Bacillus acidiproducens sp. nov., vineyard soil isolates that produce lactic acid

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Two novel spore-forming lactic acid bacteria, strains SL213^T and SL1213, were isolated from vineyard soils in Korea. Cells of both isolates were rod-shaped bacilli and contained subterminal, ellipsoidal spores. Strains were facultatively anaerobic, catalase-positive, oxidase-negative and motile with single flagella. *meso*-Diaminopimelic acid, glucose and galactose were detected in whole-cell hydrolysates. Major fatty acids found in the strains were anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{17:0}. The G + C contents of the DNA were 46.1 and 46.3 mol%. 16S rRNA gene sequences from the two strains were almost identical (99.9%) and placed them in the genus *Bacillus*, according to phylogenetic analysis. The type strains most closely related to SL213^T were *Bacillus coagulans* ATCC 7050^T and *Bacillus badius* ATCC 14574^T, with 16S rRNA gene sequence similarities of 96.9 and 95.9%, respectively. Levels of DNA–DNA relatedness between strain SL213^T and strain SL1213, *B. coagulans* ATCC 7050^T and *B. badius* ATCC 14574^T were 92.5, 49.0 and 27.5%, respectively. On the basis of 16S rRNA gene sequences and chemotaxonomic and phenotypic evidence given in this study, we report that SL213^T represents a novel species, for which the name *Bacillus acidiproducens* sp. nov. is proposed. The type strain is SL213^T (=KCTC 13078^T =JCM 14638^T).

The members of the genus *Bacillus* Cohn 1872 are Gram-positive, rod-shaped, aerobic or facultatively anaerobic, spore-forming bacteria. Within the bacilli, a spore-forming lactic acid bacillus was first described in 1932 and it was originally known as '*Lactobacillus sporogenes*' (Horowitz-Wlassowa & Nowotelnov, 1932). Results from further physiological and biochemical characterization caused the strain to be transferred into the *Bacillus coagulans* group, a change in classification that was later confirmed by genotypic studies (Becker & Pederson, 1950; De Clerck *et al.*, 2004). More recently, other spore-forming rods that produce lactic acid have been grouped into the genus *Bacillus* (Gibson & Gordon, 1974).

A unique characteristic of lactic acid-producing strains of the genus *Bacillus* is their ability to produce spores and lactic acid; the spores can stay dormant for extended periods under stressful environmental conditions, including high temperature and acidic pH. Organisms from the genus *Bacillus* have been isolated frequently and from almost every environment, such as marine areas (Bae *et al.*, 2005; Gugliandolo *et al.*, 2003; Yoon *et al.*, 2003), airborne

dust (Shivaji *et al.*, 2006) and soil (Albert *et al.*, 2005; Gatson *et al.*, 2006; Heyrman *et al.*, 2005; Logan *et al.*, 2002; Palmisano *et al.*, 2001). In contrast, isolation of spore-forming, lactic acid-producing *Bacillus* strains has been reported only rarely, from products of milk (Hammer, 1915), fermented foods, plants (Alkan *et al.*, 2007), sewage sludge (Kotay & Das, 2007) and soil (Patel *et al.*, 2006). In this study, we report the isolation and characterization of novel bacteria from vineyard soil in Korea that form spores, produce lactic acid, are facultatively anaerobic and grow at temperatures up to 55 °C. After polyphasic characterization, the isolates were classified in a single novel species of the genus *Bacillus*.

Isolation of spore-forming lactic acid bacteria was performed according to the method described by Yanagida *et al.* (1997). One gram of a soil sample was mixed with 9 ml 0.75% NaCl solution. The mixed solution was heated for 30 min at 60 °C, after which one drop of the suspension was spread onto GYP agar containing 1% CaCO₃ (Yanagida *et al.*, 1997). The culture was incubated under anaerobic conditions with a gas phase of H₂/CO₂/N₂ (7:5:88, by vol.), in a Forma anaerobic chamber, at 30 °C for 2–3 days. After incubation, acid-producing bacteria were identified by the appearance of clear zones around

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SL213^T and SL1213 are EF379274 and EF392690.

colonies, and were then selected and purified by repeated isolation. Two novel spore-forming, lactic acid bacteria, designated SL213^T and SL1213, were maintained on tryptic soy agar (TSA, Difco) at 30 °C. Subsequent analysis was performed to determine 16S rRNA gene sequences, phenotypic characteristics and DNA–DNA relatedness to type strains of related species based on 16S rRNA gene sequence similarity.

Cells grown on TSA were used to determine physiological characteristics of the isolates. Gram staining was performed using a Gram stain set (Difco). Cell morphology and size were examined using phase-contrast microscopy (Nikon 80i). For determination of flagellum type, cells were negatively stained with 1 % (w/v) uranyl acetate according to the method described by Chang *et al.* (2002) and observed with a model CM-20 transmission electron microscope (Philips). Some physiological characteristics were determined using API 20E and API 50CH galleries (bioMérieux) according to the instructions of the manufacturer. Catalase activity was detected by placing drops of 3 % (v/v) H₂O₂ on cultures growing on GYP and observing oxygen bubbles. Oxidase activity was determined using an Oxy-swab (bioMérieux). Production of lactic acid was confirmed using a D-lactic acid/L-lactic acid kit (R-biopharm; Roche Diagnostics). The pH range for growth was checked using 500 ml flasks containing 250 ml tryptic soy broth (TSB) with a pH of 4–8 (in 0.5 pH unit increments) at 37 °C. TSB containing 100 mM Na₂HPO₄/NaH₂PO₄ was used as buffer. Growth was checked by monitoring optical density at 595 nm (Bio-Rad). To determine the optimum growth temperature and tolerance of NaCl, cells were cultured in TSB for 2 days at temperatures ranging from 15 to 60 °C and in NaCl concentrations up to 7 % (w/v). Motility was observed in TSB solidified with 0.4 % agar.

Purified cell-wall preparations were obtained as described by Schleifer & Kandler (1972). Amino acid and sugar analyses of the whole-cell hydrolysate were performed as described by Stanek & Roberts (1974), using TLC on cellulose plates (Merck) with the solvent system of Rhuland *et al.* (1955).

Cells of strains SL213^T and SL1213 stained Gram-positive and were facultatively anaerobic rods that contained subterminal ellipsoidal spores. Single cells were observed as slightly curved rods, 4.0–4.5 µm long and 0.8–1.2 µm in diameter. Isolates were motile with single flagella and produced lactic acid and grew in TSB containing 5 % NaCl. Strains were negative for oxidase, indole production and nitrate reduction, but positive for catalase and acid production from ribose, mannitol and sucrose. Isolates were more similar to *Bacillus coagulans* ATCC 7050^T than to *Bacillus badius* ATCC 14574^T in their physiological properties (De Clerck *et al.*, 2004). These results are summarized in the species description and in Table 1.

In the analysis of whole-cell hydrolysates, *meso*-diaminopimelic acid and glucose and galactose were detected in the

isolates. The predominant cell-wall amino acid and sugars found in the isolates agreed with results for the type strain of *B. coagulans* (De Clerck *et al.*, 2004).

The isolates and several *Bacillus* reference strains were subjected to cellular fatty acid methyl ester analysis to confirm the genus classification. All the tested strains were grown on TSA (pH 7.0) for 24 h at 37 °C. Fatty acids were extracted and analysed according to the standard protocol of the Microbial Identification System (MIDI Microbial ID). Extracts were analysed using a gas chromatograph (model 6890N and autosampler 7683; Agilent) and identified using the Microbial Identification Sherlock software package.

The cellular fatty acid profiles of the strains are presented in Table 2. The predominant fatty acids of the two novel isolates and *B. coagulans* ATCC 7050^T were anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0}, iso-C_{15:0} and C_{16:0}, comprising approximately 95 % of the fatty acids extracted. The major fatty acids of the isolates were similar to those reported for *B. coagulans* ATCC 7050^T in previous studies (De Clerck *et al.*, 2004). Among the analysed reference strains, *B. badius* ATCC 14574^T differed significantly from the novel isolates in that the only predominant fatty acid was iso-C_{15:0} (51.43 %) and the minor components were present in smaller amounts. The cellular fatty acid profiles indicate that strains SL213^T and SL1213 were more similar to *B. coagulans* ATCC 7050^T than to *B. badius* ATCC 14574^T in some major groups, although cellular fatty acids alone do not give good differentiation within the bacilli.

Nearly complete 16S rRNA gene sequences for strains SL213^T and SL1213 were determined according to Chang *et al.* (2001). The sequences were aligned manually with representatives of the genus *Bacillus* and related taxa using known 16S rRNA secondary structure information. Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967) and maximum-parsimony (Fitch, 1971) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining tree based on 1000 resamplings.

A preliminary comparison of the nucleotide sequences with sequences in GenBank indicated that the isolates were closely related to members of the genus *Bacillus*. A rooted phylogenetic tree showing the relationship between strains SL213^T and SL1213 and representatives of the genus *Bacillus* is shown in Fig. 1. The strains formed a highly significant monophyletic clade with *B. coagulans* ATCC 7050^T and *B. badius* ATCC 14574^T. The clade was also confirmed in other treeing algorithms, supported by a highly significant bootstrap value of 100 % (not shown). On the basis of pairwise 16S rRNA gene sequence similarities, the closest phylogenetic relative of strain

Table 1. Differential physiological characteristics of strains SL213^T and SL1213 and closely related type strains

Strains: 1, strains SL213^T and SL1213; 2, *B. coagulans* ATCC 7050^T; 3, *B. badius* ATCC 14574^T. +, Positive; –, negative; w, weakly positive. All strains are motile, positive for catalase and negative for oxidase, reduction of nitrate and nitrite, hydrolysis of casein, DNA and Tween 80, formation of H₂S and indole and activities of phosphatase, urease, phenylalanine deaminase and ornithine and lysine decarboxylases. Data were obtained in this study.

Characteristic	1	2	3
Cell length (µm)	4.0–4.5	5.5–6.0	5.0
Temperature for growth (°C)			
Range	25–55	30–55	15–50
Optimum	37	37	30
Colony colour	White or ivory	White	Cream–white
Spore shape	Ellipsoidal	Ellipsoidal	Round
Spore location	(Sub)terminal	(Sub)terminal	Central
Anaerobic growth	+	+	–
Growth in NaCl at:			
5 %	+	+	–
7 %	–	+	–
Lactic acid production	+	+	–
Hydrolysis of gelatin	–	–	+
Acetoin production (Voges–Proskauer test)	+	+	–
β-Galactosidase (ONPG)	–	+	–
Methyl red test	–	+	–
Acid production from:			
Glycerol	+	+	–
L-Arabinose	+	–	–
Ribose	+	–	–
D-Xylose	+	–	–
Galactose	+	+	–
Glucose	+	+	–
Fructose	+	+	–
Mannose	+	+	–
Rhamnose	+	–	–
Mannitol	+	–	–
Methyl α-D-glucoside	+	–	–
N-Acetylglucosamine	+	+	–
Aesculin	–	+	–
Salicin	–	w	–
Lactose	–	+	–
Sucrose	+	–	–
Trehalose	–	+	–
Starch	–	w	–
Turanose	–	+	–
D-Lyxose	+	–	–
D-Tagatose	+	–	–
Gluconate	+	+	–

SL213^T was *B. coagulans* ATCC 7050^T (96.9 %), followed by *B. badius* ATCC 14574^T (95.9 % similarity). The sequences of SL213^T and SL1213 showed high similarity (99.9 %), suggesting that the two strains belong to the same species. It was evident from 16S rRNA gene sequence analysis that the isolates represented a novel genomic species in the genus *Bacillus*, as no strains of species with validly published names showed more than 97 % 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994). The 16S rRNA gene sequences for strains SL213^T (1379 nt)

and SL1213 (1378 nt) were thus clearly discriminated from those of type strains of species of *Bacillus*.

For the determination of DNA G+C (mol%) content, DNA was prepared according to Chang *et al.* (2002) and analysed by reversed-phase HPLC as described by Tamaoka & Komagata (1984). The relative G+C contents were calculated by using standard DNA from *Escherichia coli* KCTC 2441^T. The DNA G+C contents of strain SL213^T and SL1213 were respectively 46.3 and 46.1 mol%. These

Table 2. Cellular fatty acid composition of strains SL213^T and SL1213 and closely related type strains

Strains: 1, SL213^T; 2, SL1213; 3, *B. coagulans* ATCC 7050^T; 4, *B. badius* ATCC 14574^T. Data were obtained in this study. All of the strains were grown on TSA (pH 7.0) for 24 h at 37 °C. Values are percentages of fatty acids; —, not detected.

Fatty acid	1	2	3	4
iso-C _{14:0}	1.57	2.06	1.53	1.46
C _{14:0}	1.46	2.39	1.57	1.82
iso-C _{15:0}	5.28	6.53	7.25	51.43
anteiso-C _{15:0}	53.39	48.21	49.01	5.85
C _{15:0}	—	—	—	1.32
C _{16:1} ω7c alcohol	—	—	—	3.14
iso-C _{16:0}	7.06	9.24	8.98	5.44
C _{16:1} ω11c	—	—	—	4.01
C _{16:0}	5.32	7.39	2.98	3.24
iso-C _{17:1} ω10c	—	—	—	6.02
Summed feature*	—	—	—	2.68
iso-C _{17:0}	1.04	1.23	—	3.52
anteiso-C _{17:0}	23.10	21.36	26.10	3.50
Unknown	1.11	1.59	1.35	3.72

*Comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B.

values are consistent with a taxonomic position of the strains within the genus *Bacillus* (Shida *et al.*, 1997).

The novel strains and some *Bacillus* reference strains were subjected to genomic relatedness analysis. DNA–DNA hybridization was determined by the microplate hybridization method as described by Ezaki *et al.* (1989). Hybridization was carried out with photobiotin-labelled probes using a Fluoroskan Ascent Fluorescent plate reader (Thermo Life Science) with three replications for each sample at 45 °C. The mean genomic relatedness between

the two isolates was 92.5 %, indicating that strains SL213^T and SL1213 represent a single species of *Bacillus*. The type strains of other *Bacillus* species were more distantly related to strain SL213^T. The genomic relatedness between SL213^T and *B. coagulans* ATCC 7050^T was 49 % (96.9 % 16S rRNA gene sequence similarity). Strain SL213^T and *B. badius* ATCC 14574^T showed 27.5 % genomic relatedness (95.9 % 16S rRNA gene sequence similarity). These values were below the 70 % threshold suggested for species delineation (Wayne *et al.*, 1987), and indicate that strain SL213^T represents a novel species within the genus.

On the basis of the molecular and phenotypic evidence presented in this study, we propose that strains SL213^T and SL1213 be classified in a novel species of the genus *Bacillus*, *Bacillus acidiproducens* sp. nov.

Description of *Bacillus acidiproducens* sp. nov.

Bacillus acidiproducens (a.ci.di.pro.du'cens. N.L. n. *acidum* an acid; L. part. adj. *producens* producing; N.L. part. adj. *acidiproducens* acid-producing).

Cells are facultatively anaerobic, Gram-positive-staining rods (0.8–1.2 × 4.0–4.5 µm) that contain subterminal ellipsoidal spores. Motile by single polar flagella. Colonies grown on TSA are round, flat and white or ivory (5.0 mm in diameter). Grows at 25–55 °C; optimum growth at 37 °C. Grows at pH 6.0–7.5; optimum growth at pH 7.0. Grows in 5 % NaCl. Positive for catalase, acetoin and lactic acid production. Negative for oxidase, reduction of nitrate and nitrite, hydrolysis of gelatin, casein, DNA and Tween 80, β-galactosidase (ONPG) and methyl red test, formation of H₂S and indole and activities of phosphatase, urease, phenylalanine deaminase and ornithine and lysine decarboxylases. Utilizes glycerol, L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, methyl α-D-glucoside, N-acetylglucosamine, sucrose, D-lyxose, D-tagatose and gluconate. The diagnostic amino

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strains SL213^T and SL1213 and closely related taxa. Solid circles indicate that the corresponding nodes were also obtained in the maximum-parsimony tree. Bootstrap values from 1000 replications are shown as percentages at each node in the tree. Bar, 0.01 substitutions per nucleotide position.

acid is *meso*-diaminopimelic acid and the major cell-wall sugars are glucose and galactose. Major fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 46.3 mol%.

The type strain, SL213^T (=KCTC 13078^T =JCM 14638^T), was isolated from vineyard soil in Daejeon, Korea. Strain SL1213, isolated from the same source, is a second strain of the species.

Acknowledgements

We are grateful to Dr Jean P. Euzéby (Society for Systematic and Veterinary Bacteriology, France) for his advice on nomenclatural queries. This work was supported by grants KHS4120711 and BDM0200726 from the Ministry of Science and Technology (MOST), and the KRIBB Research Initiative Program, Republic of Korea. We are indebted to the Korea Basic Science Institute (KBSI), Republic of Korea, for the transmission electron microscope.

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