**Chryseobacterium carnipullorum** sp. nov., isolated from raw chicken

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Three Gram-staining-negative, rod-shaped, non-sporo-forming, non-motile, oxidase-positive, yellow pigmented and aerobic bacterial isolates designated 8_R23573, 9_R23581T and 10_R23577 were isolated from raw chicken at a broiler processing plant in Bloemfontein, South Africa. A polyphasic taxonomic approach was used to determine their exact taxonomic identities. Phylogenetic analysis of the 16S rRNA gene sequences showed that the three strains belonged to the genus *Chryseobacterium*, exhibiting the highest similarities to *Chryseobacterium shigense* DSM 17126T (98.6–99.2 %) and *Chryseobacterium luteum* DSM 18605T (98.3–98.7 %). The most abundant quinone was menaquinone MK-6 and the predominant cellular fatty acids were iso-15:0, iso-17:1ω9c, iso-17:0 3-OH and summed feature 3 (iso-16:1ω7c and/or iso-15:0 2-OH), which supported the affiliation of the strains to the genus *Chryseobacterium*. The DNA G+C contents of the strains were 36.9, 36.7 and 36.6 mol% respectively. The DNA–DNA hybridization results gave relatedness values ranging from 78.8 to 87.2 % among the three strains and 23.4 to 56.1 % to the two nearest phylogenetic neighbours *C. shigense* DSM 17126T and *C. luteum* LMG 23785T. On the basis of the data from this polyphasic study, the three strains are concluded to represent a novel species of the genus *Chryseobacterium* for which the name *Chryseobacterium carnipullorum* sp. nov. is proposed. The type strain is 9_R23581T (=LMG 26732T=DSM 25581T).

The genus *Chryseobacterium* belongs to the family *Flavobacteriaceae* (Bernardet et al., 2002; Bernardet, 2011). It was described by Vandamme et al. (1994) to accommodate six species formerly classified within the genus *Flavobacterium*, namely *Chryseobacterium balustinum*, *Chryseobacterium gleum*, *Chryseobacterium indologenes*, *Chryseobacterium indoltheticum*, *Chryseobacterium meningosepticum* and *Chryseobacterium scophthalmum* and was later amended (Kämpfer et al., 2009). The type species is *C. gleum*. Members of the genus *Chryseobacterium* include psychrotolerant and proteolytic spoilage micro-organisms that are widely distributed in food sources such as milk, fish, meat and poultry (Hugo et al., 2003; de Beer et al., 2005, 2006) and occur in a variety of ecological niches such as soil, water, sludge, plants, and human and fish clinical specimens (Hugo et al., 2003; Bernardet et al., 2005, 2011; Hugo & Jooste, 2012).

In 2006 the genus *Chryseobacterium* consisted of only 10 validly named species. Since then the genus has undergone significant and rapid expansion and, at the time of writing, it comprises 61 species with validly published names (Bernardet et al., 2006) and more continue to be described (Euzéby, 2012). This can be ascribed to the readily available and improved phenotypic, chemotaxonomic and molecular identification methods used in the polyphasic taxonomic approach.

During the course of the characterization of chryseobacterial strains isolated from raw chicken in a previous study (de Beer, 2005) at a poultry processing plant in Bloemfontein, the Free State Province, South Africa, three isolates designated 8_R23573, 9_R23581T and 10_R23577 were characterized using a polyphasic approach. For short-term maintenance, the isolates were freeze-dried on 5 mm diameter filter paper discs in sealed Petri dishes and stored at −20 °C. Before use the strains were reactivated in 10 ml nutrient broth (CM67; Oxoid). Purity was checked by streaking on nutrient agar (CM003; Oxoid) and Gram-staining. Incubation was at 25 °C for 48 h. The pure cultures on nutrient agar slants were stored at 4 °C for shorter-term maintenance and restreaked every 4–6 weeks. For long-term maintenance, the cultures were freeze-dried in ampoules and stored at −20 °C.

The isolates were subjected to whole-cell PCR amplification of the 16S rRNA gene using an Eppendorf
Mastercycler Temperature Gradient Personal Thermal Cycler and the primers 27F and 1492R (Lane, 1991). The amplicons were purified using a Biospin Gel Extraction kit (Bioflux) according to manufacturer’s instructions. Sequencing was done with an ABI BigDye Terminator v1.1 sequencing cycler (Applied Biosystems) using the dNTP chain-termination method according to the manufacturer’s instructions. The primers 27F and 1492R as well as 341F and 517R (Maeda et al., 2009) were used to obtain sequences. The sequences were manually edited and analysed using Gene Pro 4.8 (Drummond et al., 2009) and compared with sequences from type strains of species of the genus Chryseobacterium on GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST). The amplified sequence lengths of strains 8_R23573, 9_R23581<sup>T</sup> and 10_R23577 were 1436, 1412 and 1423 bp respectively.

Comparative gene sequence homologies of the final sequences were analysed using CLUSTALW2 multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequences were then compared with sequences retrieved from GenBank as described previously and aligned using CLUSTAL X version 2.0 (Larkin et al., 2007). Phylogenetic and molecular evolutionary analyses were conducted with MEGA software version 4 (Tamura et al., 2007) using the neighbour-joining method with the Kimura two (KP2) parameter distance measure to determine the relationship of the isolates to the type strains of species of the genus Chryseobacterium. Confidence values were estimated from bootstrap analysis of 1000 replicates. Comparative sequence analysis revealed 99.0% 16S rRNA gene sequence similarity among the strains 8_R23573, 9_R23581<sup>T</sup> and 10_R23577. This demonstrated their high phylogenetic relatedness.

Comparison of the consensus sequences with the sequences of type strains of species of the genus Chryseobacterium with validly published names showed that isolates 8_R23573, 9_R23581<sup>T</sup> and 10_R23577 had the highest similarities to Chryseobacterium shigense DSM 17126<sup>T</sup> (98.6–99.2 %) and Chryseobacterium luteum DSM 18605<sup>T</sup> (98.3–98.7 %). The type strains of species of the genus Chryseobacterium that were similar to isolate 8_R23573 were Chryseobacterium oranimense H8<sup>T</sup> (97.7 % similarity) and Chryseobacterium ureilicus F-Fue-04IIIIaaa<sup>T</sup> (97.6 % similarity). Type strains of species of the genus Chryseobacterium that shared ≥ 97.0 % similarity to isolate 9_R23581<sup>T</sup> were C. oranimense strain H8<sup>T</sup> (98.4 %), Chryseobacterium vyrstaenense LMG 22846<sup>T</sup> (98.4 %) and C. ureilicus F-Fue-04IIIIaaa<sup>T</sup> (98.1 %). Isolate 10_R23577 was also similar to two other type strains, C. oranimense strain H8<sup>T</sup> and C. ureilicus F-Fue-04IIIIaaa<sup>T</sup> at 98.2 and 98.0 % similarity, respectively.

Fig. 1 shows the neighbour-joining phylogenetic analysis of strains 8_R23573, 9_R23581<sup>T</sup> and 10_R23577 compared with closely related species of the genus Chryseobacterium. It revealed a clear affiliation of all three strains to the genus Chryseobacterium and they clustered tightly together suggesting that they could belong to the same species. These strains clustered with C. shigense DSM 17126<sup>T</sup> as their nearest phylogenetic neighbour. The stability of this phylogeny was supported by a high bootstrap resampling value of 98 %. This phylogeny was also obtained using two other treeing methods, maximum-parsimony and UPMGA (data not shown).

The reference strains used in this study, C. shigense DSM 17126<sup>T</sup> and C. luteum LMG 23785<sup>T</sup> were selected on the basis of being the nearest phylogenetic neighbours to the isolates. Phenotypic tests used were according to the minimal standards for the description of new genera and cultivable species of the family Flavobacteriaceae (Bernardet et al., 2002). The isolates were incubated aerobically and anaerobically (on nutrient agar in an anaerobic jar with a gas generating kit, Oxoid BR0038B) at 25 °C. Colonial morphology was observed on nutrient agar. Strains were streaked on nutrient agar and incubated for 48 h. Gram-staining and cell morphology were recorded according to the methods of MacFaddin (1980). The presence or absence of fruity odour was noted. The non-staining (KOH) Gram-reaction was done according to the method of Buck (1982). The production of oxidase, catalase and phosphatase was determined according to the protocol of MacFaddin (1980). The production of flexirubin-type pigments was examined according to the method of Bernardet et al. (2002). Motility was determined by phase-contrast examination of wet mounts from nutrient broth (CM67; Oxoid). Gliding motility was determined according to the protocol of Jooste (1985). Fluorescence was demonstrated on King’s medium B (Cowan, 1974).

Biochemical characteristics were determined using standardized growth suspensions from 24 h nutrient broth cultures. Cells were harvested by centrifugation at 5 143 g for 10 min using a Beckmann J2-21 centrifuge and the pellets were washed twice before being resuspended using 0.1 M phosphate buffer (pH 7.0). The suspensions were standardized in comparison with a McFarland number 2 density standard (0691326; Difco) using 0.1 M phosphate buffer (pH 7.0). Inoculations were conducted using a multi-inoculation device (Jooste, 1985).

The following range of phenotypic tests were carried out according to the protocols of Cowan (1974) and MacFaddin (1980): oxidative or fermentative metabolism of glucose; methyl red and Voges–Proskauer reactions; gluconate oxidation; potassium cyanide tolerance; malonate utilization; growth in 0–5 % (w/v) sodium chloride; growth at 5, 25, 30, 35, 37 and 42 °C; growth on cetrime agar (5284; Merck), MacConkey agar (CM0007; Oxoid) and Simmons’ citrate agar (CM135; Oxoid); reduction of 0.4 % selenite (Holmes et al., 1975); nitrate and nitrite reduction; production of acid from 10 % (w/v) glucose and lactose; alkaline reaction on Christensen’s citrate agar (Holmes et al., 1975); production of ammonia from arginine, lysine decarboxylase, ornithine decarboxylase, DNase [CM321 (Oxoid) + 0.01 % toluidine blue], β-galactosidase (ONPG), hydrogen sulphide [TSI agar method;
Fig. 1. Phylogenetic analysis of strains 8_R23573, 9_R23581\textsuperscript{T} and 10_R23577 and closely related species of the genus Chryseobacterium based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses). Riemerella anatipestifer DSM 17126\textsuperscript{T} and Chryseobacterium carnipullorum sp. nov. LMG 11054\textsuperscript{T} were used as the outgroup to root the tree. Multiple alignments were performed and evolutionary distances were computed using the Kimura two-parameter method. Clustering was determined based on 1000 replications, are given as percentages at the branching points. Bar, 0.01 substitutions per nucleotide position.

In addition to the conventional tests, all strains were profiled using the Omnilog Gen II and III identification system (Biolog Inc., 2008) according to the manufacturer’s instructions. The microtitre plates were incubated at 25 °C for 24 h. The samples were prepared using the standard method according to the protocols of Kuykendall \textit{et al.} (1988) and MIDI Inc. (2001) and identified using gas chromatography (6890N; Agilent) and version 6.1 of the Sherlock Microbial Identification System software (MIDI). Cellular fatty acid (CFA) profiles of strains 8_R23573, 9_R23581\textsuperscript{T} and 10_R23577 and the type strains of their two nearest phylogenetic neighbours, \textit{C. shigense} DSM 17126\textsuperscript{T} and \textit{C. luteum} LMG 23785\textsuperscript{T} and from other closely related species of the genus Chryseobacterium on the basis of several phenotypic characteristics.

Analyses of fatty acids and respiratory quinones and DNA–DNA hybridization were carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) Identification Service.

Cells for cellular fatty acid analysis were grown on nutrient agar slants at 25 °C for 24 h. The samples were prepared using the standard method according to the protocols of Kuykendall \textit{et al.} (1988) and MIDI Inc. (2001) and identified using gas chromatography (6890N; Agilent) and version 6.1 of the Sherlock Microbial Identification System software (MIDI). Cellular fatty acid (CFA) profiles of strains 8_R23573, 9_R23581\textsuperscript{T} and 10_R23577 and the type strains of their two nearest phylogenetic neighbours, \textit{C. shigense} DSM 17126\textsuperscript{T} and \textit{C. luteum} LMG 23785\textsuperscript{T} are shown in Table 2. The predominant CFAs for the unknown strains were iso-15:0 (42.4–43.5 %), iso-17:0 3-OH (18.9–20.1 %) and iso-17:0 3-OH (15.7–16.0 %). The presence of
large amounts of iso-15:0, iso-17:1ω9c and iso-17:0 3-OH fatty acids supported the affiliation of the three strains to the genus *Chryseobacterium* (Hugo et al., 1999; Bernardet et al., 2006, 2011).

The respiratory lipoquinones were extracted using the two-stage method described by Tindall (1990a; 1990b) followed by phase separation into hexane. They were separated by TLC on silica gel and further analysed by HPLC. The major respiratory quinone of strains 8_R23573, 9_R23581T and 10_R23577 was MK-6 (97, 97 and 98 % respectively) while MK-5 was the minor respiratory quinone (3, 3 and 2 % respectively). This was in accordance with results for all members of the genus *Chryseobacterium* (Bernardet et al., 2011).

The G+C content of the DNA of the strains was determined by HPLC (Shimadzu) according to the methods of Cashion et al. (1977) and Mesbah et al. (1989). The DNA G+C contents of strains 8_R23573, 9_R23581T and 10_R23577 were 36.9, 36.7 and 36.6 mol%. This provided further evidence of the affiliation of the strains to strains of the genus *Chryseobacterium* whose DNA G+C contents range from 29 to 39 mol% G+C (Bernardet et al., 2011).

The DNA–DNA hybridization (DDH) values were determined spectroscopically (100Bio; Cary) according to the methods of De Ley et al. (1970), Cashion et al. (1977) and Huss et al. (1983). DDH experiments were performed between strains 8_R23573, 9_R23581T and 10_R23577 and the type strains of the two most closely related species, *C. shigense* DSM 17126T and *C. luteum* LMG 23785T. The DNA–DNA reassociation values between strains 8_R23573 and 9_R23581T, 9_R23581T and 10_R23577 and 8_R23573 and 10_R23577 were 87.1 % (reciprocal 86.5 %), 87.2 % (reciprocal 86.5 %) and 81.4 % (reciprocal 85.9 %), respectively. These values indicated that the unknown bacterial strains from raw chicken were affiliated to the same species (Wayne et al., 1987; Rosselo-Mora, 2006). The DNA–DNA reassociation values for strain 8_R23573 with *C. shigense* DSM 17126T and *C. luteum* LMG 23785T were 42.1 % (reciprocal 43.8 %) and 35.7 % (reciprocal 27.4 %), respectively.

### Table 1. Differential characteristics of *Chryseobacterium carnipullorum* sp. nov. strains 8_R23573, 9_R23581T, 10_R23577 and closely related species of the genus *Chryseobacterium*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Methyl-red test</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth with 3% NaCl</td>
<td>w</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth on marine agar</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Haemolysis on blood agar</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth at 5°C</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from:</td>
<td>10% glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>10% lactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Ethanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Alkaline reaction on Christensen’s citrate agar</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Production of:</td>
<td>3-Ketolactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>W</td>
<td>w</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Brown pigment on tyrosine agar</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of malonate</td>
<td>W</td>
<td>w</td>
<td>W</td>
<td>W</td>
<td>–</td>
</tr>
<tr>
<td>Degradation of tyrosine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Reduction of nitrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>36.9</td>
<td>36.7</td>
<td>36.6</td>
<td>36.6*</td>
<td>ND†</td>
</tr>
</tbody>
</table>

*Shimomura et al. (2005).
†Behrendt et al. (2007).
Table 2. Cellulor fatty acid profiles of isolates 8_R23573, 9_R23581T and 10_R23577 and closely related type strains in the genus Chryseobacterium

<table>
<thead>
<tr>
<th>Fatty acid</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>iso-13:0</td>
<td>1.7</td>
<td>1.8</td>
<td>1.6</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>iso-15:0</td>
<td>43.5</td>
<td>45.2</td>
<td>42.4</td>
<td>38.5</td>
<td>41.5</td>
</tr>
<tr>
<td>anteiso-15:0</td>
<td>2.5</td>
<td>2.6</td>
<td>2.6</td>
<td>3.7</td>
<td>TR</td>
</tr>
<tr>
<td>iso-15:0 3-OH</td>
<td>2.6</td>
<td>2.7</td>
<td>2.6</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>iso-16:0 3-OH</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
<td>1.1</td>
</tr>
<tr>
<td>ECL 16.582</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>iso-17:0 2-OH</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
<td>1.6</td>
<td>TR</td>
</tr>
<tr>
<td>iso-17:0 3-OH</td>
<td>16.0</td>
<td>15.7</td>
<td>15.7</td>
<td>17.8</td>
<td>19.2</td>
</tr>
<tr>
<td>iso-17: 1o9c</td>
<td>18.9</td>
<td>19.7</td>
<td>20.1</td>
<td>21.0</td>
<td>17.7</td>
</tr>
<tr>
<td>iso-18: 1o5c</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>7.2</td>
<td>7.5</td>
<td>7.4</td>
<td>6.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>

*Summed feature 3, iso-16: 1o7c and/or iso-15:0 2-OH or both.

respecively. DNA–DNA hybridizations for strain 9_R23581T with C. shigense DSM 17126T and C. luteum DSM 23785T gave relatedness values of 51.3 % (reciprocal 56.1 %) and 23.4 % (reciprocal 25.6 %), respectively. The DNA–DNA reassociation values for strain 10_R23577 with C. shigense DSM 17126T, C. luteum DSM 23785T, 17126T and C. luteum DSM 23785T gave relatedness values of 45.3 % (reciprocal 43.0 %) and 32.2 % (reciprocal 28.2 %), respectively. Chryseobacterium shigense DSM 17126T and C. luteum DSM 23785T gave DNA–DNA relatedness values of 14 % (reciprocal 10.6 %; Behrendt et al., 2007). All these values clearly confirmed that the unknown strains from the South African National Research Foundation, for financial assistance.

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