Clostridium lundense sp. nov., a novel anaerobic lipolytic bacterium isolated from bovine rumen

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A strictly anaerobic, mesophilic, endospore-forming, lipolytic bacterium, designated strain R1^T, was isolated from bovine rumen fluid and characterized. Cells of this isolate were Gram-positive, non-motile rods that formed spherical terminal spores. The overall biochemical and physiological characteristics indicated that this strain should be placed in the genus *Clostridium*. The strain grew at temperatures between 25 and 47 °C (optimum, 37 °C), at pH between 5·0 and 8·5 (optimum pH 5·5–7·0) and in NaCl concentrations of 0–3 % (w/v). The isolate was not able to utilize glucose or other carbohydrates as carbon sources. The DNA G+C content was 31·2 mol%. Sequence analysis of the 16S rRNA gene of R1^T revealed that it has the closest match (98 % similarity) with *Clostridium tetanomorphum* DSM 4474^T. The highest levels of DNA–DNA relatedness of the isolate were 61·9 and 54·3 % with *Clostridium pascui* DSM 10365^T and *C. tetanomorphum* DSM 4474^T, respectively. Based on 16S rRNA gene sequence similarity, phylogenetic analysis, DNA G+C content, DNA–DNA hybridization data and distinct phenotypic characteristics, strain R1^T (=DSM 17049^T=CCUG 50446^T) was classified in the genus *Clostridium*, as a member of a novel species, for which the name *Clostridium lundense* sp. nov. is proposed.

Most of the research on lipase producers has been focused on aerobic bacteria and fungi, with much less attention being paid to anaerobes (Dighe *et al.*, 1998). Not many anaerobic lipolytic micro-organisms are known. Most of the information available on anaerobic lipase production is related to rumen. However, the number of described lipid-hydrolysing bacteria occupying the lipid-hydrolysing ecological niche in this ecosystem is very small with a few obligately anaerobic rumen bacteria described, *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens* strain S2, isolate LIP4, which cluster in the genus *Propionibacterium*, and LIP5, which is a member of the clostridial cluster XIVa (Jarvis *et al.*, 1998). Jarvis *et al.* (1999) isolated a glycerol-fermenting clostridial strain, LIP1, that possesses lipolytic activity. This strain is also a member of the clostridial cluster XIVa. Dighe *et al.* (1998) reported the isolation of a novel anaerobic lipolytic species, *Selenomonas lipolytica*, from an anaerobic lagoon receiving wastewater from an edible-oil mill. Svetlitshnyi *et al.* (1996) described a novel species, *Thermosyntropha lipolytica*, which is alkali-tolerant and thermophilic.

In the genus *Clostridium*, whose members exhibit a wide range of phenotypic characteristics (Van Dyke & McCarthy, 2002), to the authors' knowledge, only 10 members of the genus have been reported to produce lipase, *Clostridium botulinum*, *C. aurantibutyricum*, *C. novyi*, *C. sporogens* (Dighe *et al.*, 1998), *C. tetanomorphum*, *C. tetani* (Wilde *et al.*, 1989, 1997), *C. ghonii* (Sneath, 1986), *C. aerotolerans* (van Gylswyk & van der Toorn, 1987), isolate LIP5 (Jarvis *et al.*, 1998) and isolate LIP1 (Jarvis *et al.*, 1999). However, little has been reported about their actual lipolytic activity. All of these micro-organisms, except isolates LIP1 and LIP5, are included in cluster I of the genus *Clostridium*, which is known for exhibiting high levels of intracluster similarity, despite having markedly different phenotypes (Collins *et al.*, 1994).

In this paper, phylogenetic and phenotypic characterization of a novel anaerobic lipolytic micro-organism is described. Strain R1^T was isolated from bovine rumen content collected from a slaughterhouse located near Lund, Sweden.

The liquid medium (denoted medium A) used in the enrichment and as a base medium, in most cultivations of R1^T, contained the basal salts described by Markossian *et al.* (2000) supplemented with 0·1 % (w/v) yeast extract, 0·0025 % (w/v) rezasurin and 120 mg L-cysteine

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R1^T is AY858804.

A table showing the differential features of isolate R1^T and the phylogenetically closest related species of *Clostridium* and a figure showing a scanning electron micrograph of strain R1^T during exponential growth in yeast extract medium are available in IJSEM Online.

hydrochloride l^{-1}. For isolation, 2 % (v/v) olive oil was added directly to each serum bottle as a lipid source. The anaerobic conditions were as described by Ljungdahl & Wiegel (1987). Lipolytic activity of isolate R1^T was detected in rhodamine B medium consisting of medium A supplemented with 0·75 % (w/v) gum arabic, 2 % (v/v) olive oil and 0·001 % (w/v) rhodamine B (Jarvis & Thiele, 1997; Kouker & Jaeger, 1987). To obtain a solid medium, 2 % agar was added. The ability to grow under aerobic conditions was tested in rhodamine B medium. Tolerance to reducing agent was studied by inoculating medium A containing 500 mg L-cysteine hydrochloride l^{-1}. All media had a final pH of 6·8–7·2 after autoclaving. Liquid cultures were grown with shaking (120 r.p.m.) at 37 °C.

Growth of isolate R1^T on medium A containing 2 % of olive, sunflower, sesame, corn or rapeseed oil was determined by optical density measurements (OD_{600}). All oils, except olive oil, which was from Sigma, were purchased from a local supermarket. Utilization of various other organic substrates as carbon and energy sources and other physiological features were determined by using the bioMérieux API 20A test kit according to the manufacturer's instructions. Growth rates, optimum growth temperature and tolerance to pH and NaCl were determined in medium A containing 1 % (w/v) yeast extract as the sole carbon and energy source. For these studies, cells were grown at temperatures ranging from 20 to 55 °C, at initial pH values of 4–9 (adjusted with 2·5 M NaOH or 2·5 M HCl) and in 0–6 % (w/v) NaCl for 7 days.

Cell morphology and size were examined using phase-contrast microscopy with a Nikon Optiphot-2 microscope at × 1000 magnification. Gram staining was performed by using a Difco Gram stain set and cultures were grown in medium A according to standard procedures (Gerhardt *et al.*, 1994). Spore formation was also determined by microscopy after staining with malachite green (Gerhardt *et al.*, 1994). Mobility was determined under the phase-contrast microscope by making observations of samples from the liquid culture immediately after placing a cover slip over a drop of culture. Cells were also observed by a JSM-5600 LV scanning electron microscope (at × 3000–13 000 magnification). For this purpose, cells were harvested from liquid culture during their exponential phase of growth, washed twice with water and dehydrated through a graded series of ethanol and isopropyl alcohol aqueous solutions. Cells were then mounted onto 12 mm cover slips, dried overnight in a vacuum desiccator and then gold-palladium (80/20) coated.

Genomic DNA was extracted and purified according to Arahal *et al.* (2002). Universal primers 28F (5′-AGAGTTTGATCCTGGCTCAG-3′; positions 8–28 using *Escherichia coli* numbering) and 1512R (5′-ACGGCTACCTTGTTACGACT-3′; positions 1512–1493 using *E. coli* numbering) were used to amplify the 16S rRNA gene (Weisburg *et al.*, 1991). PCR products were purified by using the QIAquick PCR purification kit (Qiagen). DNA sequencing was performed on both strands by using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) according to the manufacturer's instructions with an ABI Prism 3100 DNA analyser. GenBank and Ribosomal Database Project databases were used to search for 16S rRNA gene similarities (Maidak *et al.*, 2000). Phylogenetic analysis based on the 16S rRNA gene sequence was performed with the aid of the DNAMAN 4.03 software package, using the neighbour-joining and Jukes–Cantor distance correction methods (Saitou & Nei, 1987). When constructing the phylogenetic tree, only sequences from the type strains of species whose names have been validly published were taken into account. The 16S rRNA gene sequence of *C. tetanomorphum* DSM 4474^T was determined by the authors. An almost-complete 16S rRNA gene sequence (1439 bp; GenBank accession no. AY858804) of isolate R1^T was used in the analysis.

DNA–DNA hybridization between isolate R1^T and closely related type strains, as well as the determination of its DNA G+C content, was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Comparison of the partial 16S rRNA gene sequence of isolate R1^T with those available in GenBank databases indicated that this isolate clustered with members of the genus *Clostridium*, cluster I (Fig. 1). The isolate had the highest identity with *C. tetanomorphum* (98 %) followed by

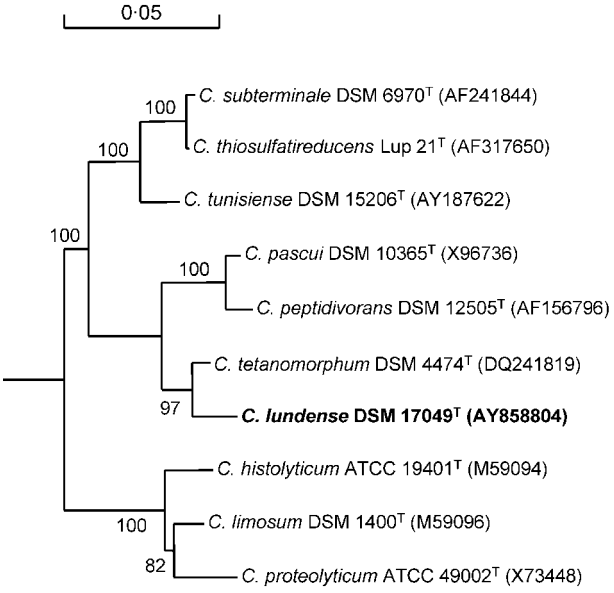

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences indicating the position of isolate R1^T within the radius of representative members of the genus *Clostridium* (cluster I) according to Collins *et al.* (1994). Representative members of cluster II (*Clostridium histolyticum* ATCC 19401^T, *Clostridium limosum* DSM 1400^T and *Clostridium proteolyticum* ATCC 49002^T) were included as outgroup bacteria. GenBank accession numbers are given in parentheses. Bar, 5 substitutions per 100 nt.

Clostridium pascui (96 %) and *Clostridium peptidivorans* (94 %).

Several morphological and taxonomic features of isolate R1^T were investigated and compared with those of *C. tetanomorphum* based on the results of the phylogenetic analysis (Supplementary Table S1 available in IJSEM Online). Isolate R1^T cells are rod-shaped, Gram-positive, 0·56 × 2·8–4·5 μm in size. During exponential growth, cells exhibited varying lengths and growth occurred by binary fission of cells (Supplementary Fig. S1 available in IJSEM Online). The formation of spherical terminal spores was observed in cultures during the stationary phase of growth causing deformation of cell morphology. This strain is a strictly anaerobic micro-organism, since no growth occurred in the presence of oxygen.

Isolate R1^T is mesophilic, exhibiting growth between 25 and 47 °C, with optimum growth at 37 °C. However, the growth rate at 45 °C was not significantly lower. The isolate grew optimally in the absence of NaCl but it tolerated up to 3 % (w/v) NaCl. The cells were able to grow in a pH range of 5·0–8·5 with a broad optimal pH for growth between 5·5 and 7·0. The growth temperature range of isolate R1^T was similar to that of the type strain of *C. tetanomorphum*, but differed from that of the type strain of *C. pascui*, which grows between 10 and 43 °C. The optimum temperature was the same for all strains.

Isolate R1^T also showed phenotypic differences when compared with *C. tetanomorphum* (Supplementary Table S1 available in IJSEM Online). Although lipase and indole production are common characteristics, utilization of glucose, xylose, maltose, sorbitol and salicin and hydrolysis of aesculin are not (Wilde *et al.*, 1997). Regarding *C. pascui*, neither R1^T nor those described as belonging to this species by Wilde *et al.* (1997) utilize glucose or other sugars, except for the type strain, which uses ribose weakly.

The DNA G + C content was 31·2 mol%, which differs from that of the type strains of *C. tetanomorphum* DSM 4474^T and *C. pascui* DSM 10365^T at 28 (Wilde *et al.*, 1989) and 27 mol% (Wilde *et al.*, 1997), respectively. The DNA–DNA relatedness was 61·9 and 54·3 % with *C. pascui* DSM 10365^T and *C. tetanomorphum* DSM 4474^T, respectively. DNA–DNA relatedness between the novel isolate and reference bacteria was significantly lower than the recommended value of $\geqslant$70 %, which is accepted as the definition of distinct species (Wayne *et al.*, 1987).

Based on the analysis of morphological, physiological and phylogenetic characteristics, isolate R1^T should be classified in a novel species of the genus *Clostridium*, for which the name *Clostridium lundense* sp. nov. is proposed.

Description of *Clostridium lundense* sp. nov.

Clostridium lundense (lund.en′se. N.L. neut. adj. *lundense* from Lund, relating to the city where the type strain was isolated).

Cells are rod-shaped, 0·56 × 2·8–4·5 μm in size, Gram-positive and non-motile. Cells form associations of two or more cells in the stationary phase of growth. Colonies are circular, 1 mm in diameter, with entire margins and convex and have a cream colour when grown on peptone/yeast extract/glucose medium. Forms spores that are spherical, terminal and deform the cell shape. Obligately anaerobic, catalase-negative, indole-positive, glucosidase-positive, sulphide-production-positive. Gelatin is not hydrolysed but aesculin is. Growth occurs at temperatures between 25 and 47 °C, with optimum growth at 37 °C. Growth occurs in 0–3 % (w/v) NaCl but is optimal in the absence of NaCl. Growth occurs at pH 5·0–8·5 with a broad optimal pH for growth between 5·5 and 7·0. The strain does not utilize glucose, xylose, maltose, sorbitol, salicin, mannitol, lactose, sucrose, arabinose, glycerol, mannose, melezitose, raffinose, rhamnose or trehalose. The strain shows lipolytic activity; it hydrolyses olive, sesame and corn oils. The DNA G + C content is 31·2 mol%.

The type strain, R1^T (= DSM 17049^T = CCUG 50446^T), was isolated from bovine rumen fluid.

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