Bacillus velezensis sp. nov., a surfactant-producing bacterium isolated from the river Vélez in Málaga, southern Spain

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Two Gram-positive, endospore-forming bacterial strains, CR-502^T and CR-14b, which produce surfactant molecules are described. Phenotypic tests and phylogenetic analyses showed these strains to be members of the genus *Bacillus* and related to the species *Bacillus atrophaeus*, *Bacillus mojavensis*, *Bacillus subtilis*, *Bacillus vallismortis* and *Bacillus amyloliquefaciens*, although they differ from these species in a number of phenotypic characteristics. DNA–DNA hybridization confirmed that they show less than 20 % hybridization with the above-mentioned species and therefore represent a novel species of *Bacillus*. The DNA G+C content is 46·4 mol% in strain CR-502^T and 46·1 mol% in strain CR-14b. The main fatty acids in strain CR-502^T are 15 : 0 anteiso (32·70 %), 15 : 0 iso (29·86 %) and 16 : 0 (13·41 %). The main quinone in strain CR-502^T is MK-7 (96·6 %). In the light of the polyphasic evidence gathered in this study, it is proposed that these strains be classified as a novel species of the genus *Bacillus*, with the name *Bacillus velezensis* sp. nov. The type strain (CR-502^T = CECT 5686^T = LMG 22478^T) was isolated from a brackish water sample taken from the river Vélez at Torredelmar in Málaga, southern Spain.

In recent years *Bacillus* species have come to play an increasingly important role in applied microbiology. Nowadays some species are used in the production of enzymes, antibiotics, insecticides, surfactants and fine biochemicals, including flavour enhancers and food supplements (Banat *et al.*, 2000; Harwood, 1989; Pinchuk *et al.*, 2002).

We describe here a novel species of the genus *Bacillus*, for which we propose the name *Bacillus velezensis* sp. nov. The species includes two strains that were isolated during a wide-ranging research programme aimed at discovering novel bacterial strains capable of synthesizing new lipopeptides with surfactant and/or antimicrobial activity. To this end we sampled a number of different saline and non-saline environments during 1999 and 2000 and selected potential surfactant-producer strains by the drop-collapsing test (Bodour & Miller-Maier, 1998; Jain *et al.*, 1991). The two strains described here synthesize substantial yields of lipopeptides related to the surfactin family, the most active biosurfactant group reported to date. These products, which are currently being studied, also appear to exert antimicrobial activity.

Strain CR-14b was found in a sample taken at the mouth of the river Vélez at Torredelmar in the province of Málaga (southern Spain) in April 1999. Strain CR-502^T was isolated from a sample taken from the same river mouth in October 2000. The isolation medium was MY (Moraine & Rogovin, 1966) supplemented with 7·5 % w/v salts (Rodríguez-Valera *et al.*, 1981). Both strains grew best on tryptic soy agar (TSA) and were therefore kept and routinely grown on this medium at 32 °C.

Strains CR-502^T and CR-14b were phenotypically analysed together with type strains of the most closely related *Bacillus* species and other endospore-forming bacteria according to the methods described by Claus & Berkeley (1986), Cowan & Steel (1994), Harwood (1989) and Logan & Berkeley (1984). According to Nakamura *et al.* (1999) in their original description of the subspecies *Bacillus subtilis* subsp. *spizizenii*, the morphological, physiological and biochemical characteristics are the same as those of *Bacillus subtilis* subsp. *subtilis*, although there is a phenotypic difference between the subspecies in their cell-wall constituents. For this reason we only deemed it necessary to include in our study one of the two subspecies, *B. subtilis* subsp. *subtilis*. We carried out 122 phenotypic tests,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CR-502^T and CR-14b are AY603658 and AY608741 respectively.

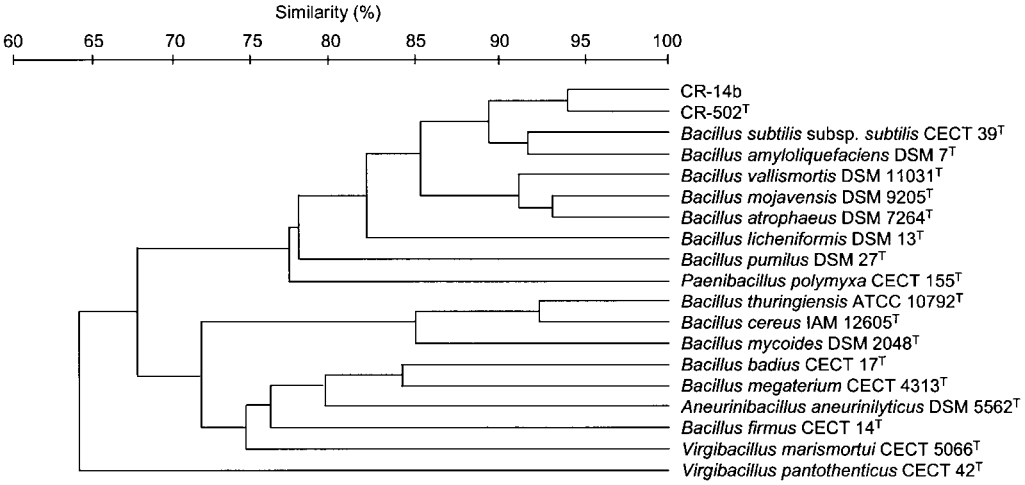

Fig. 1. Dendrogram based on phenotypic data. The simple-matching (S_{SM}) coefficient and UPGMA were used.

including both API 20E and API 50 CHB (bioMérieux), the results of which appear in the species description. Morphology and the presence of flagella and endospores in strain CR-502^T were studied by transmission and scanning electron microscopy according to the methods described by Bouchotroch *et al.* (2001).

Differential phenotypic characteristics (92 tests) were chosen to carry out a cluster analysis using the simple-matching coefficient (S_{SM}) (Sokal & Michener, 1958) and UPGMA (Sneath & Sokal, 1973). Computer analysis was undertaken with the TAXAN program (Information Resources Group, Maryland Biotechnology Institute, University of Maryland, College Park, MD 20742, USA). The estimated test error (Sneath & Johnson, 1972) was no more than 3 % in any of the tests. No test was found to be so irreproducible (test variance $\geqslant 0{\cdot}2$) as to be excluded from the analysis. As can be seen in Fig. 1, the two strains grouped between themselves at a 94 % similarity level and shared the highest similarity (about 90 %) with the cluster formed by *B. subtilis* subsp. *subtilis* and *Bacillus amyloliquefaciens*. Table 1 shows the main features that distinguish *B. velezensis* from other phenotypically and phylogenetically related taxa. The two novel strains are characterized by their capacity to produce acids from glycogen, lactose, methyl α-D-glycoside and D-raffinose but not from D-turanose. They do not hydrolyse

Table 1. Characteristics which distinguish *Bacillus velezensis* sp. nov. from other related species of *Bacillus*

1, *B. velezensis* sp. nov. CR-502^T; 2, *B. subtilis* subsp. *subtilis* CECT 39^T; 3, *B. atrophaeus* DSM 7264^T; 4, *B. mojavensis* DSM 9205^T; 5, *B. vallismortis* DSM 11031^T; 6, *B. amyloliquefaciens* DSM 7^T. Data from this study and from Nakamura (1989), Priest *et al.* (1987) and Roberts *et al.* (1994, 1996). ND, Not determined.

Characteristic	1	2	3	4	5	6
Pigmentation	Creamy white	Creamy white	Creamy white	Creamy white	Dark brown	Creamy white
Oxidase	+	+	−	+	+	+
Acid in API system from:						
Glycogen	+	−	−	−	−	ND
Lactose	+	−	−	−	−	+
Melibiose	−	+	−	−	−	+
Methyl α-D-glycoside	+	+	−	−	−	+
D-Raffinose	+	+	−	+	−	+
D-Turanose	−	+	−	−	−	+
Hydrolysis of:						
Tween 20	−	+	+	+	+	+
Tween 80	−	+	+	+	+	ND
DNA	−	+	+	+	+	−
Arginine dihydrolase	−	+	−	−	−	−
ONPG	+	+	+	+	+	−

DNA, Tween 20 or Tween 80. They produce *O*-nitrophenyl *β*-D-galactopyranoside (ONPG) but not arginine dihydrolase.

DNA was purified using the method of Marmur (1961). The G+C content was determined from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) obtained at 260 nm with a Perkin-Elmer UV-Vis Lambda3B spectrophotometer programmed for temperature increases of 1·0 °C min^{-1}. T_m was determined by the graphic method described by Ferragut & Leclerc (1976) and the G+C content was calculated from this temperature using the equation of Owen & Hill (1979). The T_m value of the reference DNA from *Escherichia coli* NCTC 9001^T was taken to be 74·6 °C in 0·1× SSC (Owen & Pitcher, 1985). The G+C content of the two strains was 46·4 mol% for strain CR-502^T and 46·1 mol% for strain CR-14b. These G+C values fall within the wide range of 41·5–47·5 mol% accepted for *B. subtilis* (Claus & Berkeley, 1986; Harwood, 1989).

The 16S rRNA genes were amplified by PCR using standard protocols (Saiki *et al.*, 1988). The forward primer, 16F27 (5′-AGAGTTTGATCATGGCTCAG-3′), annealed at positions 8–27 and the reverse primer, 16R1488 (5′-CGGTTACCTTGTTAGGACTTCACC-3′) (both from Pharmacia), annealed at the complement of positions 1511–1488 (*E. coli* numbering according to Brosius *et al.*, 1978). We also used an intermediate primer designed by us, 5′-CGGATCGTAAAGCTCTGTTG-3′. This primer annealed at positions 401–421. The PCR products were purified using the Qiaquick spin-gel extraction kit (Qiagen). Direct sequence determinations of PCR-amplified DNAs were carried out with the ABI PRISM dye-terminator, cycle-sequencing, ready-reaction kit (Perkin-Elmer) and an ABI PRISM 377 sequencer (Perkin-Elmer) according to the manufacturer's instructions. The sequences obtained were compared to reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center of Biotechnology Information database using the BLAST search. Phylogenetic analysis was performed using MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignments of data by CLUSTAL X (Thompson *et al.*, 1997). Distances and clustering were determined with the neighbour-joining and maximum-parsimony methods. The stability of clusters was ascertained by bootstrap analysis (1000 replications). The phylogenetic tree, constructed using the neighbour-joining method, is shown in Fig. 2. The maximum-parsimony algorithm gave a similar result (data not shown). The sequences of strains CR-502^T and CR-14b formed a phylogenetic branch with the species with which they also showed the highest phenotypic similarity. The highest similarity values between our strains and these species were 99 % (*B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* and *Bacillus vallismortis*) and 98 % (*Bacillus mojavensis* and *Bacillus atrophaeus*).

DNA–DNA hybridization was conducted following the methods of Lind & Ursing (1986) with the modifications of Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001). Strain

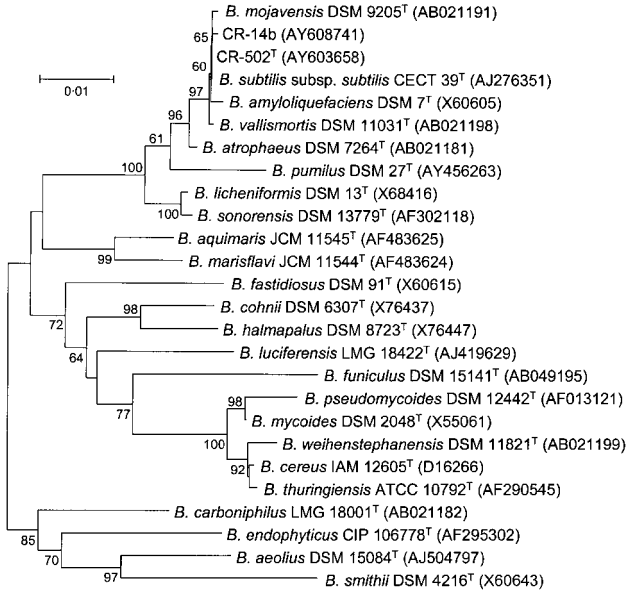

Fig. 2. Phylogenetic relationships between the two strains of *Bacillus velezensis* sp. nov. and other *Bacillus* species. The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 60 % are shown (1000 replications). Bar, 1 % estimated sequence divergence.

CR-502^T was not related to the other type strains of related species, showing less than 20 % hybridization with them (Table 2).

Fatty-acid analysis of strain CR-502^T was undertaken by the analytical services of Microbial Identification Systems (Williston, VT 05495, USA), using the MID/Hewlett Packard Microbial Identification System (MIS), which relies upon high-resolution GC to obtain the fatty-acid profile. The results of their chemotaxonomic analyses are

Table 2. G+C content and DNA–DNA hybridization between strain CR-502^T and other related taxa

Values are mean results of at least three independent determinations, which generally did not differ by more than 5 %. Data from this study and from Claus & Berkeley (1986), Roberts *et al.* (1994, 1996), Nakamura (1989) and Priest *et al.* (1987).

Strain	Hybridization with CR-502^T (%)	G+C content (mol%)
CR-502^T	100	46·4
CR-14b	81·9	46·1
B. subtilis subsp. *subtilis* CECT 39^T	13·3	41·5–47·5
B. mojavensis DSM 9205^T	8·3	43
B. atrophaeus DSM 7264^T	9·3	41–43
B. vallismortis DSM 11031^T	9·3	43
B. amyloliquefaciens DSM 7^T	18·6	43–44·35

Table 3. Main differences in cellular fatty-acid composition (%) between *B. velezensis* sp. nov. CR-502^T and type strains of other related species of *Bacillus*

Strains: 1, *B. velezensis* sp. nov. CR-502^T; 2, *B. mojavensis* NRRL B-14698^T; 3, *B. subtilis* NRRL NRS-744^T; 4, *B. amyloliquefaciens* ATCC 23350^T; 5, *B. atrophaeus* NRRL NRS-213^T; 6, *B. licheniformis* ATCC 14580^T; 7, *B. vallismortis* NRRL B-14890^T. Data from this study and Roberts *et al.* (1994, 1996). −, Not detected.

Fatty acid	1	2	3	4	5	6	7
13 : 0 iso	0·87	−	−	−	−	−	−
14 : 0	2·96	−	−	−	−	−	−
14 : 0 iso	1·08	0·98	1·13	2·46	1·44	1·31	−
15 : 0 anteiso	32·70	42·51	40·19	36·48	51·36	37·75	37·5
15 : 0 iso	29·86	22·33	29·27	30·50	15·02	28·87	24·60
16 : 0	13·41	2·05	3·14	4·52	1·99	3·91	2·71
16 : 0 iso	1·31	2·56	2·36	4·40	3·10	4·42	4·06
16 : 1ω5*c*	−	1·74	1·52	2·14	1·72	1·53	0·64
16 : 1ω9*c*	−	0·69	0·23	0·62	1·16	0·91	0·45
16 : 1ω11*c*	4·42	−	−	−	−	−	−
17 : 0 iso	7·67	8·92	9·59	9·01	4·97	6·99	14·43
17 : 0 anteiso	4·27	12·53	9·38	7·06	14·83	11·30	12·07
17 : 1ω7*c* iso	−	3·45	1·72	1·67	1·99	1·23	1·55
17 : 1ω10*c* iso	1·44	−	−	−	−	−	−

given in the species description and in Table 3. Strain CR-502^T showed a specific fatty-acid profile characterized mainly by its high 16 : 0 fatty-acid content and the presence of 16 : 1ω11*c*, 13 : 0 iso, 14 : 0 and 17 : 1ω10*c* iso.

Quinones were identified by HPLC at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). As in other *Bacillus* strains, the main quinone in strain CR-502^T was menaquinone with seven isoprene units, MK-7 (96·6 %), plus a minor proportion of MK-6 (3·4 %).

Accordingly, on the basis of differences in phenotypic and chemotaxonomic characteristics and genetic distinctiveness, strains CR-502^T and CR-14b should be recognized as representing a novel species of the genus *Bacillus*, for which we propose the name *Bacillus velezensis*.

Description of *Bacillus velezensis* sp. nov.

Bacillus velezensis (vel.e.zen′sis. N.L. adj. masc. *velezensis* pertaining to Vélez, named thus for being first isolated from the river Vélez in Málaga, southern Spain).

The cells are Gram-positive rods, 0·5 × 1·5–3·5 μm, occurring both singly and in pairs and occasionally in short chains. Endospores are ellipsoidal and lie in paracentral or subterminal positions in non-swollen sporangia. They do not contain parasporal crystals or accumulate poly-β-hydroxybutyric acid. They are motile by peritrichous flagella. On TSA medium the bacteria grow in creamy-white, rough colonies with slightly irregular edges. In liquid TSB medium a thin film is formed at the surface whilst the rest of the medium is uniformly cloudy, showing no strands or clumps. They are capable of growing in NaCl concentrations of up to 12 % w/v. They grow within the temperature range of 15 to 45 °C and at pH values of between 5 and 10. They are chemo-organotrophic. Catalase and oxidase are produced. Their metabolism is respiratory, with oxygen as terminal electron acceptor. They do not grow in anaerobiosis in the presence of nitrate or fumarate. They produce acids (without gas) from aesculin, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, glycerol, D-glucose, glycogen, inositol, lactose, maltose, mannitol, D-mannose, methyl α-D-glycoside, D-raffinose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. They do not produce acids from adonitol, D-arabinose, D-arabitol, L-arabitol, 2- or 5-ketogluconate, dulcitol, erythritol, D- or L-fucose, galactose, β-gentiobiose, gluconate, inulin, D-lyxose, D-melezitose, melibiose, methyl α-D-mannoside, methyl β-D-xyloside, *N*-acetylglucosamine, rhamnose, L-sorbose, D-tagatose, D-turanose, xylitol or L-xylose. They reduce nitrate and produce H_2S from L-cysteine. Vogues–Proskauer and ONPG are positive. They hydrolyse blood, starch, gelatin and casein. They grow in media without yeast extract. Indol, lysine decarboxylase, ornithine decarboxylase, urease, arginine dihydrolase, tryptophan deaminase, phenylalanine deaminase and growth on lysozyme (0·001 %, w/v) are negative. They do not hydrolyse Tween 20, Tween 80, DNA or tyrosine. They are susceptible to amoxicillin (25 μg), amoxicillin/clavulanic acid (30 μg), cephalotin (30 μg), chloramphenicol (30 μg), colistin (10 μg), doxycycline (30 μg), erythromycin (15 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacin (5 μg), novobiocin (30 μg), rifampicin (30 μg), trimetroprim/sulfamethoxazole (1·25/23·75 μg) and vancomycin (30 μg). They are resistant to aztreonam (30 μg). The DNA G+C content is 46·1–46·4 mol% (T_m method).

The type strain is strain CR-502^T (=CECT 5687^T=LMG 22478^T), which was isolated from a brackish water sample taken at the mouth of the river Vélez at Torredelmar, Málaga, southern Spain. The description of the type strain is the same as that of the species. Additionally, this strain is able to produce acids from starch and sucrose and produce dihydroxyacetone. It is resistant to ceftazidime (30 μg). Contrary to strain CR-14b, the type strain does not use citrate as sole source of carbon and energy and does not produce lecithinase. The major fatty acids are 15 : 0 anteiso (32·70 %), 15 : 0 iso (29·86 %) and 16 : 0 (13·41 %). The main quinone is MK-7 (96·6 %). Its DNA G+C content is 46·4 mol% (T_m method).

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