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ORIGINAL PAPER

Reclassification of *Nocardioides basaltis* Kim et al. 2009 as a later synonym of *Nocardioides salarius* Kim et al. 2008, and emendation of the species description

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Abstract The taxonomic relationship between *Nocardioides salarius* CL-Z59^T and *Nocardioides basaltis* J112^T was established. The 16S rRNA gene sequences of the type strains showed 99.6 % similarity. The ΔT_m for genomic DNA–DNA hybridization of *N. salarius* CL-Z59^T and *N. basaltis* J112^T was 0.6–1.6 °C, indicating that both strains belong to a single species. Phenotypic and chemotaxonomic characteristics showed no pronounced differences between the two species. Based on the results of the polyphasic approach, it is proposed that *N. basaltis* J112^T is a later heterotypic synonym of *N. salarius* CL-Z59^T. An emended description of the species *N. salarius* is given.

Keywords *Nocardioides salarius* · *Nocardioides basaltis* · Heterotypic synonym · *Actinobacteria*

Introduction

Nocardioides salarius was described by Kim et al. (2008) for a novel marine actinobacterium isolated from seawater enriched with zooplankton. At nearly the same time (4 months later), *Nocardioides basaltis* was described by Kim et al. (2009) for a novel actinobacterium isolated from a black sand beach. Since the publication of these two studies, seventeen *Nocardioides* species have been newly proposed (list of prokaryotic names with standing in nomenclature; http://www.bacterio.cict.fr). However, the taxonomic relationship between *N. salarius* CL-Z59^T and *N. basaltis* J112^T has not yet been examined, although the type strains of both species shared high similarity (i.e. 99.6 %) of their 16S rRNA gene sequences. The level of 16S rRNA gene sequence similarity is higher than the thresholds of 97 % (Rosselló-Mora and Amann 2001) or 98.7–99 % (Stackebradt and Ebers 2006) considered to be the point at which genomic DNA–DNA relatedness experiments should be mandatory for confirming separate species. Literature data had already shown that *N. salarius* CL-Z59^T and *N. basaltis* J112^T gave congruent results in most phenotypic features (24 out of 26 enzymatic reactions in API ZYM and API 20NE kits; 17 out of 19 in utilization of sole carbon sources; optimum pH, optimum temperature and NaCl range for growth; Kim et al. 2008, 2009). Few phenotypic traits were different between the two species, such as pH range (pH 6–10 and 5.5–8.0 for *N. salarius* CL-Z59^T and *N. basaltis* J112^T, respectively), upper limit of temperature range (35 and 37 °C for *N. salarius* CL-Z59^T and *N. basaltis* J112^T, respectively), optimum NaCl for growth (3 and 1–2 % for *N. salarius* CL-Z59^T and *N. basaltis* J112^T, respectively) and decompositions of certain substrates (Kim et al. 2008, 2009), which needed to be closely

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examined under the same test conditions. However, a difference of DNA G+C content between *N. salarius* CL-Z59^T (73.3 mol% by HPLC analysis; Kim et al. 2008) and *N. basaltis* J112^T (68 mol% by thermal denaturation and fluorometry; Kim et al. 2009) seemed to be rather large to assign both species into a single species (Rosselló-Mora and Amann 2001). In this study, *N. salarius* CL-Z59^T and *N. basaltis* J112^T were compared using a polyphasic analysis to clarify the precise taxonomic relationship of the two species.

Materials and methods

Strains and culture conditions

The type strains of *N. salarius* CL-Z59^T (=KCCM 42320^T) and *N. basaltis* J112^T (=KCTC 19365^T) were obtained from the Korean culture center of microorganisms (KCCM) and the Korean collection for type culture (KCTC), respectively. Unless otherwise specified, all characteristics of *N. salarius* CL-Z59^T and *N. basaltis* J112^T were based on cultures grown aerobically on marine agar 2216 (MA; Difco) at 30 °C for 3 days.

Phylogenetic analysis of 16S rRNA gene sequences

The 16S rRNA gene sequences of *N. salarius* CL-Z59^T (GenBank accession no. DQ401092) and *N. basaltis* J112^T (EU143365) were aligned by the jPHYDIT program (Jeon et al. 2005) with the sequences of the type strains of other phylogenetically related species obtained from GenBank and Ribosomal Database Project II (Cole et al. 2007). Accurate alignment of multiple sequences was made manually according to the 16S rRNA secondary-structure information implemented in the jPHYDIT program. Phylogenetic trees were obtained by use of the neighbour-joining (Saitou and Nei 1987) and the maximum-parsimony (Fitch 1971) methods using MEGA 4 program (Tamura et al. 2007). An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes and Cantor (1969). The robustness of the tree topologies was assessed by bootstrap analyses based on 1,000 replications for the neighbour-joining and the maximum-parsimony methods.

DNA–DNA relatedness

Genomic DNA of *N. salarius* CL-Z59^T and *N. basaltis* J112^T was extracted using a commercial kit (Genomic DNA extraction kit; Bioneer). The relatedness of genomic DNAs between *N. salarius* CL-Z59^T and *N. basaltis* J112^T was made using a fluorometric method (Gonzalez and Saiz-Jimenez 2005): the optimum temperature for renaturation (T_{or}) was calculated using $T_{or} = 0.51$ (%GC) + 47 (De Ley et al. 1970). The melting temperature (T_m) at which 50 % of the initial double stranded molecules denatured into single-stranded DNA for *N. salarius* CL-Z59^T DNA and *N. salarius* CL-Z59^T/*N. basaltis* J112^T hybrid DNA was compared and the difference (ΔT_m) calculated (Goodfellow et al. 2012) for triplicate samples assayed on two different days.

Genomic G+C content

The genomic DNA G+C content of *N. salarius* CL-Z59^T and *N. basaltis* J112^T was determined by two different methods. First, the fluorometric method of Gonzalez and Saiz-Jimenez (2002) was employed using genomic DNA in the absence of formamide. Melting curves of genomic DNA and their T_m were obtained for triplicate samples using iQ iCycler real-time thermocycler (Bio-Rad). The G+C content value was calculated using the equation %GC = 1.98 T_m—106.91 (Gonzalez and Saiz-Jimenez 2002). Genomic DNAs of *Pedobacter roseus* CL-GP80^T (41.3 mol%; Hwang et al. 2006), *Maritalea myrionectae* CL-SK30^T (52.7 mol%; Hwang et al. 2009) and *Cucumibacter marinus* CL-GR60^T (62.9 mol%; Hwang and Cho 2008) were used to calibrate the fluorometric method. In addition, the DNA G+C content for *N. salarius* CL-Z59^T and *N. basaltis* J112^T was determined by HPLC analysis (Tamaoka and Komagata 1984) carried out by the identification service of the KCCM in Seoul, Korea.

Cellular fatty acids

The fatty acid methyl esters in whole cells of *N. salarius* CL-Z59^T grown on MA at 30 °C for 3 days were analyzed with gas chromatography (Agilent technologies 6890) according to the instructions of the Microbial Identification System (MIDI; version 3.10) with the TSBA40 database (Sasser 1990) at the

KCCM. The culture conditions and the analysis method were the same as those employed in Kim et al. (2009) for analysis of *N. basaltis* J112^T. Under these conditions, both strains appear to be in early exponential phase (data not shown).

Phenotypic tests

To compare phenotypic characteristics of *N. salarius* CL-Z59^T and *N. basaltis* J112^T, the following experiments were performed in duplicate for both strains at the same time, with repeat experiments on different days. The pH range (pH 5.0–10.5 at intervals of 0.5 pH unit) for growth was determined by assessing changes in OD_{600} in pH-buffered marine broth 2216 (MB; Difco) using 10 mM MES (Sigma) for pH 5.0–6.0, 10 mM TAPS (Sigma) for pH 6.5–9.0 and 100 mM $NaHCO_3/Na_2CO_3$ for pH 9.5–10.5 (Manaia et al. 2003; Yumoto et al. 2004) at 30 °C for up to 10 days. Upper limit of temperature for growth was tested on the basis of colony formation on MA plates incubated at 35, 37 and 40 °C. The NaCl requirement for optimal growth was determined using synthetic ZoBell broth with various NaCl concentrations (1–5 % at intervals of 1 %, w/v) as described by Kim et al. (2008). Decomposition of casein and L-tyrosine was determined according to the protocols described by Smibert and Krieg (1994). Degradation of starch and Tween 80 was investigated as described by Hansen and Sørheim (1991). In addition, other enzyme activities were assayed in duplicate using the API ZYM kit (bioMérieux) according to the manufacturer's instructions except that the cell suspension was prepared as described by Kim et al. (2008). Carbon utilization was tested using a modified basal broth medium containing 0.2 % carbon source as described by Kim et al. (2008). Carbon utilization was scored as negative when growth was equal to, or less than, that in the negative control with no carbon source. Growth was measured by monitoring changes in the OD_{600} every 3 days for 9 days incubation at 30 °C.

Results and discussion

A pairwise comparison of the 16S rRNA gene sequences of *N. salarius* CL-Z59^T and *N. basaltis* J112^T showed they differed by only five bases among 1,411 locations, resulting in a similarity of 99.6 %. Phylogenetic analyses of 16S rRNA gene sequences of *N. salarius* CL-Z59^T and *N. basaltis* J112^T revealed that they formed a robust clade within the genus *Nocardioides* (Fig. 1). Genomic DNA–DNA relatedness experiments showed the ΔT_m between *N. salarius* CL-Z59^T DNA and *N. salarius* CL-Z59^T/*N. basaltis* J112^T hybrid DNA was 1.6 °C (Fig. 2) or less (i.e. 0.6 °C; data not shown). The ΔT_m values of 0.6–1.6 °C were obviously lower than the criterion of 5 °C recommended for the delineation of bacterial species (Wayne et al. 1987; Rosselló-Mora and Amann 2001), indicating that *N. salarius* CL-Z59^T and *N. basaltis* J112^T belonged to a single species.

The genomic DNA G+C content of *N. salarius* CL-Z59^T and *N. basaltis* J112^T was comparable (74.8 and 73.4 mol%, respectively; Table 1) in the HPLC analysis. Using the fluorometric method, both type strains showed an identical T_m value of 89.6 °C (data not shown) corresponding to the DNA G+C content of 70.5 mol% (Gonzalez and Saiz-Jimenez 2002). It was notable that the DNA G+C content of *N. basaltis* J112^T measured by Kim et al. (2009) and the present study (68 and 70.5 mol%, respectively) using the fluorometric method was consistently lower than that by the HPLC analysis (73.4 mol% in the present study). This discrepancy might be partially owing to the high DNA G+C content of *N. basaltis* J112^T compared to those of microbial species (30.9–66.6 mol%) previously employed for the calibration curve between T_m and DNA G+C content in Gonzalez and Saiz-Jimenez (2002). Considering the high DNA G+C content of *Nocardioides* species (67–75 mol%; Kim et al. 2008), it is recommended to use the HPLC analysis to determine DNA G+C content for novel *Nocardioides* species, until a new calibration curve between T_m and high DNA G+C content is established for the fluorometric method.

The fatty acid profile of *N. salarius* CL-Z59^T was similar with that of *N. basaltis* J112^T analyzed previously under comparable conditions (Kim et al. 2009), although there was a minor difference (<5 %) in the proportions of certain fatty acids (i.e. $C_{17:1}\omega 8c$, iso-$C_{15:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$ and 10-methyl $C_{16:0}$; Table 2). The chemotaxonomic characteristics re-evaluated in the present study do not support the conclusion that *N. salarius* CL-Z59^T and *N. basaltis* J112^T are separate species.

Most phenotypic characteristics gave the same results between *N. salarius* CL-Z59^T and *N. basaltis*

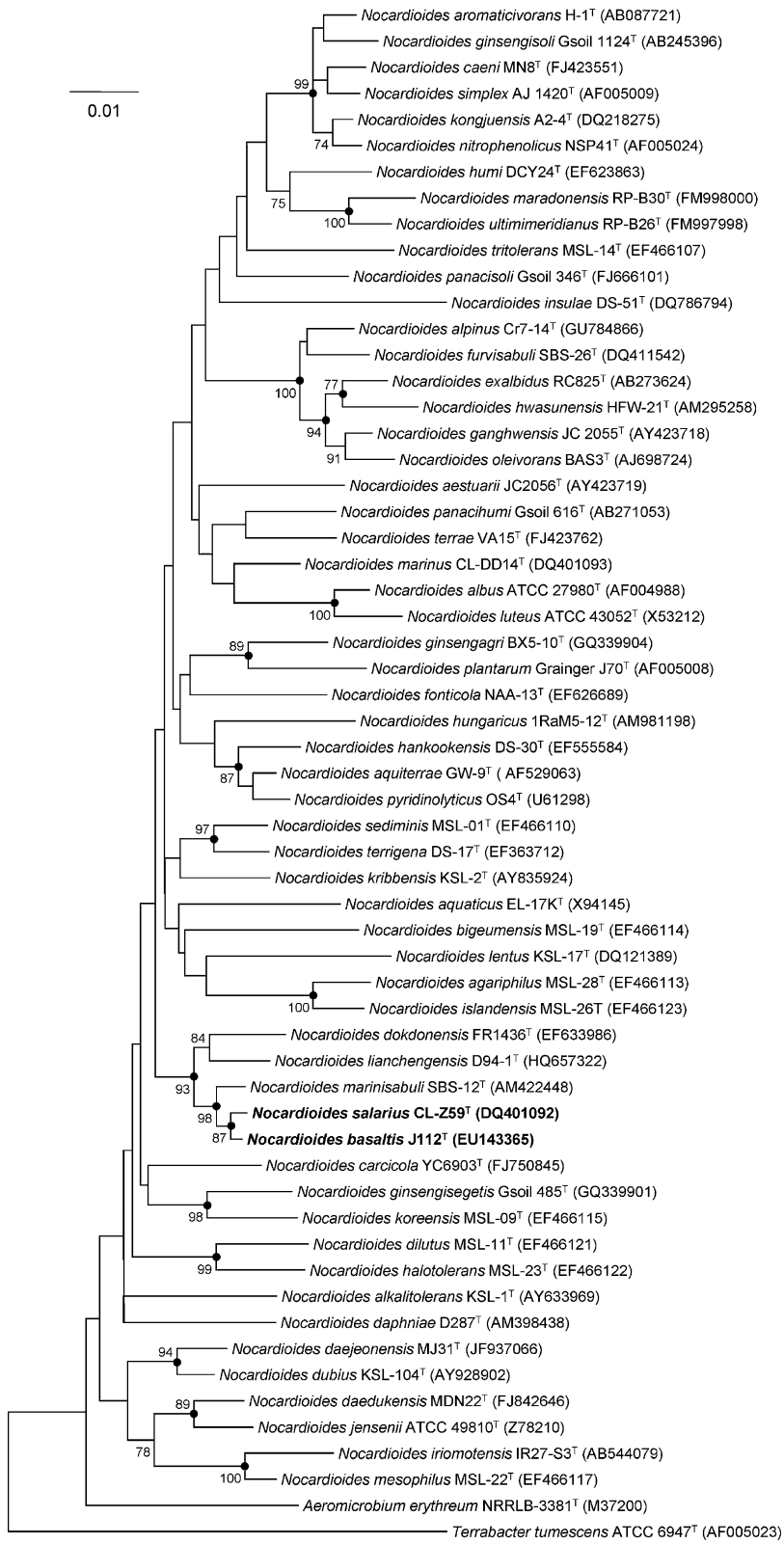

Fig. 1 Neighbour-joining tree showing the phylogenetic positions of *Nocardioides salarius* CL-Z59^T and *Nocardioides basaltis* J112^T and related species on the basis of 16S rRNA gene sequence. Only bootstrap values above 70 % are shown (1,000 resamplings) at the branching points. *Solid circles* indicate that the corresponding nodes were also obtained in the maximum-parsimony tree. *Terrabacter tumescens* ATCC 6947^T (AF005023) was used as an outgroup. *Bar*, 0.01 nucleotide substitution per site

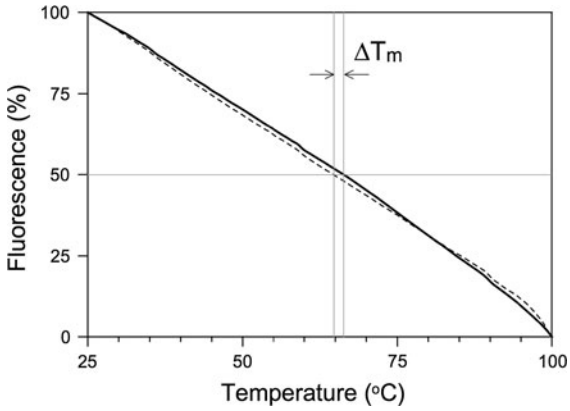

Fig. 2 Thermal denaturation of genomic DNA from *Nocardioides salarius* CL-Z59^T (*solid line*) and the *N. salarius* CL-Z59^T/*Nocardioides basaltis* J112^T hybrid DNA mix (*dotted line*). The calculated ΔT_m is 1.6 °C

J112^T (Table 1). The pH range (pH 6.0–9.0), upper limit of temperature (37 °C) and optimum NaCl concentration (2–3 %) for growth; decompositions of starch and L-tyrosine; enzyme profile of the API ZYM kit; and utilization of D-salicin as sole carbon was identical for both type strains, whereas some of these features had been shown to be differ in the original studies for *N. salarius* CL-Z59^T and *N. basaltis* J112^T (Kim et al. 2008, 2009; Table 1). In addition, utilization of L-arginine, L-asparagine, L-lysine, L-ornithine and succinate as sole carbon gave congruent results between both type strains (Table 1), characteristics which had previously been uncharacterized for *N. basaltis* J112^T (Kim et al. 2009). Although a few phenotypic characteristics were different between both type strains (i.e. decompositions of casein and Tween 80; utilization of D-ribose as sole carbon; Table 1), *N. salarius* CL-Z59^T and *N. basaltis* J112^T shared common characteristics in most phenotypic properties, as shown in the earlier studies (Kim et al. 2008, 2009).

Based on the phenotypic, phylogenetic, chemotaxonomic and genomic DNA–DNA relatedness results obtained from the present study, it is proposed to reclassify *N. basaltis* Kim et al. (2009) as a later heterotypic synonym of *N. salarius* Kim et al. (2008).

Table 1 Phenotypic properties of *Nocardioides salarius* CL-Z59^T and *Nocardioides basaltis* J112^T

Characteristic	1	2
pH range for growth	6.0–9.0 (6–10)	6.0–9.0 (5.5–8.0)
Temperature for growth at:		
35 °C	+ (+)	+ (+)
37 °C	+ (ND)	+ (+)
40 °C	− (−)	− (−)
Optimum NaCl for growth (%)	2–3 (3)	2–3 (1–2)
Decomposition of:		
Casein	+ (+)	− (ND)
Starch	− (w)	− (−)
Tween 80	+ (+)	− (−)
L-Tyrosine	+ (+)	+ (−)
API ZYM test:		
Acid phosphatase	+ (+)	+ (−)
α-Chymotrypsin	− (+)	− (−)
Cystine arylamidase, trypsin	+ (+)	+ (w)
Valine arylamidase	− (+)	− (−)
Alkaline phosphatase, esterase (C4), esterase lipase (C8), α-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase	+ (+)	+ (+)
N-Acetyl-β-glucosaminidase, α-fucosidase, α- and β-galactosidases, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase	− (−)	− (−)
Utilization of sole carbon:		
L-Arginine, L-ornithine	+ (+)	+ (ND)
L-Asparagine, L-lysine, succinate	− (−)	− (ND)
D-Ribose	+ (+)	− (−)
D-Salicin	+ (−)	+ (+)
DNA G+C content (mol%)	74.8[a] (73.3[a])	73.4[a] (68[b])

Strains: 1 *N. salarius* CL-Z59^T; 2 *N. basaltis* J112^T. Data were obtained in this study. Values in parentheses were from the studies of Kim et al. (2008) for *N. salarius* CL-Z59^T and Kim et al. (2009) for *N. basaltis* J112^T. +, positive; –, negative

w Weakly positive; *ND* not determined

[a] Data were determined by the HPLC analysis

[b] A datum was determined by the fluorometric method

Emended description of *N. salarius* Kim et al. (2008)

The characteristics of this species are as described by Kim et al. (2008, 2009); with the following amendments. L-Tyrosine is hydrolyzed, but starch is not. Decompositions of Tween 80 and casein are variable depending on the strain. According to the API ZYM test, positive for acid phosphatase, cystine arylamidase and trypsin but negative for valine arylamidase and α-chymotrypsin.

Table 2 Cellular fatty acid contents of *Nocardioides salarius* CL-Z59^T and *Nocardioides basaltis* J112^T

Fatty acid	1	2
Saturated		
$C_{14:0}$	Tr	Tr
$C_{16:0}$	1.2	1.3
Unsaturated		
$C_{17:1}\omega 8c$	Tr	4.3
$C_{18:1}\omega 7c$	2.9	1.7
$C_{18:1}\omega 9c$	1.9	2.8
Branched		
Iso-$C_{12:0}$	Tr	Tr
Iso-$C_{14:0}$	2.1	3.5
Iso-$C_{15:0}$	4.3	Tr
Iso-$C_{16:0}$H	3.6	3.7
Iso-$C_{16:0}$	64.4	70.3
Iso-$C_{17:0}$	3.4	Tr
Anteiso-$C_{17:0}$	3.3	Tr
Iso-$C_{18:0}$	1.3	2.7
10-Methyl		
$C_{16:0}$	4.6	Tr
$C_{17:0}$	1.0	3.2
Summed feature 3 ($C_{16:1}\omega 7c$/iso–$C_{15:0}$2-OH)	3.6	1.7

Strains: 1 *N. salarius* CL-Z59^T (this study); 2 *N. basaltis* J112^T (data from Kim et al. 2009). Fatty acids were analyzed for cells grown on marine agar at 30 °C for 3 days. Values are percentages of total fatty acids

Tr Trace amount (<1 %)

L-Arginine, L-ornithine and D-salicin are utilized as sole carbon source but L-asparagine, L-lysine and succinate are not utilized. Utilization of D-ribose as sole carbon source is variable depending on the strain. The DNA G+C content is 73.3–74.8 mol% as determined by HPLC. The type strain is CL-Z59^T (=KCCM 42320^T =DSM 18239^T). A reference strain is J112 (=KCTC 19365 = JCM 14945).

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