Bacillus silvestris sp. nov., a new member of the genus Bacillus that contains lysine in its cell wall

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Author for correspondence: Erko Stackebrandt. Tel: +49 531 2616352. Fax: +49 531 2616418. e-mail: erko@dsmz.de

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A Gram-positive, aerobic, rod-shaped, peritrichously flagellated, round-endospore-forming bacterium was isolated from a forest soil near Braunschweig, Lower Saxony, Germany, and designated strain HR3-23T (T = type strain). Morphologically, strain HR3-23 shows the characteristics of a member of the genus Bacillus. The spore position is terminal in a swollen sporangium. Comparative analysis of the 16S rDNA sequence shows strain HR3-23T to be most closely related to Caryophanon fenue (95.8% 16S rRNA similarity) and to Bacillus sphaericus (95.4% 16S rRNA similarity). Phylogenetically, the isolate clusters among species of Bacillus RNA group 2. The DNA G+C content of isolate HR3-23T is 39.3 mol%, the peptidoglycan type is A4a (L-LYS-D-Glu), the major respiratory lipoquinone is menaquinone MK-7 and the predominant fatty acid is of the iso-C, type. Based on the morphological, chemotaxonomic, physiological and phylogenetic properties, a new species, Bacillus silvestris, is proposed; strain HR3-23T is the type strain (= DSM 12223T).

Keywords: Bacillus silvestris, Bacillus RNA group 2, 16S rDNA analysis, phylogeny, chemotaxonomy

INTRODUCTION

The genus Bacillus, as defined traditionally, comprises Gram-positive, rod-shaped, endospore-forming bacteria with an aerobic or facultatively aerobic metabolism. The recognition that this genus is a diverse assemblage that is a grouping of convenience (Sneath, 1986) was furthermore demonstrated a few years later by comprehensive analyses of 16S rDNA nucleotide sequences of members of this genus (Ash et al., 1991; Farrow et al., 1992, 1994; Rainey & Stackebrandt, 1993; Rainey et al., 1994). These findings provided the basis for the dissection of the genus Bacillus to describe several new genera containing former Bacillus species, such as Alicyclobacillus (Witoszkey et al., 1992), Aneurinibacillus (Shida et al., 1996), Brevibacillus (Shida et al., 1996), Paenibacillus (Ash et al., 1993) and Virgibacillus (Heyndrickx et al., 1998). Analysis of 16S rDNA has developed into a fast and reliable method for phylogenetic affiliation of a novel isolate to one of these genera.

The rRNA group 2 of the genus Bacillus (Ash et al., 1991), comprises round-spore-forming members of the genus Bacillus, containing l-lysine or d-ornithine at position three of the peptide subunit (Stackebrandt et al., 1987). Analysis of 16S rRNA of Gram-positive bacteria from other genera indicated that this Bacillus group also contains non-Bacillus-type organisms, such as members of Caryophanon and Planococcus (Stackebrandt et al., 1987; Farrow et al., 1994), Filibacter (Clausen et al., 1985) and Sporosarcina (Pechman et al., 1976; Farrow et al., 1992).

In this work, we describe a new round-spore-forming species of the genus Bacillus that was isolated from forest soil. Phylogenetically and chemotaxonomically, it is related to members of Bacillus RNA group 2. The isolate can be distinguished from other species of this...
group by the primary structure of the 16S rDNA and its phenotypic properties.

METHODS

Isolation of and cultural conditions for strain HR3-23T. A soil sample was taken from a beech forest soil near Braunschweig, Germany. The sample (10 g) was placed into a Petri dish which was then stored in a jar with moistened paper cloths. Following incubation at 40 °C for 15 min, the soil was subsequently suspended in 100 ml germination solution. The germination solution consisted of 10⁻¹ concentrated glucose/yeast extract/malt extract medium (0.4 g glucose, 0.4 g yeast extract, 1.0 g malt extract, 0.2 g CaCO₃ in 1 l distilled water) with the addition of 1.0 g L-asparagine. After autoclaving, the solution was allowed to cool down to room temperature. A filter-sterilized cycloheximide solution (0.1 g dissolved in 10 ml water) was added.

Two subsequent tenfold dilutions of the soil suspension in germination solution were set up. The 10⁻³ dilution step was stirred at room temperature for 15 min. Aliquots of 100 µl were then streak-plated on different isolation media and incubated at different temperatures.

Strain HR3-23T was isolated from a plate with Difco tryptic soy broth agar (Difco; amended with 3.0 g yeast extract 1⁻¹ and 14 g agar 1⁻² prior to autoclaving), inoculated aerobically for 1 d at 25 °C. Purity of the culture was confirmed by serial plating onto the same medium. Good growth of the isolate was also observed on the R-medium of Yamada & Komagata (1972).

Determination of physiological characteristics. The following physiological tests were carried out according to Gordon et al. (1973): catalase and oxidase reactions, anaerobic growth, Voges-Proskauer (VP) test, temperature range for growth (5-35 °C in steps of 5 °C), presence of lecithinase, resistance to lysozyme, growth in the presence of NaCl (0, 2, 5, 7 and 10 %), growth at pH 5.7, formation of acid from D-glucose, L-arabinose, D-xylose, D-mannitol, D-fructose, formation of gas from glucose, hydrolysis of starch, decomposition of Tween 80, use of citrate and propionate, reduction of nitrate, production of indole, deamination of phenylalanine, decomposition of casein and tyrosine, liquefaction of gelatin and presence of arginine dihydrolase.

Hydrolysis of aesculin was tested according to Lanji (1989) in a medium consisting of 10.0 g Bacto-peptone, 1.0 g sodium citrate, 1.0 g aesculin and 0.05 g iron citrate in 1 l water at pH 6.8-7.0.

A loopful of cell material of strain HR3-23T was used to set up an overnight culture on a plate of Difco tryptic soy broth agar, incubated at 25 °C. From this culture, a suspension corresponding to McFarland standard 1 (OD₅₅₀ of 0.25; bioMérieux) was prepared in 1 ml sterile water. One drop of this suspension was added to each of the test tubes.

Growth in the VP test, NaCl tolerance and the presence of lecithinase were negative under the conditions described by Gordon et al. (1973), even after prolonged incubation. Therefore, these tests were repeated, adding half a volume of a 0-1% (w/v) sterile yeast extract solution to the culture suspension prior to inoculation. As growth failed or was too poor for a sound evaluation, these tests were repeated using the following media. For the VP test, a medium consisting of 5.0 g glucose, 5.0 g peptone from tryptic-digested casein, and 5.0 g KH₂PO₄ in 1 l water, pH 7.2 was used. For the test of growth in the presence of different NaCl concentrations, culture tubes of Difco tryptic soy broth (prepared with the addition of yeast extract as described above) were set up containing various amounts of NaCl.

The lecithinase test was repeated with Difco tryptic soy broth agar medium to which 5 ml egg yolk (egg yolk emulsion; Oxoid) was added after the medium had cooled down following autoclaving.

Physiological reactions were also tested using the substrate panel of the API 50CH system (bioMérieux).

Microscopic investigations

Primary morphological characterization was done by light microscopy, including phase-contrast observations. The size and ultrastructure of cells and spores were determined by electron microscopy.

Light microscopy. For the investigation of cell morphology, 2% (w/v) agar (dissolved in water)-covered slides were prepared. A drop of culture grown in TSB medium (trypticase soy broth; Becton Dickinson) overnight was placed on the agar. Flagella were stained according to the method of Heimbrook et al. (1989).

Transmission electron microscopy (TEM). Cells were centrifuged, washed with PBS (0.145 M NaCl, 0.15 M sodium phosphate; Sambrook et al., 1989) and fixed on ice for 1 h with a fixation solution containing 3% (v/v) glutaraldehyde and 5% (v/v) formaldehyde. After three washings with PBS, samples were embedded in 1:5% (w/v) agar in water; the solidified agar was cut into small cubes and dehydrated on ice with a graded series of acetone (10, 30, 50 and 70% for 30 min each). At the 70% acetone dehydration step, the samples were treated with 2% (w/v) uranyl acetate in 70% acetone (overnight). Samples were then further dehydrated with 90 and 100% acetone (30 min on ice) and once in 100% acetone at room temperature for 1 h.

For infiltration with epoxy resin (using Spurr's resin formula; Spurr, 1969), samples were initially treated according to the following protocol: one part 100% acetone with one part resin for 6 h; one part acetone and two parts resin overnight; pure resin for another 2 d with several changes. Samples were then transferred to gelatin capsules which were filled with pure resin. Polymerization was at 70 °C for 8 h. After trimming of the samples, ultrathin sections cut with glass knives were collected onto Formvar-coated copper grids (300 mesh) and air-dried. Counter-staining was done with a Leica Ultrostainer (uranyl acetate at 40 °C for 30 min, lead citrate at 20 °C for 3 min). Samples were examined in a Zeiss EM910 TEM at an acceleration voltage of 80 kV and at calibrated magnifications.

Scanning electron microscopy (SEM). A culture was grown on the isolation agar medium for 24 h. Agar blocks were cut from the plate, fixed in 2.5% (v/v) glutaraldehyde and dehydrated on ice in a series of increasing acetone concentrations (10, 30, 50, 70, 90 and 100% for 10 min each). Preparations were dried at the critical point of liquid CO₂, sputter-coated with gold (10 nm thickness) and examined in a Zeiss model DSM 982 SEM.

Analysis of chemotaxonomic characteristics. Cell wall and lipid analysis were carried out as described previously (Groth et al., 1996) using the methods of Schleifer & Kandler (1972), MacKenzie (1987), Stead et al. (1992), Collins et al. (1977), Minnikin et al. (1979, 1975) and Collins & Jones (1980).
Isolation of DNA for determination of the G+C value (mol %) and for PCR-amplification of the 16S rRNA gene. Genomic DNA was extracted by an enzymic lysis procedure as described by Rainey et al. (1996). G+C contents were determined by HPLC (Mesbah et al., 1989). An aliquot of the isolated DNA was also used for PCR-mediated amplification of the 16S rDNA (Rainey et al., 1996). The PCR products were purified by using a QIAquick PCR purification kit (Qiagen) and directly sequenced using previously described protocols (Rainey et al., 1996). The sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems).

Phylogenetic analysis. The 16S rDNA sequence of isolate HR3-23T was initially aligned to the sequences of a general selection of different bacteria of the DSMZ identification database and, after a first phylogenetic analysis, with members of and relatives to the RNA group 2 of the genus Bacillus [as defined by Ash et al. (1991) and Farrow et al. (1994)] using the ae2 editor (Maidak et al., 1997). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). Phylogenetic dendrograms according to the method of De Soete (1983) and also dendrograms obtained by the use of the neighbour-joining method contained in the PHYLIP package (Felsenstein, 1993) were constructed. Bootstrap analysis (Felsenstein, 1985) was used to evaluate the tree topology of the neighbour-joining data by performing 500 resamplings.

RESULTS

Morphology

Strain HR3-23T developed whitish shiny colonies on agar media. It formed peritrichously flagellated rods with a length of 0.9-2.0 μm and a width of 0.5-0.7 μm (Fig. 1a, b). Round terminal spores having a diameter of 0.5-0.7 μm were observed in swollen sporangia after growth in TSB. The ultrastructural investigation of the
spore surface showed a cauliflower-like fine structure (Fig. 1c). Remaining parts of the lysed vegetative cell wall were still found to be attached to a few of the investigated spores (Fig. 1d). In the images obtained from the ultrathin sections of cells, the typical bilayered bacterial endospore cell wall structure was observed (Fig. 1e).

Physiological characteristics

Growth of isolate HR3-23T occurred at 10–40 °C with an optimum of 20–30 °C. The following results were obtained after cultivation at 28 °C. The organism did not grow at pH 5-7. Growth was observed on modified Difco tryptic soy broth agar (see Methods) in the presence of 0, 2 and 5% (w/v) NaCl; no growth was observed at these NaCl concentrations in medium according to Gordon et al. (1973). The isolate was positive for catalase and negative for oxidase. Anaerobic growth did not occur. No acid was produced from D-glucose, L-arabinose, D-xylose, D-mannitol and D-fructose. From the 49 substrates provided in the API 50CH strip, acid was produced only from sucrose after 3 d incubation.

Production of gas from glucose was not observed and the pH of carbohydrate-containing media was not changed. From the tests of the API 50CH auxiliary panel, only utilization of glycerol (after 48 h) and ribose (after 5 d) was indicated by an increased turbidity.

The VP test and egg yolk lecithinase test were negative, indole was not formed. No hydrolysis of casein, gelatin, starch, Tween 80 or aesculin was observed. Strain HR3-23T did not utilize citrate or propionate and did not degrade tyrosine. Deamination of phenylalanine was negative and nitrate was not reduced to nitrite. No growth occurred in the presence of lysozyme. Arginine dihydrolase activity was not detected.

Phylogenetic analysis and DNA base composition

A 1449 nt stretch of the 16S rRNA gene of isolate HR3-23T was sequenced, representing 94.0% of the Escherichia coli gene sequence (Brosius et al., 1978). Similarity values calculated for the sequence of strain HR3-23T and other bacterial sequences indicated a membership to the genus Bacillus. The similarity values were above 91% for members of the Bacillus RNA group 2 as defined by Ash et al. (1991) and Farrow et al. (1992). The highest values were found with members of the genus Caryophanon, i.e. Caryophanon temne NCDO 23224T (95.8%) and Caryophanon latum NCIMB 9533T (95.6%) and with Bacillus sphaericus NCDO 1767 (95.4%). The similarity of strain HR3-23T to the 16S rDNA sequence of the type strain of the species B. sphaericus, ATCC 14577T, was determined to be 94.2%. The sequences of NCDO 1767 and ATCC 14577T showed a similarity value of 98.5% to each other.

Irrespective of treeing algorithms, isolate HR3-23T branched adjacent to the pair B. sphaericus, Bacillus fusiformis and members of the genus Caryophanon. This branching order is also supported by high bootstrap values (Fig. 2; see also Table 1). The only variation was found in the branching order of members of the genera Planococcus and Kurthia. When the algorithm of De Soete (1983) was used, their position changed to form a lineage adjacent to B. sphaericus and related species (data not shown).

Chemotaxonomic properties

The fatty acid profile of isolate HR3-23T was characterized by the predominance of iso-C15:0 (44.2%), followed by iso-C16:1 (18.7%) fatty acids. Smaller amounts were found for iso-C14:0 (2.9%), anteiso-C15:0 (5.6%), iso-C16:0 (6.2%), C16:1 (3.1%), iso-C17:0 (5.1%), anteiso-C17:0 (3.0%), iso-C17:0 (7.8%), anteiso-C17:1 (2.8%) and C17:0 (0.9%). The peptidoglycan of isolate HR3-23T contained lysine, glutamic acid and alanine, representing peptidoglycan type A4\(\rightarrow\) (L-Lys-D-Glu) as described by Schleifer & Kandler (1972). Isoprenoid quinones were MK-7, MK-6 and MK-8 at a peak area ratio of 84:4:1. Polar lipids comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, minor amounts of phosphatidylserine and one unknown phospholipid.
Table 1. Chemotaxonomic properties of strain HR3-23T and related organisms from Bacillus RNA group 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Accession no.</th>
<th>Cell wall type*</th>
<th>Menaquinone system†</th>
<th>G + C (mol %)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X70321</td>
<td>L-Lys-d-Asp†</td>
<td>MK-7†</td>
<td>36.0–38.0†</td>
</tr>
<tr>
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<td>X70320</td>
<td>L-Lys-d-Asp†</td>
<td>MK-7†</td>
<td>36.0–38.0†</td>
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<tr>
<td>3</td>
<td>X70371</td>
<td>L-Lys-d-Glu‡</td>
<td>MK-7‡</td>
<td>44.0‡</td>
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<tr>
<td>4</td>
<td>X60644</td>
<td>L-Lys-d-Glu‡</td>
<td>MK-7‡</td>
<td>39.7–39.8‡</td>
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<td>X60634</td>
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<td>MK-7‡</td>
<td>39.7–40.5‡</td>
</tr>
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<td>MK-7‡</td>
<td>38.5–38.7‡</td>
</tr>
<tr>
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<td>MK-7‡</td>
<td>40.0–41.5‡</td>
</tr>
<tr>
<td>8</td>
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<td>Orn-d-Glu§</td>
<td>MK-7§</td>
<td>35.9–36.1§</td>
</tr>
<tr>
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<td>L-Lys-d-Glu§</td>
<td>MK-7, MK-8§</td>
<td>39–41§</td>
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<td>MK-7, MK-8§</td>
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<td>NA</td>
<td>35–36§</td>
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<td>MK-7†</td>
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<td>MK-7</td>
<td>39–3</td>
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<tr>
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<td>MK-6§</td>
<td>44.0–45.6§</td>
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</tbody>
</table>

NA, Data not available.

* Data from: 1, Shaw & Keddie (1983); 2, Stackebrandt et al. (1987); 3, Claus & Fritze (1989); 4, Ranftl & Kandler (1970); 5, Hao et al. (1985); 6, Kocur (1986).

† Data from: 1, Shaw & Keddie (1983); 2, Maiden & Jones (1984); 3, Fahmy et al. (1985); 4, D. Fritze, personal communication; 5, Hess et al. (1979); 6, Claus & Fahmy (1986); 7, Hao et al. (1985); 8, Kocur (1986); 9, Collins & Jones (1979); 10, Holt et al. (1994).

‡ Data from: 1, Shaw & Keddie (1983); 2, Maiden & Jones (1984); 3, Fahmy et al. (1985); 4, Claus & Fahmy (1986); 5, Hao et al. (1985); 6, Kocur (1986); 7, Priest et al. (1988); 8, Trentini (1986).

The G + C content of the DNA of isolate HR3-23T was 39.3 mol %.

DISCUSSION

Morphological and phylogenetic analyses have clearly demonstrated that strain HR3-23T is a member of the genus Bacillus. Based upon the results of physiological properties, strain HR3-23T appears to be most similar to Bacillus fastidiosus, but this phenotypic relationship is due to the significant number of negative test results obtained for the isolate and B. fastidiosus (Claus Berkeley, 1986). In addition, while B. fastidiosus requires a medium containing allantoin or uric acid (Claus & Berkeley, 1986), contains peptidoglycan with meso-diaminopimelic acid as the diagnostic amino acid and is phylogenetically a member of the Bacillus RNA group 1, strain HR3-23T contains peptidoglycan with lysine as the diagnostic amino acid and clusters with the Bacillus RNA group 2 and its non-Bacillus-type relatives.

The phylogenetic relationship among the round-spore-forming bacilli and their non-spore-forming relatives has been extensively investigated and discussed by Farrow et al. (1994). Their findings regarding the branching order within the RNA group 2 bacilli were confirmed by the present study. The sequence of Filibacter limicola was not included in the study of Farrow et al. (1994), but membership of this species in Bacillus RNA group 2 had previously been demonstrated by oligonucleotide cataloguing (Stackebrandt et al., 1987). In contrast to the few branching points which are supported by a high bootstrap value, most of the branching points of the more deeply branching lineages are not statistically significant, hence the order at which these lineages evolved remains uncertain. One of the few clusters supported by high bootstrap values embraces the genus Caryophanon and some Bacillus species, including the novel isolate HR3-23T. From a phylogenetic point of view, the latter strain and Caryophanon species share a common ancestry which strengthens the evolutionary origin of the morphologically different Caryophanon species from Bacillus-type ancestors.

Although the taxonomic status of the RNA group 2 bacilli has been discussed (Farrow et al., 1994), no formal proposal has as yet been forwarded which transfers the L-lysine- and ornithine-containing bacilli...
into one or, because of the phylogenetic intermixing with non-
Bacillus-type genera, several new genera. Hence, any strain worthy of species rank within RNA
group 2, exhibiting taxonomic properties that would
identify this taxon as a member of the genus Bacillus,
should still be described as a species of this genus.
Based upon phylogenetic, chemotaxonomic and mor-
phological properties, strain HR3-23T represents a
member of this genus; based upon phylogenetic
distance and physiological properties, strain HR3-23T
represents a new species. No DNA–DNA
hybridization studies were carried out, as the binary
16S rDNA similarity values obtained for strain HR3-
23T and its closest phylogenetic neighbours were low
even to exclude high DNA–DNA reassociation
values that would indicate membership to one of these
species (Stackebrandt & Goebel, 1994).

Description of Bacillus silvestris sp. nov.

Bacillus silvestris (sil.ves’tris. N.L. masc. n. silva forest,
referring to the place of isolation).

Gram-positive, rod-shaped, peritrichously flagellated,
endospore-forming, strictly aerobic organism. Round
spores lie terminally in a swollen sporangium. The rods
measure 0–9–20 µm by 0.5–0.7 µm; spore diameters
are 0.5–0.7 µm. The peptidoglycan contains lysine,
glutamic acid and alanine, representing peptidoglycan
type A4z. Polar lipids comprise phosphatidylglycerol,
diphosphatidylglycerol, phosphatidylethanolamine,
minor amounts of phosphatidylserine and one un-
known phospholipid. The dominant isoprenoid quin-
one is of the MK-7 type. The fatty acid profile is
characterized by the predominance of iso-C\textsubscript{15:0}
(44.2 %), followed by iso-C\textsubscript{16:0} (18.7 %) fatty acids.
Further compounds are: iso-C\textsubscript{15:1} (7.8 %), iso-C\textsubscript{16:0}
(6.2 %), anteiso-C\textsubscript{15:0} (5.6 %), iso-C\textsubscript{17:0} (5.1 %), C\textsubscript{16:1}
(3.1 %), anteiso-C\textsubscript{16:0} (3.0 %), iso-C\textsubscript{14:0} (2.9 %),
anteiso-C\textsubscript{17:1} (2.8 %) and C\textsubscript{17:0} (0.9 %). Temperature
range for growth is 10–40 °C with an optimum between
20 and 30 °C. Catalase-positive, oxidase-negative, VP
test-negative, no pH change in the growth medium.

Reactions towards the substrates provided in the
API 50CH panel indicate that glycerol and ribose are
utilized as sole carbon sources, but acid is not
produced. No acid from and no utilization of D-
ribose, arbutin, aesculin, salicin, methyl α-D-glucoside,
maltose, lactose, melezitose, inulin, melezitose,
D-raffinose, starch, glycogen, xylitol, β-
gentiobiose, D-turanose, D-lyxose, D-tagatose, D-
and L-fucose, D- and L-arabinose, glucose, 2-ketogluconate
and 5-ketogluconate. Casein, gelatin, starch, Tween 80
and aesculin are not hydrolysed. Citrate or propionate
are not utilized and tyrosine is not degraded.
Deamination of phenylalanine and the egg yolk
lecithinase test are negative. Nitrite is not produced
from nitrate, indole is not formed. The strain does not
grow at pH 5.7 or in the presence of lysozyme. It grows
in the presence of 0, 2 and 5 % (w/v) NaCl in modified
Difco tryptic soy broth agar. Arginine dihydrolase
is not present. The G+C content of the DNA is 39.3
mol %, as determined by HPLC. Phylogenetically,
the strain is HR3-23T (= DSM 12223T).

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