

Classification of *Ralstonia pickettii*-like isolates from the environment and clinical samples as *Ralstonia insidiosa* sp. nov.

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Thirteen *Ralstonia pickettii*-like isolates from the environment (water, soil and activated sludge) and human clinical samples (including respiratory secretions of cystic fibrosis patients) were investigated in a polyphasic taxonomic study that employed 16S rDNA sequence analysis, DNA–DNA hybridization, determination of DNA base composition, whole-cell protein analysis, biochemical characterization and PCR-based assays. All isolates were classified as a novel *Ralstonia* species, for which the name *Ralstonia insidiosa* sp. nov. is proposed. The type strain, LMG 21421^T (= CCUG 46789^T), was isolated from the sputum of a patient with acute lymphoblastic leukaemia. *R. insidiosa* can be differentiated from other species of the genus *Ralstonia* and phenotypically similar species (including the *Burkholderia cepacia* complex and *Achromobacter xylosoxidans*) by a variety of biochemical tests, whole-cell protein analysis and several PCR-based assays. Some outstanding issues in the taxonomy of the genus *Ralstonia* are also discussed.

INTRODUCTION

The genus *Ralstonia* was proposed in 1995 to accommodate the generically misplaced species *Burkholderia pickettii*, *Burkholderia solanacearum* and *Alcaligenes eutrophus* (Yabuuchi *et al.*, 1995). Since its proposal, a number of additional *Ralstonia* species have been described: *Ralstonia basilensis* (Steinle *et al.*, 1998), *Ralstonia gilardii* (Coenye *et al.*, 1999), *Ralstonia paucula* (Vandamme *et al.*, 1999), *Ralstonia oxalatica* (Sahin *et al.*, 2000), *Ralstonia mannitolilytica* (De Baere *et al.*, 2001), *Ralstonia campinensis* (Goris *et al.*, 2001), *Ralstonia metallidurans* (Goris *et al.*, 2001) and *Ralstonia taiwanensis* (Chen *et al.*, 2001). Thus, at the time of writing, the genus contains 11 species with validly published names.

Species of the genus *Ralstonia* occupy diverse ecological niches. *Ralstonia solanacearum* is an important phytopathogen that has an unusually broad host range and causes

bacterial wilt on a variety of economically important crops (Hayward, 1991). *Ralstonia eutropha*, *R. oxalatica*, *R. basilensis*, *R. campinensis* and *R. metallidurans* are environmental organisms; strains that belong to several of these species have considerable importance as possible agents for the bioremediation of soil and water contaminated with heavy metals or chlorinated organic compounds (Steinle *et al.*, 1998; Goris *et al.*, 2001). *Ralstonia pickettii* has been isolated from various clinical sources (Riley & Weaver, 1975; McNeil *et al.*, 1985) and has been found to be responsible for pseudobacteraemia (Verschraegen *et al.*, 1985). Strains of *R. mannitolilytica* (formerly known as '*Pseudomonas thomasi*' and *R. pickettii* biovar 3) have also been isolated from various clinical sources, including nosocomial recurrent meningitis (Philips *et al.*, 1972; De Baere *et al.*, 2001; Vaneechoutte *et al.*, 2001). *R. gilardii*, *R. paucula* and *R. taiwanensis* have been isolated both from human clinical samples and from the environment (Coenye *et al.*, 1999; Vandamme *et al.*, 1999; Chen *et al.*, 2001; Wauters *et al.*, 2001). Recent studies have demonstrated that *R. pickettii*, *R. mannitolilytica*, *R. gilardii* and *R. taiwanensis* can also be recovered from the respiratory tract of cystic fibrosis (CF) patients (Chen *et al.*, 2001; Coenye *et al.*, 2002a, b).

Here we report on the polyphasic taxonomic analysis of 13 strains that were initially identified as *R. pickettii*, *Ralstonia* sp. or *Burkholderia cepacia* complex. The strains

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Abbreviations: BDB, blood disease bacterium; CF, cystic fibrosis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Ralstonia insidiosa* LMG 21421^T is AF488779.

Figures showing normalized whole-cell protein profiles and PCR analysis with primer pair Rp-F1/R38R1 of *Ralstonia* strains are available as supplementary material in IJSEM Online.

were isolated from various environmental samples (river and pond water, soil and activated sludge) and from clinical samples, including the respiratory secretions of CF patients. The results of our study allow us to classify these 13 isolates as members of a novel *Ralstonia* species, for which we propose the name *Ralstonia insidiosa* sp. nov.

METHODS

Bacterial strains and growth conditions. The *R. insidiosa* strains used in this study, several of which have been deposited in the culture collections of the BCCM/LMG (Laboratorium voor Microbiologie Gent, Belgium) and CCUG (University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden), are listed in Table 1. Type and reference strains of all other *Ralstonia* species have been described previously (Coenye *et al.*, 1999; Vandamme *et al.*, 1999; Chen *et al.*, 2001; De Baere *et al.*, 2001; Goris *et al.*, 2001). Strains were grown aerobically on Mueller–Hinton broth (Becton Dickinson) supplemented with 1.8% (w/v) agar and incubated for 24 h at 32 °C, unless otherwise indicated.

SDS-PAGE of whole-cell proteins. Preparation of whole-cell proteins of all *R. insidiosa* strains and SDS-PAGE were performed as described previously (Pot *et al.*, 1994). Strains were grown for 48 h on trypticase soy agar (BBL) and incubated at 37 °C. Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis were performed using GelCompar 4.2 software (Applied Maths). Protein patterns from type and reference strains of all other *Ralstonia* species were available from previous studies (Coenye *et al.*, 1999; Vandamme *et al.*, 1999; Chen *et al.*, 2001; De Baere *et al.*, 2001; Goris *et al.*, 2001).

16S rDNA sequencing. Preparation of DNA, PCR amplification of the 16S rRNA gene and 16S rDNA sequencing were performed as described previously (Coenye *et al.*, 2002a). A phylogenetic tree based on the neighbour-joining method (Saitou & Nei, 1987) was constructed by using Kodon software (Applied Maths).

Determination of DNA base composition and DNA–DNA hybridization. DNA preparation, determination of DNA base composition by HPLC (Coenye *et al.*, 2001) and DNA–DNA

hybridizations with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989) were performed as described previously. The hybridization temperature was 50 °C.

PCR assays. PCR assays were performed in 25 µl reaction mixtures that contained 2 µl DNA solution, 1 U *Taq* polymerase (Gibco-BRL), 250 mM (each) dNTP (Gibco-BRL), 1.5 mM MgCl₂, 1 × PCR buffer (Gibco-BRL) and 20 pmol (each) oligonucleotide primer. Specific primers for *R. pickettii* (Rp-F1/Rp-R1) and *R. mannitolilytica* (Rm-F1/Rm-R1) were developed in a previous study (Coenye *et al.*, 2002b). Amplification was carried out using a PTC-100 programmable thermal cycler (MJ Research). After initial denaturation was performed for 2 min at 94 °C, 30 amplification cycles were completed, each consisting of 1 min at 94 °C, 1 min at the appropriate annealing temperature and 1 min 30 s at 72 °C. A final extension step of 10 min at 72 °C was applied. Negative PCR controls, containing all reaction mixture components except template DNA, were included for every experiment. The annealing temperatures used were 55 °C (for the identification of *R. insidiosa* and *R. pickettii*) or 57 °C (for the identification of *R. mannitolilytica*).

Evaluation of PCR assays. For evaluation of the PCR assay, 108 isolates were tested. These included 13 *R. insidiosa* strains (listed in Table 1), 71 strains that represented all other *Ralstonia* species and 24 type or reference strains that represented phylogenetically related species, including all members of the *B. cepacia* complex (*B. cepacia* genomovars I, III and VI, *Burkholderia multivorans*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia ambifaria*, *Burkholderia anthina* and *Burkholderia pyrrocinia*), *Burkholderia gladioli*, *Pandoraea apista*, *Pandoraea norimbergensis*, *Pandoraea pnomenus*, *Pandoraea sputorum*, *Pandoraea pulmonicola*, *Achromobacter xylosoxidans*, *Achromobacter denitrificans*, *Alcaligenes faecalis*, *Bordetella avium*, *Bordetella hinzii*, *Bordetella bronchiseptica*, *Bordetella pertussis* and *Bordetella parapertussis*.

Phenotypic characterization. For conventional biochemical testing, bacteria were grown on Mueller–Hinton agar plates for 24–48 h and then inoculated into the test reagent. Oxidase and catalase tests were performed with 1% tetramethyl *p*-phenylenediamine dihydrochloride and 3% hydrogen peroxide, respectively. Lysine decarboxylase, ONPG and oxidation–fermentation sugars (sucrose and lactose) were obtained from Remel. These test reactions were incubated at 37 °C and examined daily for 7 days except for ONPG,

Table 1. List of *Ralstonia insidiosa* sp. nov. strains studied

Culture collections are abbreviated as: ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Sweden; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Belgium.

| Strain | Source | Deposited by |
|--|--------------------------------------|--------------|
| AU2944 ^T =LMG 21421 ^T =CCUG 46789 ^T | Sputum, patient with leukaemia (USA) | D. Shelly |
| C6137=LMG 18101=CCUG 38754=HI2202 | Sputum, CF patient (Canada) | D. Henry |
| FC262=LMG 18111=CCUG 38748=ATCC 49129=HI2126 | Unspecified clinical sample (USA) | D. Henry |
| R-4241=HI2131 | Sputum, CF patient (Canada) | D. Henry |
| KA1=LMG 18324 | Activated sludge (Japan) | T. Saito |
| KS9=LMG 18343 | Farm soil (Japan) | T. Saito |
| KR4=LMG 18330 | River water (Japan) | T. Saito |
| FR2t1=LMG 18321 | River water (Japan) | T. Saito |
| A1=LMG 18319 | Activated sludge (Japan) | T. Saito |
| K1=LMG 18323 | River water (Japan) | T. Saito |
| KR6=LMG 18332 | River water (Japan) | T. Saito |
| TP6=LMG 18363 | Pond water (Japan) | T. Saito |
| CCUG 38964=R-4041 | Water (Sweden) | E. Falsten |

which was examined at 24 and 48 h. The RapID NF Plus (Remel) and API 20 NE (bioMérieux) commercial identification systems were used, according to the manufacturers' instructions. Growth on *B. cepacia* selective agar (BCSA) that contained 600 U polymyxin B ml⁻¹, 10 µg gentamicin ml⁻¹ and 2.5 µg vancomycin ml⁻¹ was determined at 32 °C.

RESULTS AND DISCUSSION

Taxonomic structure and phylogenetic position of *R. insidiosa*

The reproducibility of SDS-PAGE of whole-cell proteins was checked by preparing protein extracts in duplicate. The correlation level between patterns obtained with different extracts of the same strain was >93% (data not shown). By visual comparison of the protein patterns, all *R. insidiosa* isolates were virtually indistinguishable. However, after numerical analysis of the patterns, the *R. insidiosa* isolates clustered in two subgroups (see supplementary figure available in IJSEM Online). This is due to slight but reproducible distortions and variations in the protein pattern (mainly in the region between 40 and 60 kDa), which can have a profound effect on numerical analysis, as discussed previously (Vandamme *et al.*, 1997, 1999; Goris *et al.*, 2001). Reference strains of other *Ralstonia* species were characterized by distinct protein patterns and occupied separate positions in the dendrogram (see supplementary figure available in IJSEM Online).

The 16S rRNA gene of *R. insidiosa* LMG 21421^T (GenBank no. AF488779) has high similarity to the 16S rRNA genes of reference strains of *R. pickettii* (97.8%) and *R. mannitolilytica* (97.5%) (Fig. 1). Similarity to the 16S rRNA genes of other *Ralstonia* species (including *R. solanacearum*) is <97.0% (Fig. 1). This clearly indicates that this organism belongs to the genus *Ralstonia*. The DNA G + C content

of *R. insidiosa* strains LMG 21421^T and LMG 18319 was 64.3 and 63.9%, respectively. This DNA base ratio of 63.9–64.3% is within the range of the genus *Ralstonia* [63–70% (Yabuuchi *et al.*, 1995; Goris *et al.*, 2001)].

DNA–DNA hybridization between *R. insidiosa* strains from both protein-electrophoretic subgroups (LMG 21421^T and LMG 18319) revealed a DNA–DNA binding value of 100%. DNA–DNA binding values with the type strain of *R. pickettii* (47% with LMG 21421^T and 48% with LMG 18319) and the type strain of *R. mannitolilytica* (45% with both LMG 21421^T and LMG 18319) were low. This clearly demonstrates that the isolates in both electrophoretic subgroups represent a single, novel *Ralstonia* species.

Identification of *R. insidiosa*

For the development of species-specific PCR primers, 16S rDNA sequences of all *Ralstonia* species and representatives of related genera were retrieved from GenBank and aligned using MegAlign software (DNASStar). Based on this alignment, reverse primer R38R1 (5'-CACACCTAA-TATTAGTAAGTGCG-3') was developed. Primer R38R1 was used in combination with the previously developed forward primer Rp-F1 (5'-ATGATCTAGCTTGCTAGAT-TGAT-3') (Coenye *et al.*, 2002b) and allowed the specific amplification of a 403 bp 16S rDNA fragment from all *R. insidiosa* strains, without cross-reactivity with any other *Ralstonia*, *Burkholderia*, *Pandoraea*, *Achromobacter* or *Bordetella* species tested (data not shown). None of the *R. insidiosa* strains reacted with primer pair Rm-F1/Rm-R1 (developed for the identification of *R. mannitolilytica*), but all *R. insidiosa* strains cross-reacted with primer pair Rp-F1/Rp-B1 (developed for the identification of *R. pickettii*) (data not shown). Therefore, the combined use of these three PCR assays will allow the sensitive and specific identification of *R. insidiosa*, *R. pickettii* and *R. mannitolilytica*.

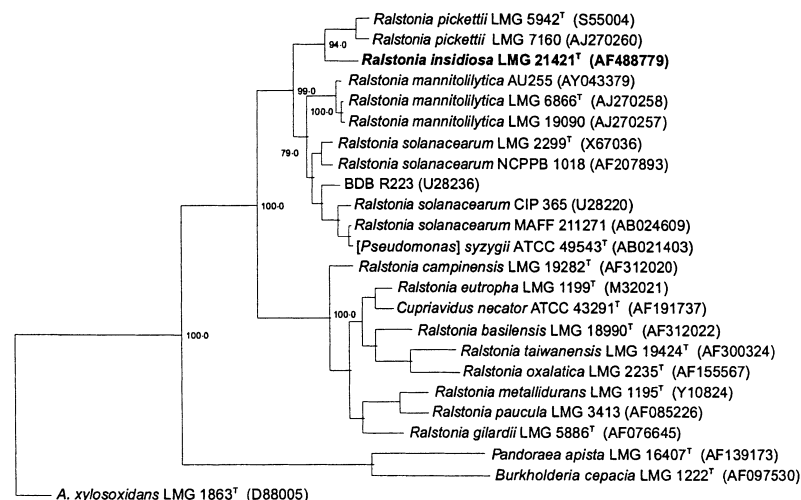


Fig. 1. Phylogenetic tree based on 16S rDNA sequence data, showing the position of *R. insidiosa* within the genus *Ralstonia*. GenBank accession numbers are shown in parentheses. Bar, 10% sequence dissimilarity.

All *R. insidiosa* strains grew at 28, 32 and 37 °C and on BCSA, showed oxidase, catalase, lipase, phosphatase, proline aminopeptidase, pyrrolidonyl aminopeptidase and γ -L-glutamyl aminopeptidase activities, and assimilated glucose, gluconate, caprate, adipate, malate and citrate. All *R. insidiosa* strains reduced nitrate. Activities of lysine decarboxylase, arginine dihydrolase, α -glucosidase, β -glucosidase, tryptophan aminopeptidase and *N*-benzylarginine aminopeptidase were not observed. None of the *R. insidiosa* strains assimilated arabinose, mannose, mannitol or maltose. Indole production or the production of acid from glucose, sucrose or lactose was not observed. None of the isolates showed β -galactosidase activity when tested with the API 20 NE or RapID NF Plus systems, but tube ONPG tests gave weakly positive results after 48 h incubation. Assimilation of *N*-acetylglucosamine and phenylacetate and the presence of urease activity were strain-dependent characteristics.

With the API 20 NE system, *R. insidiosa* strains were identified as *A. xylosoxidans* ('good identification', profile 1040477) or *R. pickettii* ('good identification', profile 5240477 or 'low discrimination', profile 1240575). With the RapID NF Plus system, all *R. insidiosa* strains were identified as *Shewanella putrefaciens* ('adequate identification', profile 430616 or 'questionable identification', profile 434616). Phenotypically, *R. insidiosa* can be differentiated from its closest neighbours *R. pickettii* and *R. mannitolilytica* by lack of assimilation of arabinose, mannitol and *N*-acetylglucosamine. Differentiation of *R. insidiosa* from *R. solanacearum* is possible by the lack of assimilation of phenylacetate and caprate in the latter. Characteristics that

are useful for differentiation of *R. insidiosa* from other *Ralstonia* species are shown in Table 2. The absence of lysine decarboxylase activity allows differentiation from most members of the *B. cepacia* complex (except *B. multivorans* and *B. cepacia* genomovar VI); differentiation from the two latter species is possible (Coenye *et al.*, 2001) due to the lack of acid production from lactose in *R. insidiosa*. Several *R. insidiosa* strains were identified as *A. xylosoxidans* by using the API 20 NE system, but unlike *A. xylosoxidans* (von Graevenitz, 1995), *R. insidiosa* does not produce acid from glucose. All *R. insidiosa* strains were identified as *S. putrefaciens* with the RapID NF Plus system. However, *R. insidiosa* can easily be differentiated from *S. putrefaciens* by the lack of citrate and malate assimilation in the latter species (Venkateswaran *et al.*, 1999).

Outstanding issues in the taxonomy of the genus *Ralstonia*

Comparative 16S rDNA sequence analysis has suggested that there are two sublineages within the genus *Ralstonia* (De Baere *et al.*, 2001); the present phylogenetic analysis confirms this (Fig. 1). The *R. pickettii* lineage comprises *R. pickettii*, *R. mannitolilytica*, *R. solanacearum* and *R. insidiosa*. The *R. eutropha* lineage comprises *R. eutropha*, *R. gilardii*, *R. paucula*, *R. oxalatica*, *R. taiwanensis*, *R. basilensis*, *R. metallidurans* and *R. campinensis*.

It has previously been reported that the phytopathogenic organisms [*Pseudomonas*] *syzygii* (causal agent of Sumatra disease of cloves) and the 'blood disease bacterium' (BDB, causal agent of blood disease of bananas) are closely related to *R. solanacearum* (Taghavi *et al.*, 1996; Anzai *et al.*, 2000);

Table 2. Characteristics that are useful for the differentiation of *R. insidiosa* from other *Ralstonia* species

Species: 1, *R. insidiosa*; 2, *R. pickettii*; 3, *R. mannitolilytica*; 4, *R. solanacearum*; 5, *R. gilardii*; 6, *R. paucula*; 7, *R. taiwanensis*; 8, *R. eutropha*; 9, *R. campinensis*; 10, *R. basilensis*; 11, *R. metallidurans*; 12, *R. oxalatica*. Data for *R. insidiosa* were obtained during this study; data for *R. oxalatica* are from Gillis *et al.* (1995); data for all other *Ralstonia* species are from Coenye *et al.* (1999), Vandamme *et al.* (1999), Chen *et al.* (2001), De Baere *et al.* (2001) and Goris *et al.* (2001). Reactions are scored as: +, >90% of strains reacted positively; -, <10% of strains reacted positively; v, 10–90% of strains reacted positively; w, weakly positive reaction following 48 h incubation.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-----------------------------|---|---|---|---|---|---|---|---|---|----|----|----|
| Positive reaction with: | | | | | | | | | | | | |
| Primers Rp-F1/R38-R1 | + | - | - | - | - | - | - | - | - | - | - | - |
| Primers Rp-F1/Rp-R1 | + | + | - | - | - | - | - | - | - | - | - | - |
| Primers Rm-F1/Rm-R1 | - | - | + | - | - | - | - | - | - | - | - | - |
| Nitrate reduction | + | + | - | + | - | - | + | + | + | - | + | + |
| Urease activity | v | v | + | - | - | + | - | - | + | - | - | v |
| Assimilation of: | | | | | | | | | | | | |
| Arabinose | - | + | + | - | - | - | - | - | - | - | - | - |
| Mannitol | - | - | + | - | - | - | - | - | - | - | - | - |
| <i>N</i> -Acetylglucosamine | - | + | + | - | - | - | - | + | - | - | - | v |
| Phenylacetate | + | + | - | - | - | + | + | + | + | + | + | + |
| Caprate | + | + | + | - | - | + | + | + | + | + | + | v |
| Citrate | + | + | + | + | - | + | v | + | - | + | + | + |
| Adipate | w | w | - | - | - | + | - | + | + | + | + | v |

this is confirmed in our study (Fig. 1). However, previous studies using DNA–DNA hybridization, 16S rDNA sequencing and AFLP (amplified fragment length polymorphism) fingerprinting have indicated that *R. solanacearum* is taxonomically diverse (Palleroni & Doudoroff, 1971; Hayward, 1991; Taghavi *et al.*, 1996; Poussier *et al.*, 2000) and a thorough reassessment of the relationships between the phylogenetic subdivisions within *R. solanacearum*, and between *R. solanacearum*, [*P.*] *syzygii* and the BDB, seems necessary before any taxonomic changes are made.

The species *Cupriavidus necator* was proposed by Makkar & Casida (1987). This organism (represented by a single isolate, ATCC 43291^T) is highly resistant to copper and is a non-obligate predator of bacteria in soil. Based on 16S rDNA sequence analysis, this organism clearly belongs to the genus *Ralstonia*, with *R. eutropha* as its closest relative (99.3% sequence identity; Fig. 1). However, its DNA G + C content was reported to be 57 ± 1 mol%, which is significantly below the range reported for the genus *Ralstonia*; additional taxonomic data will be required to accurately determine the status of this organism.

A survey of GenBank revealed the presence of several 16S rDNA sequences derived from as-yet-unclassified *Ralstonia* species. Most of these have been collected from soil or water polluted with various organic compounds or heavy metals. Alignment of these 16S rDNA sequences with those of representatives of all *Ralstonia* species revealed that several of these isolates probably belong to existing *Ralstonia* species, while others were more distantly related and probably represent novel taxa (data not shown). Polyphasic taxonomic studies including DNA–DNA hybridization will ultimately be required to determine the status of these isolates and to determine whether strains with potential bioremediation applications are distinct from pathogens of humans and plants.

Description of *Ralstonia insidiosa* sp. nov.

Ralstonia insidiosa (in.si.di.o'sa. L. fem. adj. *insidiosa* deceitful, dangerous, referring to the fact that these seemingly harmless environmental organisms can be isolated from, and possibly cause infection in, humans).

Cells are Gram-negative, non-sporulating, aerobic, non-fermentative, motile rods. Growth occurs at 28, 32 and 37 °C and on BCSA. Catalase, oxidase, lipase, phosphatase, proline aminopeptidase, pyrrolidonyl aminopeptidase and γ-L-glutamyl aminopeptidase activities are present. No lysine decarboxylase, arginine dihydrolase, gelatinase, α-glucosidase, β-glucosidase, tryptophan aminopeptidase or *N*-benzylarginine aminopeptidase activity is detected. No indole production or production of acid from glucose, sucrose or lactose occurs. Assimilates glucose, gluconate, caprate, adipate, malate and citrate but not arabinose, mannose, mannitol or maltose. Reduces nitrate. Characteristics that differentiate *R. insidiosa* from other *Ralstonia*

species are summarized in Table 2. The DNA base composition is 63.9–64.3 mol% G + C.

The type strain, LMG 21421^T (=CCUG 46789^T), was isolated from the sputum of a patient with acute lymphoblastic leukaemia in the USA in 2001. In addition to the characteristics described for the species, the type strain shows no urease activity, does not assimilate *N*-acetylglucosamine and assimilates phenylacetate.

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