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**Jennifer Loveland-Curtze · Peter P. Sheridan**
**Kevin R. Gutshall · Jean E. Brenchley**

# Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the *Arthrobacter* subgroup and description of *Arthrobacter psychrolactophilus*, sp. nov.

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**Abstract** During our work on psychrophilic microorganisms we obtained a large collection of new isolates. In order to identify six of these, we examined their growth properties, cell wall compositions, and their 16S rRNA gene sequences. The results showed that all of the isolates are gram-positive, aerobic, contain lysine in their cell walls, and belong to the high mol% G+C *Arthrobacter* subgroup. Phylogenetic analysis of the 16S rRNA genes grouped five isolates obtained from a small geographical region into a monophyletic clade. Isolate B7 had a 16S rRNA sequence that was 94.3% similar to that of *Arthrobacter polychromogenes* and 94.4% similar to that of *Arthrobacter oxydans*. Primary characteristics that distinguish isolate B7 from the *Arthrobacter* type strain (*Arthrobacter globiformis*) and *A. polychromogenes* include lack of growth at 37°C, growth at 0–5°C, the ability to use lactose as a sole carbon source, and the absence of blue pigments. Because of these differences, isolate B7 was chosen as a type strain representing a new *Arthrobacter* species, *Arthrobacter psychrolactophilus*. The sixth isolate, LV7, differed from the other five because it did not have the rod/coccus morphological cycle and was most closely related to *Arthrobacter agilis*.

**Key words** *Arthrobacter psychrolactophilus* · Psychrophilic · Cell wall · Rod/coccus morphology · 16S rRNA phylogeny

## Introduction

Research in our laboratory has focused on the isolation of psychrophilic microorganisms and the characterization of their cold-active enzymes, particularly β-galactosidases. During this work we have isolated numerous organisms from geographically distant habitats ranging from Pennsylvania farmlands to Antarctica. Many of these psychrophiles are gram-positive, non-spore-forming rods. Four isolates (B7, D2, D5, and D10) were physiologically characterized and assigned to the genus *Arthrobacter* because they are strict aerobes, have rod/coccus morphological cycles, and contain lysine in their cell walls (Loveland et al. 1994; DePrada et al. 1996).

*Arthrobacter* species are members of the high mol% G+C actinomycete-coryneform bacteria (Stackebrandt and Woese 1981; Jones and Collins 1986; Jones and Keddie 1992). Criteria for distinguishing closely related genera has traditionally relied on morphological characteristics such as a rod/coccus cycle and the composition of the cell wall peptidoglycan, especially the identity of the diamino acid. This grouping, however, contains a diverse collection of bacterial taxa, not all of which form irregular rods or contain the same diamino acid in their cell walls (Jones and Collins 1986). In addition, reliance on chemotaxonomic characteristics yields little insight into the evolutionary relationships among these organisms. Previous work, primarily by Stackebrandt and his colleagues, has catalogued and compared the 16S rRNA sequences of several members of the high mol% G+C gram-positive organisms (Stackebrandt and Fiedler 1979; Koch et al. 1994, 1995; Rainey et al. 1994; Stackebrandt et al. 1995). The results of these extensive studies demonstrate that phylogenetic intermixing of genera can arise and illustrate the difficulty of using chemotaxonomic features alone for identification (Rainey et al. 1994; Koch et al. 1995; Stackebrandt et al. 1995).

The heterogeneity of the different coryneform bacteria is also illustrated by the comparison of the 16S rRNA sequences in the Ribosomal Database Project (RDP; (Maidak et al. 1994). Sequences from most of the aerobic and some of the facultative coryneform taxa cluster in a collection designated by the RDP as the *Arthrobacter* group, which encompasses the *Microbacterium* subgroup, the *Clavibacter* assemblage, the *Arthrobacter* subgroup, and the *Der-*

J. Loveland-Curtze · P. P. Sheridan · K. R. Gutshall
J. E. Brenchley (✉)
Department of Biochemistry and Molecular Biology,
The Pennsylvania State University, 209 South Frear,
University Park, PA 16802, USA
e-mail: jeb7@psu.edu,
Tel.: +1-814-8637794, Fax: +1-814-8653330

**Table 1** Comparison of the amino acid composition in cell walls of the isolates B7, D5, D2, D10, RG1, LV7, and *Arthrobacter globiformis*. The values for *A. globiformis* are an average of four amino acid determinations, and the total picomol were 33,381. The values for the isolates B7, D5, and D2 are averages of two amino aacid determinations. The total picomol were 21,079 for isolate B7, 31,697 for isolate D5, and 33,519 for isolate D2. The total picomol were 24,168 for isolate D10, 41,663 for isolate RG1, and 25,636 for isolate LV7. Minor amounts of other amino acids are not reported

| | Amino acid composition (% of total picomol) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Isolate | LYS | DAP | ORN | ALA | THR | SER | GLY | GLU | LEU |
| *A. globiformis* | 13.4 | 0.5 | 0.1 | 44.0 | 2.3 | 2.9 | 5.1 | 11.0 | 5.0 |
| B7 | 14.8 | 1.4 | 0.07 | 50.0 | 5.1 | 3.7 | 4.1 | 10.7 | 2.5 |
| D5 | 13.3 | 1.4 | 0.01 | 50.1 | 5.6 | 4.0 | 4.0 | 10.4 | 2.5 |
| D2 | 13.9 | 1.8 | 0.06 | 46.4 | 6.0 | 3.8 | 4.7 | 10.8 | 3.1 |
| D10 | 11.8 | 0.0 | 0.0 | 42.9 | 11.2 | 2.4 | 4.0 | 10.8 | 4.4 |
| RG1 | 12.9 | 0.0 | 0.0 | 38.8 | 10.7 | 2.6 | 4.1 | 11.4 | 4.6 |
| LV7 | 9.2 | 0.0 | 0.0 | 40.6 | 8.8 | 2.2 | 6.0 | 10.6 | 4.8 |

*matophilus* subgroup. Traditional genus designations lie within these groups. For example, the *Arthrobacter* subgroup includes the genera *Arthrobacter, Brevibacterium, Micrococcus, Renibacterium*, and *Rothia*. The major taxonomic properties distinguishing these genera have been cell shape, the presence or absence of a rod/coccus morphological cycle, and the presence of either lysine or mesodiaminopimelic acid as the predominant diamino acid in the cell wall (Table 1).

Considering the difficulty of accurately placing organisms in the *Arthrobacter* subgroup into appropriate genera, we undertook the current study to identify our isolates. The first goal was to further characterize our psychrophilic isolates B7, D2, D5, and D10 by determining whether their phylogenetic placement was consistent with the previous physiological data. Second, we wanted to determine and compare the properties of two new isolates, RG1 and LV7. Third, we examined whether the diamino acids present in the cell walls of our isolates corresponded with their phylogenetic placement and if our findings would clarify whether features such as presence of a rod/coccus cycle and lysine in the cell wall are useful for identifying members of the *Arthrobacter* subgroup. And fourth, we addressed the question of whether the isolates were similar to previously characterized organisms or if they represented new and distinct species by comparing their chemotaxonomic and phylogenetic placements with those previously examined. In order to characterize these isolates, we examined their growth and morphology, analyzed their cell walls, and sequenced their 16S rRNA genes. We report here the results for these six isolates, which show that they are indeed distinct from previously reported species and that five form a clade within the *Arthrobacter* subgroup, whereas one, although within the *Arthrobacter* subgroup, is more closely related to *Arthrobacter agilis* [recently reclassified from *Micrococcus agilis* (Koch et al. 1995)].

## Materials and methods

### Isolation and cultivation

Isolates B7, D2, D5, and D10 were obtained from soil samples from whey-enriched farm fields (Loveland et al. 1994; DePrada et al. 1996), and strain RG1 was obtained from a limestone quarry. Isolate LV7 was obtained from a cyanobacterial mat sample from a lake (designated "Lake Vestal") located near the Miers and Adams glaciers in Antarctica. Portions of frozen mat were inoculated into Instant Ocean medium [1% peptone, 0.2% lactose, and 75% strength seawater, which was reconstituted from Instant Ocean salts (Aquarium Systems, Mentor, Ohio, USA)] and incubated at 5°C. Cells growing in this enrichment were purified by restreaking on Instant Ocean agar [1% peptone, 0.2% lactose, 75% strength seawater, which was reconstituted from Instant Ocean salts (Aquarium Systems), and 1.5% agar] and incubated at 5°C. The *A. globiformis* ATCC 8010 type strain was obtained from L. E. Casida, Jr., (Pennsylvania State University).

Trypticase soy broth (TSB) and trypticase soy agar (TSA) with no added carbohydrate were used for determining cell morphology and for determining the temperature ranges for growth, except for the isolate obtained from Lake Vestal. Isolate LV7 was grown in either 75% or 100% seawater medium prepared using Instant Ocean salts. BactoPeptone (1%; Difco, Detroit, Mich., USA) and 0.2% lactose or glucose were added to the seawater medium.

For determining carbohydrate utilization and vitamin requirements, isolates were grown in M9 medium (Miller 1972) containing 0.2% carbohydrate and/or 1 ml Medium Eagle Vitamin Solution (Gibco BRL, Gaithersburg, Md., USA) per 100 ml. To check for utilization of nicotine and production of pigment, isolate B7 was cultured on nicotine agar composed of 0.4% nicotine, 0.2% $KH_2HPO_4$, 0.5% KCl, 0.01% yeast extract, 0.0025% $MgSO_4$, 0.0025% $FeSO_4$, 1.5% agar, and tap water, with the pH adjusted to 6.8. Isolate B7 was grown on peptone-yeast agar (1% peptone, 2% glycerol, 0.6% KCl, and 2% agar) to determine production of the blue pigments characteristic of *A. polychromogenes*.

### Enzyme determinations

General enzyme production was determined using API Coryne test strips (Bio-Merieux Vitek, Hazelwood, Mo., USA). Gelatin hydrolysis was detected by inoculating cells onto nutrient agar containing 5% gelatin and, after growth, by flooding the agar with saturated ammonium sulfate to highlight the zones of hydrolysis.

### Cell wall characterization

Cell wall extracts were prepared according to the short method described by Schleifer and Kandler (1972). The extracts were then hydrolyzed completely, and quantitative amino acid analyses were done at The Hershey Medical Center of the Pennsylvania State University or at The University of Michigan Protein and Carbohydrate Structure Facility.

### Scanning electron microscopy

Isolate B7 was grown in TSB at 25°C, and cells were collected by filtration through a Millipore Swinney disk holder containing a polycarbonate filter with a pore size of 0.2 μm. The cells were prepared using the methods of Kormendy (1975) and were examined using a JEOL JSM 5400 scanning electron microscope.

### Fatty acid determinations

The fatty acid composition for isolate B7 was determined by both MIDI (Microbial ID, Newark, Del., USA) and by IEA (Industrial

and Environmental Analysts, Essex Junction, Vt., USA). The results of the independent measurements were similar, and the averages of the two determinations are reported.

### DNA base composition

The mol% G+C was measured for purified chromosomal DNA extracted from isolate B7 by determining the melting temperature as described by Mandel and Marmur (1968). Purified chromosomal DNA from *Escherichia coli* ATCC 23848 was used as an independent control.

### 16S rRNA sequencing and comparisons

The genomic DNA was isolated using standard molecular methods (Sambrook et al. 1989) except in the case of isolate LV7, in which modifications of these methods were used (Giovannoni et al. 1990). The 16S rRNA genes were amplified using the polymerase chain reaction (PCR) from chromosomal DNA with Ready-To-Go beads (Pharmacia) and the universal primers 8 FPL and 1492 RPL (Pace et al. 1986). The products were sequenced at the Pennsylvania State University Nucleic Acid Facility on an ABI 370 sequencing apparatus. The 16S rRNA gene sequences were aligned using the ESEE program (Cabot 1987–1990) with those from the Ribosomal Database Project (RDP) (Maidak et al. 1994) and from a BLAST search of the NCBI database. Alignment was based on 1,454 nucleotide positions, and the isolate gene sequences corresponded to *E. coli* positions 25–1449 (B7), 67–1449 (D2), 70–1449 (D10), 47–1449 (LV7), 28–1345 (RG1), and 255–1345 (D5). Although the sequence from isolate D5 was part of the clade containing isolates B7, D2, D10, and RG1, its sequence was not included in the final analyses to avoid tree branch distortion due to its abbreviated length (1,092 nucleotides).

Phylogenetic analyses were performed using the PAUP (Swofford 1993) and PHYLIP (Felsenstein 1993) programs. Trees generated by the PAUP program were congruent with those generated by the PHYLIP program. The CLUSTAL V program package was used to calculate the percent sequence similarity. Parameters for pairwise comparisons were a K-tuple value of 2 and a gap penalty of 5. Multiple alignment parameters were a gap penalty of 10 and a gap length penalty of 10. The percent sequence similarities were not affected by changes in the multiple alignment parameters, indicating that the data were robust. The 16S rRNA gene sequences were placed with GenBank under accession nos. AF134179 (isolate B7), AF134181 (isolate D2), AF134182 (isolate D5), AF134180 (isolate D10), AF134183 (isolate RG1), and AF134184 (isolate LV7).

## Results

### Physiology and morphology

Physiological characterization showed that all the isolates grow at 0–5°C and do not grow at 37°C; thus, they are psychrophiles as defined by Neidhardt et al. (1990). Isolates B7, D2, and D5 grow at 30°C, but not at 31°C. Isolates D10 and RG1 grow at 25°C, but not at 30°C, whereas isolate LV7 fails to grow above 24°C. All isolate colonies are circular and convex, and exhibit varying degrees of yellow pigmentation depending upon the growth medium used and the incubation temperature. The isolates are gram-positive, obligate aerobes.

Isolates B7, D2, D5, D10, and RG1 all have a marked rod/coccus cycle. Scanning electron micrographs (Fig. 1) of isolate B7 cells harvested during exponential and stationary phases show the presence of pleiomorphism and fragmentation of club-like rods into short rods and coccoid cells. These results confirm earlier studies of the rod/coccus cycle for isolates B7, D2, D5, and D10 (Loveland et al. 1994; DePrada et al. 1996) in which isolates B7, D2, and D5 showed average cell lengths of 1.4, 1.2, and 1.2, respectively, during exponential growth and 0.5, 0.6, and 0.6, respectively, during stationary phase. In contrast to these results, isolate LV7 failed to show a rod/coccus cycle. When isolate LV7 cells were grown in Instant Ocean broth containing 1% peptone, the cells were very short rods or large cocci, often forming irregular clusters, during all phases of the growth cycle. The morphology of LV7 cells is similar to that observed for organisms that placed in the genus *Micrococcus*.

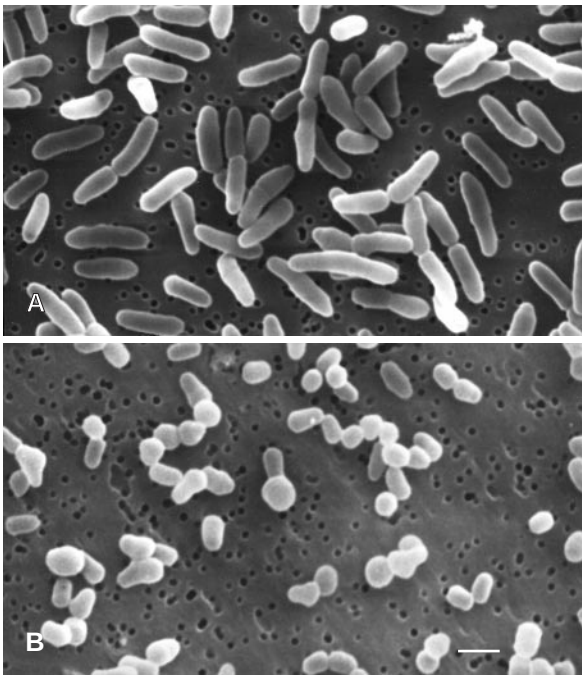


**Fig. 1 A, B** Scanning electron micrographs showing the rod/coccus cycle of isolate B7 cells growing in trypticase soy broth. **A** Club-shaped cells harvested at 230 Klett units. **B** Short rods and coccoid cells harvested at 450 Klett units (*bars* 1 μm)

Members of both the *Arthrobacter* and the *Brevibacterium* genera have a rod/coccus cycle. A major chemotaxonomic feature of these genera is the type of diamino acid found in the peptidoglycan interpeptide bridge. Lysine is typically found in the cell wall of *Arthrobacter* species, whereas *meso*-diaminopimelic acid (DAP) is present in *Brevibacterium* species. In order to distinguish between these genera, we analyzed the cell wall compositions of our isolates. The results showed that all contain lysine rather than DAP as the predominant diamino acid in their cell walls (Table 1). These results are consistent with the traditional taxonomic placement of isolates B7, D2, D5, D10, and RG1 within the genus *Arthrobacter.*

Measurements of the base composition of DNA purified from isolate B7 gave a value of 60.6 mol% G+C. This value is consistent with results obtained by sequencing

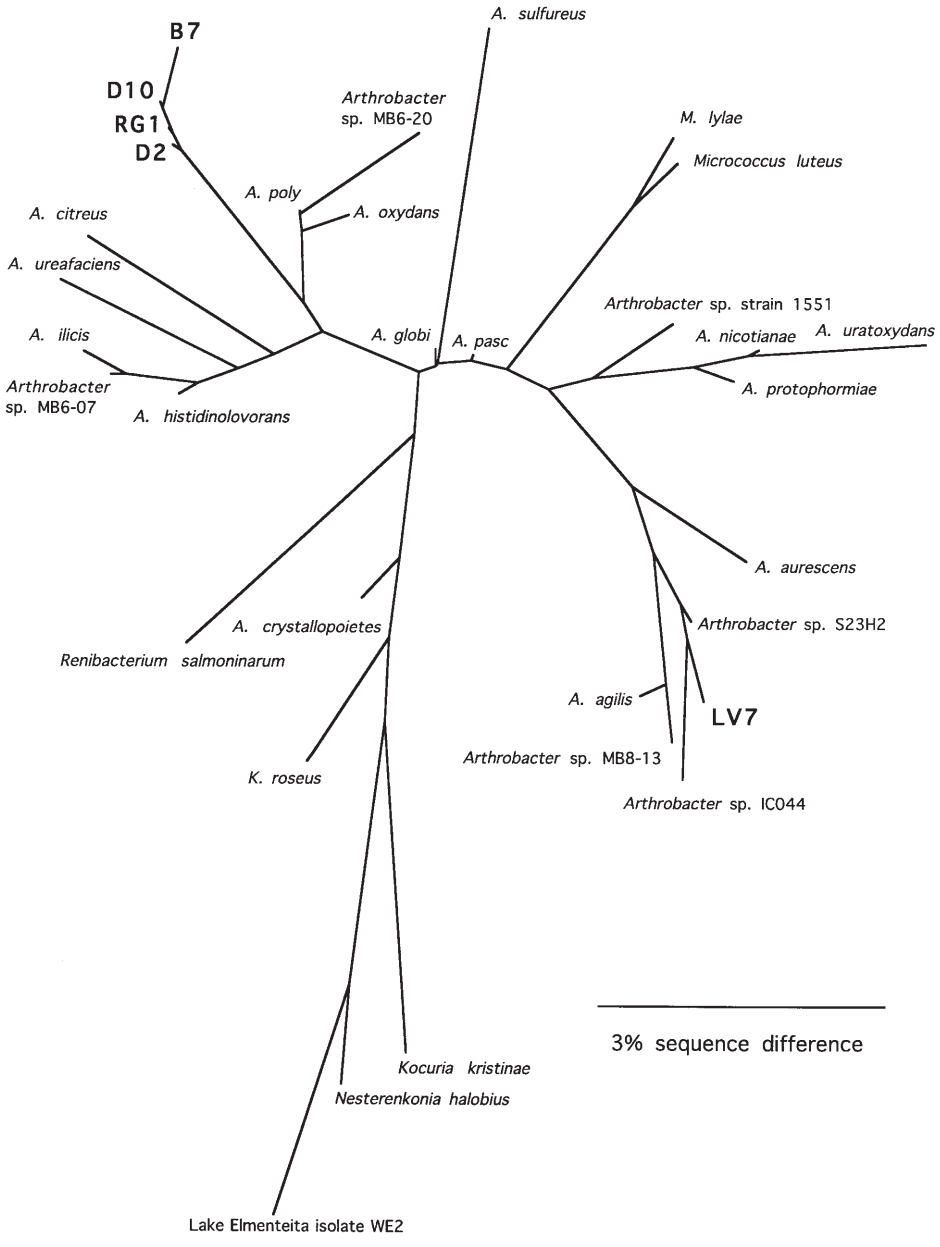


**Fig. 2** Phylogenetic tree derived from maximum-likelihood analysis (Felsenstein 1993) of 16S rRNA gene sequences of isolates B7, D2, D10, RG1, and LV7. Sequences were aligned with 16S rRNA sequences obtained from the Ribosomal Database Project (Maidak et al. 1994) and from a BLAST search of the NCBI database. Alignment was based on 1,454 nucleotide positions, and the sequences of the isolates corresponded to *Escherichia coli* positions 25–1449 (isolate B7), 67–1449 (isolate D2), 70–1449 (isolate D10), 47–1449 (isolate LV7), and 28–1345 (isolate RG1). Organisms used in the analysis but not shown on the tree are *Arthrobacter nicotinovorans*, *Arthrobacter ramosus*, and *Kocuria erythromyxa*. (*A. globi Arthrobacter globiformis*, *A. poly Arthrobacter polychromogenes*, and *A. pasc Arthrobacter pascens*)

three fragments carrying β-galactosidase genes cloned from isolate B7. The fragments contained 3,908, 2,239 and 1,422 nucleotides and had a DNA G+C content of 63, 61, and 59 mol%, respectively (Trimbur et al. 1994; Gutshall et al. 1995, 1997). These values are also within the range of 59–70 mol% G+C reported for *Arthrobacter* species.

## Phylogenetic relationships

To determine their phylogenetic relationships, the 16S rRNA genes of all six isolates were amplified using the PCR and were sequenced. Maximum-likelihood analyses of these sequences with *Arthrobacter* species obtained from the RDP and from a BLAST search of the NCBI database (using *Kocuria kristinae* as the outgroup) confirmed our original placement of isolates B7, D2, D5, and D10 (Loveland et al. 1994; DePrada et al. 1996) within the *Arthrobacter* genus. The results are also consistent with the physiological studies, suggesting that isolates D2, D5, D10, and B7 are distinct from the type strain, *A. globiformis* 8010 (Loveland et al. 1994; DePrada et al. 1996). In addition, isolates D2, D10, B7, and RG1 formed a clade distinct from the remainder of the subgroup (Fig. 2). Isolate LV7, however, lies outside this clade and branches from the *A. agilis* lineage.

To analyze the relationships of these organisms further, the percent sequence similarities of the 16S rRNA genes were calculated using the CLUSTAL V program of the MegAlign package (Table 2). The results quantify the clustering of isolates D2, D10, B7, and RG1 and docu-

**Table 2** Percent similarity table of 16S rRNA gene sequences of isolates B7, D2, D10, RG1, and LV7 generated with the CLUSTAL V program of the MegAlign package. Parameters for pairwise comparisons were a K-tuple value of 2 and a gap penalty of 5. Multiple alignment parameters were a gap penalty of 10 and a gap length penalty of 10. Similarity values were not sensitive to changes in the values of the multiple alignment parameters, indicating that the data is robust (*Kckris Kocuria kristinae, Aglobi Arthrobacter globiformis, Apoly Arthrobacter polychromogenes, Acit Arthrobacter citreus, Ahist Arthrobacter histidinolovorans, Ailic Arthrobacter ilicis, Aoxy Arthrobacter oxydans, 6–20 Arthrobacter* sp. MB6–20, *Mlut Micrococcus luteus, Aaures Arthrobacter aurescens, Agil Arthrobacter agilis, S23 Arthrobacter* sp. S23H2, and *ICO Arthrobacter* sp. ICO44). 16S rRNA sequences from the following organisms were included in the analysis but are not presented in the table: *Arthrobacter* sp. MB8–13, *Arthrobacter protophormiae, Arthrobacter uratoxydans, Arthrobacter nicotianae, Arthrobacter* sp. strain 1551, *Micrococcus lylae, Arthrobacter pascens, Arthrobacter ramosus, Arthrobacter sulfureus, Arthrobacter ureafaciens, Arthrobacter* sp. MB6–07, *Arthrobacter nicotinovorans, Renibacterium salmoninarum, Arthrobacter crystallopoietes, Kocuria erythromyxa, Kocuria roseus,* Lake Elmenteita isolate WE2, and *Nesterenkonia halobius*

| | B7 | D10 | RG1 | D2 | LV7 | Kckris | Aglobi | Apoly | Acit | Ahist | Ailic | Aoxy | 6–20 | Mlut | Aaures | Agil | S23 | ICO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B7 | 0.00 | | | | | | | | | | | | | | | | | |
| D10 | 99.3 | 0.00 | | | | | | | | | | | | | | | | |
| RG1 | 99.1 | 96.3 | 0.00 | | | | | | | | | | | | | | | |
| D2 | 98.7 | 99.2 | 96.5 | 0.00 | | | | | | | | | | | | | | |
| LV7 | 93.1 | 93.2 | 91.0 | 92.8 | 0.00 | | | | | | | | | | | | | |
| Kckris | 89.7 | 90.2 | 89.0 | 89.7 | 91.9 | 0.00 | | | | | | | | | | | | |
| Aglobi | 92.3 | 92.6 | 92.0 | 92.7 | 95.2 | 92.5 | 0.00 | | | | | | | | | | | |
| Apoly | 94.3 | 94.7 | 94.5 | 95.0 | 94.9 | 92.6 | 96.2 | 0.00 | | | | | | | | | | |
| Acit | 91.2 | 91.7 | 91.2 | 92.1 | 93.4 | 91.8 | 93.7 | 94.8 | 0.00 | | | | | | | | | |
| Ahist | 93.0 | 93.3 | 92.3 | 93.4 | 92.9 | 91.7 | 94.2 | 95.6 | 94.3 | 0.00 | | | | | | | | |
| Ailic | 92.7 | 93.0 | 92.6 | 93.1 | 93.9 | 90.9 | 92.8 | 94.6 | 93.2 | 96.7 | 0.00 | | | | | | | |
| Aoxy | 94.4 | 94.6 | 94.4 | 95.0 | 94.7 | 92.4 | 96.0 | 98.7 | 94.1 | 95.8 | 94.7 | 0.00 | | | | | | |
| 6–20 | 92.5 | 92.3 | 91.3 | 92.4 | 89.5 | 90.7 | 91.8 | 94.5 | 90.8 | 93.8 | 90.5 | 94.3 | 0.00 | | | | | |
| Mlut | 87.9 | 88.5 | 86.8 | 88.0 | 87.4 | 84.7 | 91.2 | 89.8 | 87.1 | 88.6 | 87.6 | 84.6 | 87.6 | 0.00 | | | | |
| Aaures | 94.0 | 93.7 | 93.3 | 94.0 | 95.7 | 92.6 | 94.5 | 96.1 | 94.8 | 96.4 | 97.5 | 96.2 | 92.0 | 88.7 | 0.00 | | | |
| Agil | 93.8 | 94.2 | 93.6 | 93.9 | 96.1 | 91.5 | 93.2 | 93.6 | 93.2 | 94.1 | 95.6 | 93.6 | 89.8 | 88.7 | 95.7 | 0.00 | | |
| S23 | 94.1 | 93.9 | 92.8 | 93.4 | 98.9 | 89.6 | 94.6 | 94.9 | 93.5 | 93.5 | 94.8 | 95.4 | 89.0 | 89.2 | 96.5 | 96.7 | 0.00 | |
| ICO | 90.3 | 90.1 | 88.8 | 90.0 | 95.0 | 87.5 | 92.0 | 91.6 | 91.1 | 91.1 | 91.6 | 92.0 | 90.8 | 87.1 | 93.6 | 93.1 | 95.3 | 0.00 |

ment that they form a particularly tight clade within the genus *Arthrobacter*. The similarity between the type strain *A. globiformis* and isolate B7 is 92.3%, which is less than that found between some *Arthrobacter* species or even between separate genera, i.e., *A. globiformis* and *K. kristinae* (Table 2). The existence of this monophyletic clade is consistent with the physiological and morphological distinctiveness of these psychrophilic isolates.

## Comparison of isolate LV7 with phylogenetic neighbors

Isolate LV7 contains lysine in its cell wall, as do the other isolates; however, it does not have an observable rod/coccus cycle and is phylogenetically related to *A. agilis* (previously *Micrococcus agilis*) (Koch et al. 1995), which also lacks a morphological cycle. Isolate LV7 appears to have very short rods or large cocci (which often form irregular clusters) throughout its life cycle. It has been suggested that the micrococci are locked in the coccus part of the morphological cycle. However, it is possible that some growth condition may exist in which organisms such as *A. agilis* and isolate LV7 show a rod/coccus cycle.

Although phylogenetically closely related, isolate LV7 and *A. agilis* differ in habitat, growth requirements, and colony pigmentation. While isolate LV7 was isolated from an Antarctic mat sample, *A. agilis* has been isolated from human skin, soil, and water (Kocur 1986), although micrococci are most often found on mammalian skin (Kocur et al. 1991). *A. agilis* produces a red pigment, whereas isolate LV7 forms colonies that are cream-colored to yellow depending upon growth conditions. Isolate LV7 grows well in a medium with 3.5% salt. Although some micrococci (e.g., some strains of *Micrococcus luteus* and *Micrococcus lylae*) can tolerate as much as 10% NaCl in nutrient broth, *A. agilis* does not grow in medium containing 5.0% NaCl (Koch et al. 1995).

## Comparison of isolate B7 with phylogenetic neighbors

The phylogenetic analysis of the isolate B7 cluster identifies its nearest neighbors as two closely related species, *A. polychromogenes* and *A. oxydans*. The main features distinguishing *A. polychromogenes* from *A. oxydans* are the production of blue pigments in carbohydrate-peptone-yeast media and growth on nicotine agar. However, in DNA reassociation studies, Stackebrandt and Fiedler (1979) have found high degrees of homology between *A. polychromogenes* and *A. oxydans* and have suggested that *A. polychromogenes* could be a subspecies of *A. oxydans*. To determine whether isolate B7 is physiologically distinct, we compared the key characteristics reported for these species and the type strain, *A. globiformis*, with

**Table 3** Distinguishing characteristics for isolate B7 and related species. The table summarizes results reported here plus those of Keddie et al. (1986), Loveland et al. (1994), Schippers-Lammertse et al. (1963), and Sguros (1955)

| Organism studied | | | | |
|---|---|---|---|---|
| Trait examined | Isolate B7 | *A. globiformis* | *A. polychromogenes* | *A. oxydans* |
| Growth at 37°C | No | Yes | Yes | Not reported |
| Reported temperature range | 0–30°C | 10–37°C | 10–37°C | Not reported |
| Colony pigmentation | Cream to yellow | White to cream | Blue/green | Pearl gray to yellow |
| Growth/color on nicotine | No | No | No | Yes/deep blue |
| Biotin requirement | No | No | Required for blue pigmentation | Required for growth |
| Presence of serine in cell wall | No | No | Yes | Yes |
| Nitrate reductase | No | No | Yes | Strong nitrate reduction |
| Gelatinase of gelatin liquefaction | Yes | Very slow | Rapid liquefaction | Slow liquefaction |
| Habitat organism studied | Soil | Soil | Airborne infection | Air/tobacco leaves |

those of isolate B7 (Table 3). Unlike *A. polychromogenes*, isolate B7 exhibits no blue pigmentation when cultivated on peptone-yeast glycerol agar, and unlike *A. oxydans* it does not grow on nicotine agar. Isolate B7 differs from *A. globiformis* ATCC 8010 type strain and *A. polychromogenes* in its growth range of 0–30°C. Both of these other species grow at 37°C, and *A. globiformis* does not grow at 0–5°C. The lowest reported growth temperature for *A. polychromogenes* is 10°C (Schippers-Lammertse et al. 1963; Loveland et al. 1994).

In addition, the cell wall amino acid composition differs among these species. Both *A. oxydans* and *A. polychromogenes* have serine in their peptidoglycan, whereas the type strain (Keddie et al. 1986) and isolate B7 do not. (Table 3). Biotin is required by *A. oxydans* for growth in mineral salts medium (Keddie et al. 1986) and by *A. polychromogenes* for pigment formation (Schippers-Lammertse et al. 1963), but is not required by our isolate (Loveland et al. 1994). Isolate B7 and the type strain ATCC 8010 also differ from the other species in their habitat and inability to produce nitrate reductase.

An additional characteristic distinguishing isolate B7 from other closely related species is its ability to use lactose as a sole carbon source, whereas *A. globiformis* cannot (Loveland et al. 1994). Although *A. polychromogenes* and *A. oxydans* have not been tested for their ability to use lactose, this sugar is generally not listed as a carbon source for *Arthrobacter* species. In their description of the *Arthrobacter* genus, Keddie et al. (1986) list 48 carbon sources used by 90% of the species in this genus, but lactose is not among them.

## Discussion

Our first goal was to determine whether the chemotaxonomic placement of isolates B7, D2, D5, and D10 in the genus *Arthrobacter* was consistent with a phylogenetic analysis. The results clearly show that these isolates are closely related to members of the *Arthrobacter* genus. An additional goal was to examine the cell wall composition and phylogenetic relationships of the new isolates RG1 and LV7. We found that these isolates also contain lysine in their cell wall and that RG1 shows a distinct rod/coccus cycle. Isolate RG1 also clusters phylogenetically near the others in the *Arthrobacter* genus. LV7, however, is distinct in that it was isolated from an aqueous (rather than soil) environment, it does not show a rod/coccus cycle, and it is separate from the other isolates phylogenetically.

Because the *Arthrobacter* subgroup is mixed, we were interested in the question of whether the chemotaxonomic traits of a rod/coccus cycle and the presence of lysine as the diamino acid were reliable for identifying aerobic, gram-positive organisms. The combination of physiological and phylogenetic results with isolates B7, D2, D5, D10, and RG1 shows that the presence of both the rod/coccus morphological cycle and lysine in the cell wall provides sound criteria for designating an organism to the genus *Arthrobacter*. The presence of lysine in the cell wall and the absence of an obvious rod/coccus cycle, such as was found with isolate LV7, however, could lead to misassignment without further data because other genera contain organisms that are short rods or cocci and contain lysine in their cell walls. Lysine and *meso*-diaminopimelic acid are the only linkages found in the third position among the organisms assigned to the *Arthrobacter* subgroup.

The answer to our question of whether any of the isolates is/are sufficiently distinct from known strains to warrant designation as a new species is more complicated. One approach to distinguish a new species from the most closely related characterized organism is to establish a quantitative cut-off, e.g., less than 97% identity of the 16S rRNA gene sequences (Stackebrandt and Goebel 1994). Establishing an absolute numerical standard for all organisms without taking into consideration phylogenetic clusters, evolutionary rates, and ecological habitats, however, is difficult (Ward 1998). Thus, a strict quantitative assessment could underestimate the number of new species, whereas not using phylogenetic distances as a guide could lead to a proliferation of new generic and species designations.

## Analysis of isolate LV7

Inspection of the *Arthrobacter* subgroup phylogenetic tree (Fig. 2) and the sequence similarity table (Table 2) indicates that isolate LV7 is an *Arthrobacter* species branching from the *A. agilis* lineage. Interestingly, isolate LV7 and *A. agilis* are similar in their preferred temperature for growth and in their production of β-galactosidase. *M. agilis* has been reported to differ from other micrococci because it is "psychrophilic" (Kocur et al. 1991); this species does not grow at 37°C and has an optimum at 20–30°C (isolate LV7 has a maximum of less than 23–24°C). According to the description of the species, *A. agilis* produces β-galactosidase; the only other *Micrococcus* species reported to have this enzyme are *Micrococcus halobius* (now named *Nesterenkonia halobius*) and *Micrococcus kristinae* (now called *K. kristinae*) (Stackebrandt et al. 1995).

Again, one could examine whether isolate LV7 should be designated as a new species. The 16S rRNA gene sequence similarity between isolate LV7 and *A. agilis* (96.1%) is close to the 97% value proposed for considering an isolate as a distinct species (Stackebrandt and Goebel 1994). However, the genus *Arthrobacter* is not monophyletic and has other genera interspersed within it (*Micrococcus* and *Renibacterium*). The reclassification of *M. agilis* to *A. agilis* (Koch et al. 1995) required revising the description of the genus *Arthrobacter* to include a statement indicating that one species (*A. agilis*) forms only coccoid cells. While an argument could be made to designate isolate LV7 as a new *Arthrobacter* species, isolate LV7 does not fit the emended description of the genus *Arthrobacter* since it neither has an obvious rod/coccus cycle nor does it only form true coccoid cells. Furthermore, since we have not cloned any genes from this isolate, there has not been a compelling reason for a complete characterization. There is no fundamental reason to name a new species that could require a further revision of the description of the *Arthrobacter* genus.

## Classification of isolates B7, D2, D5, D10, and RG1

Since isolates B7, D2, D5, D10, and RG1 form a monophyletic clade, it is interesting that they were obtained from a very small geographic area. Isolates B7, D2, D5, and D10 were from whey-enriched soil from farms within 20 miles of each other. Isolate RG1 was obtained from a Pennsylvania limestone quarry located within 30 miles of the farm fields. It will be interesting to determine whether members of this clade are associated with a specific geographical region or are widely dispersed. Since the isolates were selected for growth at low temperature, it would also be worthwhile to see if other psychrophilic *Arthrobacter* species isolated from other areas will cluster with this clade. Recently, an *Arthrobacter* strain (designated "RS4") with an optimal growth temperature of below 20°C was isolated from Siberian permafrost (Shi et al. 1997). A phylogenetic analysis using the isolate RS4 partial sequence (less than 900 nucleotides) indicated that this organism could be closely related to the isolate B7 cluster (data not shown). Repeating the analysis of these sequences using only 900 nucleotides resulted in the placement of isolate RS4 closer to *A. oxydans* and *A. polychromogenes*, although it remained on the branch leading to the isolate B7 clade but basal to it.

From different directions we examined the question of whether our other isolates should be given a new species designation. For example, the phylogenetic analyses show that isolates B7, D2, D5, D10, and RG1 form a monophyletic clade distinct from the rest of the *Arthrobacter* species. If one considers this as a separate group, then a case could be made for designating isolate B7 as the representative type strain of a new *Arthrobacter* species. The 16S rRNA gene sequence similarities between isolate B7 and the two most closely related *Arthrobacter* species (*A. oxydans* and *A. polychromogenes*) are 94.4% and 94.3%, respectively (Table 2). Both of these similarities are less than those between *A. globiformis* and the three species *A. oxydans* (96.0%), *Arthrobacter aurescens* (94.5%), and *A. polychromogenes* (96.2%). In addition, the similarities between *A. polychromogenes* and five other *Arthrobacter* species [*A. aurescens* (96.1%), *A. oxydans* (98.7%), *Arthrobacter citreus* (94.8%), *Arthrobacter ilicis* (94.6%), and *Arthrobacter histidinolovorans* (95.6%)] are greater than those between isolate B7 and its nearest neighbors (Table 2). Stackebrandt and Goebel (1994) have proposed that organisms with a sequence similarity of less than 97% could be considered different species. The 16S rRNA gene sequence similarity between isolate B7 and any other *Arthrobacter* species is less than this proposed threshold, supporting the case for its designation as a new species.

Consistent with the phylogenetic differences, isolate B7 differs physiologically from other species. For example, isolate B7 differs from other *Arthrobacter* species in its ability to grow on lactose as sole carbon source and to grow at 0–5°C. Neither trait is usually associated with the genus *Arthrobacter* or the type species, *A. globiformis*, although Gounot (1976) has reported two strains of *Arthrobacter glacialis* able to grow at 0°C. In addition, isolate B7 differs from *A. globiformis, A. polychromogenes*, and *A. oxydans* in several traits including colony pigmentation, biotin requirement, cell wall composition, and nitrate reduction (Table 3). We conclude that isolate B7 represents a new *Arthrobacter* species because it has an rRNA sequence similarity of less than 97% to other known species and has physiological traits distinct from other type strains.

Based on the results reported here, we propose that members of the isolate B7 clade form a new species. We have chosen isolate B7 as the type strain because it is the most-characterized and because three β-galactosidase genes have been cloned and sequenced from this organism (Trimbur et al. 1994; Gutshall et al. 1995, 1997). We propose naming isolate B7 *Arthrobacter psychrolactophilus*, and consider it to be the type strain of aerobic organisms that form yellow colonies, contain lysine in their cell wall, have an obvious rod/coccus cycle, are able to

grow on lactose as sole carbon source, and are able to grow at 0–5°C. Further work is required to determine whether isolates D2, D5, D10, and RG1 are additional strains of *A. psychrolactophilus* or if they are sufficiently different in other features (such as DNA-DNA hybridization values) to be considered another species.

Due to the differences described in this work between isolate B7 and previously reported *Arthrobacter* species, we propose to recognize the new species *Arthrobacter psychrolactophilus.*

## Description of the new species *Arthrobacter psychrolactophilus*

*Arthrobacter psychrolactophilus* (sp. nov.) psy.chro.lac.-to'phi.lus; *psychros* Gr.adj., cold; *lac* L.n.n., milk; *philos* Gr.m.n., friend, loving; *psychrolactophilus*, a cold, milk (sugar)-loving (bacterium).

Individual cells show a distinct rod/coccus cycle and have an average cell length of 1.4 μm during exponential growth and 0.5 μm during stationary phase. Gram-positive; easily decolorized; strict aerobe. Contains lysine as the diagnostic amino acid in the peptidoglycan. Non-spore-forming. Non-motile. No vitamin requirements; grows in mineral salts medium with ammonium chloride as sole source of nitrogen.

Colonies on trypticase soy agar without dextrose are yellow; degree of pigmentation varies with growth temperature and age of the cells.

The DNA base composition is 60.6 mol% G+C.

The major cellular fatty acids are anteiso- and iso-branched fatty acids. The predominant fatty acid is anteiso-$C_{15:0}$ (~73%), followed by anteiso-$C_{17:0}$ (~13%), iso-$C_{16:0}$ (~8%), $C_{16:0}$ (~2%), and iso-$C_{15:0}$ (~1.4%). All other fatty acids are at levels below 1%.

Able to grow at 0–5°C. Growth range, 0–30°C. Generation time at 10°C in trypticase soy broth with no added carbohydrate is 4.8 h. Does not produce acid with glucose as carbon source. Can utilize lactose, sorbitol, melibiose, cellobiose, glycerol, maltose, raffinose, xylose, galactose, sucrose, and glucose as sole carbon sources. Produces catalase. Produces β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, amylase, and gelatinase. Negative for nitrate reduction, alkaline phosphatase, *N*-acetyl-glucosaminidase, urease, and DNase. Habitat: soil.

16S rRNA gene sequencing analysis places this organism near *A. oxydans* and*A. polychromogenes*, although its 16S rRNA gene sequence is sufficiently different (94.4 and 94.3% sequence similarity, respectively) as to warrant a new species designation. The 16S rRNA gene sequence is 92.3% similar to the type strain of the *Arthrobacter* genus, *A. globiformis* 8010.

Type strain: *Arthrobacter psychrolactophilus* has been deposited at the ATCC (accession no. 700733).

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