^T\) was closely related to *Vagococcus lutrae* CCUG 39187\(^T\) (96.5 % similarity) and *Enterococcus termitis* LMG 8895\(^T\) (95.8 %). The chemotaxonomic properties of strain C25\(^T\) were consistent with those of the genus *Vagococcus*; the major cellular fatty acids consisted of C\(_{16:0}\), C\(_{16:1\omega 9c}\) and C\(_{18:1\omega 9c}\), and the cell-wall peptidoglycan type was based on *meso*-diaminopimelic acid. The G+C content of the genomic DNA was 44 mol%. On the basis of phylogenetic inference, fatty acid profile, and chemotaxonomic and other phenotypic properties, strain C25\(^T\) is clearly differentiated from closely related type strains of the genus *Vagococcus* and represents a novel species in this genus, for which the name *Vagococcus humatus* sp. nov. is proposed. The type strain is C25\(^T\) (=KEMB 562-002\(^T\)=JCM 31581\(^T\)).

The genus *Vagococcus* (order *Lactobacillales*, family *Enterococcaceae*) was first described by Collins et al. [1]. *Vagococcus* species have been found in diverse environments such as chicken faeces and internal organs of pigs and cattle. Recently, *Vagococcus entomophilus* was isolated from the digestive tract of a wasp [2] and *Vagococcus acidifermentans* was isolated from an acidogenic fermentation bioreactor [3]. To date, the genus *Vagococcus* consists of nine validated species. The typical characteristics of *Vagococcus* species are Gram-positive, coccus-shaped cells, cell-wall peptidoglycan containing *meso*-diaminopimelic acid, and C\(_{18:1\omega 9c}\) and C\(_{16:0}\) as the predominant cellular fatty acids.

In this study, a Gram-stain-positive, non-motile bacterium designated strain C25\(^T\) was isolated from the soil beneath a decomposing pig carcass. On the basis of 16S rRNA gene sequence analysis, strain C25\(^T\) was considered to be a *Vagococcus*-like strain. Strain C25\(^T\) was subjected to a polyphasic taxonomic investigation, and the results indicated that it should be placed within a novel species in the genus *Vagococcus*.

Strain C25\(^T\) was originally isolated from the soil beneath a decomposing pig carcass, in Korea (37° 0’ 32.07” N 126° 59’ 10.811” E). One millilitre of the soil sample was serially diluted, and 100 µl of each dilution was spread onto nutrient agar (NA; Difco) at 30 °C. After 1 week of incubation, single colonies were isolated and purified by transferring them onto new plates and incubating them again under the same conditions. The isolated colonies were tentatively identified by using the partial 16S rRNA gene sequences using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; [4]). The isolates were routinely cultured on NA at 30 °C and preserved as a suspension in nutrient broth with 20 % (w/v) glycerol at −70 °C.

For the phylogenetic analysis of strain C25\(^T\), genomic DNA was extracted using a commercial genomic DNA extraction kit (Solgent) and PCR-mediated amplification of the 16S rRNA gene (1471 bp) and the PCR product was carried out according to Kim et al. [5]. The sequence was compiled with SeqMan software (DNASTAR). The 16S rRNA gene sequences and other phylogenetic markers of related taxa were obtained from GenBank and edited using the BioEdit program [6]. Multiple alignments were performed with the CLUSTAL X program [7]. Evolutionary distances were calculated using the Kimura two-parameter model [8]. Phylogenetic trees were reconstructed using the neighbour-joining [9], maximum-parsimony [10] and maximum-likelihood methods in the program MEGA [11] with bootstrap values based on 1000 replications [12].

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp60* and *pheS* gene sequences of strain C25\(^T\) are KX247009, LT622163 and LT622164, respectively.

Three supplementary figures are available with the online Supplementary Material.
On the basis of 16S rRNA gene sequence similarity, the closest relatives to strain C25<sup>T</sup> were Vagococcus lutrae CCUG 39187<sup>T</sup> (96.5%) and Enterococcus termitis LMG 8895<sup>T</sup> (95.8%). In the neighbour-joining phylogenetic tree (Fig. 1) strain C25<sup>T</sup> clustered with other members of the genus Vagococcus, and this was also confirmed in the maximum-likelihood tree (Fig. 2).

Partial sequences of the hsp60 and pheS genes were amplified by PCR methods according to the protocols of Goh et al. [13]. The most closely related sequences observed on the basis of individual comparisons of hsp60 and pheS genes were those of the type strains of Vagococcus carniphilus (82.0% sequence similarity) and Vagococcus fluvialis (81.1% sequence similarity), respectively. Phylogenetic analyses based on 16S rRNA and housekeeping gene sequences were performed to clarify the phylogenetic position of the investigated strains. Sequences of 16S rRNA genes and other housekeeping gene sequences were identical (Figs 3, S1 and S2, available in the online Supplementary Material).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain C25<sup>T</sup> and other related species. Bootstrap values of >50% (percentages of 1000 replications) are shown at branch points. The accession numbers of sequences used for the comparative study are included in parentheses. Bar, 0.01 substitutions per nucleotide position.
V. lutrae KCTC 5623\textsuperscript{T} and E. termitis LMG 8895\textsuperscript{T} obtained from the Korean Collection for Type Cultures (KCTC) and BCCM/LMG culture collection centre were grown under the same conditions as strain C25\textsuperscript{T} and used as reference strains for the following experiments.

The Gram reaction was performed as described by Doetsch [14]. Cell morphology and motility were examined by light microscopy (Olympus BX51). Bacteria were grown on NA at 30°C for 48 h. Catalase and oxidase tests were performed as outlined by Cappuccino and Sherman [15]. Anaerobic growth
was examined in serum bottles with sodium thioglycolate (1 g l⁻¹) added to trypticase soy broth, and the upper air layer was replaced with nitrogen. Growth on NA, tryptic soy agar (TSA; Difco), R2A agar (Difco) and Luria–Bertani agar (LB; Difco) was also evaluated at 37°C. Cells of strain C25T were Gram-stain-positive, aerobic, non-motile cocci 1–1.5 µm in diameter (Fig. S3). Growth was observed on NA, R2A agar, TSA and LB but no growth was observed under anaerobic conditions.

Physiological characteristics were determined with API 20strep, API Rapid ID 32 and API ZYM systems according to the instructions of the manufacturer (bioMérieux). Growth at 4, 10, 20, 25, 28, 30, 37, 40, 42 and 45°C and at pH 4.0–10.0 (at 1.0 pH unit intervals) was assessed in tryptic soy broth (TSB; Difco) after 2 days of incubation (Fig. S3). Growth was observed on NA, R2A agar, TSA and LB but no growth was observed under anaerobic conditions.

For fatty acid methyl ester analysis, cells were grown on TSA for 48 h at 37°C, and then two loopfuls of early stationary phase cells were collected and subjected to saponification, methylation and extraction using the methods of Kuykendall et al. [16]. The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (TSBA 6, Sherlock Version 6.0; MIDI), and then analysed by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package [17]. The overall fatty acid composition of strain C25T was very similar to those of other members of the genus Vagococcus, dominated by large amounts of anteiso-branched fatty acids. The major fatty acid components detected were C₁₆:0 and C₁₈:1ω₉c, which are in line with closely related Vagococcus species. The differences in the fatty acid contents between strain C25T and the three reference strains are detailed in Table 2.
The amino acid composition of the cell-wall peptidoglycan was determined by using TLC after hydrolysis with 6 M HCl at 100 °C for 18 h as described by Komagata and Suzuki [18]. Strain C25T had meso-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan, which was consistent with the genus Vagococcus.

For determination of the DNA G+C content, genomic DNA was extracted using a commercial genomic DNA extraction kit (Solgent) and was then enzymatically degraded into nucleosides. The nucleosides were analysed using HPLC as described previously [19, 20]. The DNA G+C content of strain C25T was 40±0.05 mol%.

The phenotypic and chemotaxonomic characteristics of strain C25T were typical of the genus Vagococcus, with the presence of meso-diaminopimellic acid as the diagnostic diamino acid of the cell-wall peptidoglycan, and major fatty acids such as C16:0 and C12:0 9c. Strain C25T could be differentiated from other members of the genus Vagococcus by several phenotypic and chemotaxonomic characteristics as shown in Tables 1 and 2. Strain C25T could also be differentiated based on the assimilation of L-arginine β-naphthylamide, L-phenylalanine β-naphthylamide, L-leucine β-naphthylamide and pyroglutamic acid β-naphthylamide. Based on the physiological and chemotaxonomic characteristics, it is evident that strain C25T should be classified as the type strain of a novel species in the genus Vagococcus, for which the name Vagococcus humatus sp. nov. is proposed.

### DESCRIPTION OF VAGOCOCUS HUMATUS SP. NOV.

Vagococcus humatus (L. masc. part. adj. humatus buried).

Cells are Gram-stain-positive, aerobic, non-motile cocci 1–1.5 μm in diameter. After 3 days of incubation at 37 °C on TSA, colonies are 0.5–1 mm in diameter, pale yellow, smooth, convex and circular with regular edges. It grows between 25 and 42 °C and with weak growth observed at 20 and 42 °C. The optimum growth temperature is 37 °C in TSB. The pH range for growth is 6.0–9.0, with an optimum pH of 7.0 in TSB. Growth occurs in the presence of up to 4 % (w/v) NaCl in TSB with optimum growth in the absence of NaCl. Using the API ZYM system, positive reactions are obtained for acid

### Table 1. Differential phenotypic characters between strain C25T and the type strains of related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 15 °C</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 50 °C</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth at pH 10</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>w</td>
</tr>
<tr>
<td>NaCl range (% w/v)</td>
<td>0–4</td>
<td>0–8</td>
<td>0–7</td>
<td>0–3</td>
</tr>
<tr>
<td>Growth with 5 % (w/v) NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Enzyme activity:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Esterase (C8)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Glucosidase (starch hydrolysis)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucosidase (ascendulhydrolysis)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

### Assimilation of:

- Glutamic acid: + + + +
- Raffinose: + + + +
- 4-Nitrophenyl-β-D-glucopyranoside: + + + +
- L-Arginine β-naphthylamide: + + + +
- L-Phenylalanine β-naphthylamide: – + + +
- L-Leucine β-naphthylamide: + – – –
- Aesculin ferric citrate: – + + +
- 6-Bromo-2-naphthyl-α-D-glucopyranoside: – – – –
- Pyrogulamic acid β-naphthylamide: + + + +
- L-Alanyl-L-alanine β-naphthylamide: – + + +
- L-Glycine β-naphthylamide: + – – –
- L-Arginine: – + – –
- D-Ribose: – + + +
- D-Mannitol: – – – –
- D-Sorbitol: – + + +
- D-Lactose (bovine origin): – + + +
- Trehalose: + + + +
- Starch: – + + –
- Urea: – + – –

DNA G+C content (mol%): Strains: 1, C25T; 2, E. termitis DSM 8895T; 3, V. lutrae KCTC 5623T; 4, V. entomophilus DSM 24 756T. Only fatty acids comprising more than 1 % in any of the strains are shown; TR, <1 %; ND, not detected.

### Table 2. Fatty acid composition of strain C25T and the type strains of related species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>1.9</td>
<td>TR</td>
<td>ND</td>
<td>TR</td>
</tr>
<tr>
<td>C14:0</td>
<td>15.5</td>
<td>4.8</td>
<td>18.6</td>
<td>18.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>35.9</td>
<td>22.6</td>
<td>45.3</td>
<td>16.0</td>
</tr>
<tr>
<td>C16:0 10-methy</td>
<td>1.9</td>
<td>ND</td>
<td>ND</td>
<td>23.6</td>
</tr>
<tr>
<td>C18:1</td>
<td>1.9</td>
<td>2.1</td>
<td>6.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:1 9c  alcohol</td>
<td>12.9</td>
<td>ND</td>
<td>9.8</td>
<td>2.1</td>
</tr>
<tr>
<td>C16:1 9c  alcohol</td>
<td>21.6</td>
<td>9.6</td>
<td>12.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>4.9</td>
<td>12.9</td>
<td>4.8</td>
<td>10.1</td>
</tr>
<tr>
<td>(C18:1 9c and/or C16:1 9c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summed feature 8*</td>
<td>1.8</td>
<td>12.9</td>
<td>ND</td>
<td>6.0</td>
</tr>
<tr>
<td>(C18:1 9c and/or C18:1 10c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system.
phosphatase, N-acetyl-β-glucosaminidase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), β-glucosidase and leucine arylamidase. Negative for esterase lipase (C8), α-fucosidase, α-galactosidase, β-galactosidase (PNGP), β-galactosidase (PNGP), β-glucuronidase, α-mannosidase, naphthol-AS-BI-phosphate ohydrolase, trypsin and valine arylamidase. In the API rapid ID32 Strep and API 20 strep systems, hippuric acid, L-leucine β-naphthylamide, trehalose, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-α-L-arabinofuranoside, 4-nitrophenyl-β-D-glucuronide, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, D-mannose, raffinose, glutamic acid, potassium nitrate, L-proline β-naphthylamide, L-leucyl-L-glycine β-naphthylamide, L-leucine β-naphthylamide, pyroglutamic acid β-naphthylamide, L-tyrosine β-naphthylamide, L-alanyl-L-alanine β-naphthylamide, L-glutamine β-naphthylamide and L-glutamyl-L-glutamic acid β-naphthylamide are assimilated, but L-arabinose, D-mannitol, D-sorbitol, L-arginine, 4-nitrophenyl-α-L-fucopyranoside, 2-naphthyl-phosphate and L-serine β-naphthylamide are not. The major fatty acids are 10-methyl C₁₆:₀, C₁₄:₀ and C₁₆:₀. The diagnostic diamino acid of the cell-wall peptidoglycan is meso-diaminopimelic acid.

The type strain, C25T (=KEMB 562-002ᵀ=JCM 31581ᵀ), was isolated from the soil beneath a decomposing pig carcass in Korea. The G+C content of the genomic DNA of the type strain is 40 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

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