Bacillus axarquiensis sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in southern Spain

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Two Gram-positive, rod-shaped, endospore-forming bacteria (strains CR-119^T and CR-95^T) were isolated from brackish sediments in the mouth of the river Vélez in Málaga, southern Spain, and subjected to a polyphasic taxonomic study. Phenotypic tests showed that these strains were related to other *Bacillus* species at a similarity level of less than 87.6%. Both strains are halotolerant, aerobic, chemoheterotrophic, motile with peritrichous flagella and biosurfactant producers. Their endospores are oval, subterminal and non-deforming structures. The predominant menaquinone in both strains is MK-7. The fatty-acid profiles of both strains contain large quantities of branched and saturated fatty acids. The major fatty acids (%) are 15:0 anteiso (32.4), 15:0 iso (16.8), 17:0 iso (13.4), 16:0 (11.5) and 17:0 anteiso (10.2) in strain CR-119^T and 15:0 anteiso (37.5), 17:0 iso (16.0) and 17:0 anteiso (15.8) in strain CR-95^T. The G + C contents of strains CR-119^T and CR-95^T are 41.0 and 42.5 mol%, respectively. RAPD analysis confirmed the low degree of similarity between the two strains and also amongst other *Bacillus* species. 16S rRNA gene analysis of strain CR-119^T showed the highest sequence similarity to be 97.4%, with *Bacillus mojavensis* and *Bacillus subtilis* subsp. *spizizenii*. In the case of strain CR-95^T, the maximum similarity value was 99.5%, with *B. mojavensis*. DNA–DNA hybridization of strains CR-119^T and CR-95^T with the above species produced values lower than 46.9%. Therefore, on the basis of phenotypic characteristics, phylogenetic data and genomic distinctiveness, we conclude that these *Bacillus* strains merit classification as novel species, for which we propose the names *Bacillus axarquiensis* sp. nov. (type strain CR-119^T = CECT 5688^T = LMG 22476^T) and *Bacillus malacitensis* sp. nov. (type strain CR-95^T = CECT 5687^T = LMG 22477^T).

The genus *Bacillus* is phylogenetically and phenotypically very heterogeneous (Claus & Berkeley, 1986) and in fact over the last decade nine new genera have been separated from this original taxon (Wisotzkey *et al.*, 1992; Ash *et al.*, 1993; Shida *et al.*, 1996; Heyndrickx *et al.*, 1998; Waino *et al.*, 1999; Nazina *et al.*, 2001; Yoon *et al.*, 2001; Fortina *et al.*, 2001). It is more than likely that future years will see a further reorganization of the genus. It currently includes around 100 species (Euzéby, 2004). Some *Bacillus* species, such as *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus*, produce biosurfactants (Arima *et al.*, 1968; Naruse *et al.*,

1990; Yakimov *et al.*, 1995), compounds that reduce surface and interfacial tension and thus have excellent detergent, emulsifying, foaming and dispersing properties. They are used extensively in the textile, pharmaceutical and cosmetics industries and also in bioremediation (Banat *et al.*, 2000).

In an attempt to find new biosurfactant-producing strains we screened a large number of contaminated areas, using the drop-collapsing test, to select micro-organisms with high surfactant powers (Jain *et al.*, 1991). We eventually chose 29 strains of biosurfactant producers, 48% of which turned out to be strains of *B. subtilis*. We consequently undertook a polyphasic taxonomic study to characterize two of the remaining strains and on the basis of our results propose two novel *Bacillus* species.

Both micro-organisms were isolated from brackish sediments in the mouth of the river Vélez in the province of

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Bacillus axarquiensis* CR-119^T and *Bacillus malacitensis* CR-95^T are respectively AY603657 and AY603656.

Supplementary figures relating to dendrograms of phenotypic characteristics and RAPD are available in IJSEM Online.

Málaga in southern Spain 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.

The isolates and reference strains of the most closely related species were examined for 112 phenotypic characteristics. Unless otherwise stated the tests were carried out in TSA medium at 32 °C incubation temperature. Flagella were stained using the method of Rhodes (1958). Spores were stained according to the Schaeffer-Fulton method with 5 days' culture on TSA medium. Growth at different temperatures (4 to 45 °C) and pH values (5 to 10) was determined on TSA medium, and at various sea-salt concentrations (0 to 15 % w/v) on TSA medium supplemented with a mixture of sea salts (Rodríguez-Valera *et al.*, 1981). Catalase and oxidase production, aerobic nitrate and nitrite reduction, H₂S production from cysteine, methyl red, phenylalanine deaminase, lecithinase production, hydrolysis of starch, casein, DNA, tyrosine, Tween 20 and Tween 80, haemolysis and growth on MacConkey agar were all assayed according to Barrow & Feltham (1993). Growth in the presence of 100 U lysozyme ml⁻¹ and in MY without yeast extract and dihydroxyacetone production were tested according to Claus & Berkeley (1986). API 20E (bioMérieux) tests were also made. Acid-production profiles from carbohydrates were obtained with an API 50CH system (bioMérieux) after growth in 50 CHB/E medium, as described by Logan & Berkeley (1984). The tubes of the API 50CH gallery were examined for gas bubbles. Antimicrobial susceptibility was tested in TSA medium according to the method of Bauer *et al.* (1966). Differential characteristics were coded in binary form: positive and negative results were coded as 1 and 0 respectively and non-comparable or missing characteristics were coded as 9. The data were submitted to cluster analysis using the simple-matching coefficient (S_{SM}) (Sokal & Michener, 1958) and clustering was determined by the unweighted-pair-group method of association (UPGMA) (Sneath & Sokal, 1973). Computer analyses were conducted with the TAXAN program (Information Resources Group, Maryland Biotechnology Institute).

DNA was purified using the method of Marmur (1961). The G + C content of the DNA was calculated 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 estimated from this temperature using the equation of Owen & Hill (1979). The T_m value of the reference DNA from *Escherichia coli* NCTC 9001 was taken to be 74.6 °C in 0.1 × SSC (Owen & Pitcher, 1985).

RAPD analysis was performed with primers OPA3 and OPL12 following the methods of Pinchuk *et al.* (2002).

DNA–DNA hybridization was conducted following the

methods of Lind & Ursing (1986) with the modifications described by Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001).

Fatty-acid analyses and quinones were identified by high-resolution GLC and HPLC respectively at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). For these analyses the cell mass of the strains was obtained after growth in TSA medium.

The 16S rRNA gene was 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'-CGGTACCTGTAGGACTTCACC-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, 5'-CGGATCGTAAAGCTCTGTTG-3', designed in our laboratory. This primer annealed at positions 401–421. The PCR products were purified using the Microcon Qiaquick spin-gel extraction kit (Qiagen). Direct sequence determinations of PCR-amplified DNAs were made with an 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 Centre of Biotechnology Information database using the BLAST search. Phylogenetic analyses were made using MEGA version 2.1 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL X (Thompson *et al.*, 1997). Distances and clustering were determined using the neighbour-joining and maximum-parsimony algorithms. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications). DNASTAR was used to determine similarity percentages.

The strains described here, CR-119^T and CR-95^T, were isolated during a wide research programme, the main objective of which was to select biosurfactant-producing bacteria from different habitats. These products, which are currently being studied, are lipopeptides related to the surfactin family.

The phenotypic characteristics of both strains are given in the species descriptions. Phenotypic features that distinguish between these two strains and between other related *Bacillus* species are set out in Table 1. The dendrogram obtained from the numerical analysis (Supplementary Fig. A) is available as supplementary data in IJSEM Online. Strain CR-95^T groups at an 87.6 % similarity level with the type strains of *Bacillus vallismortis*, *Bacillus mojavensis* and *Bacillus atrophaeus*. Strain CR-119^T is related to the above species and to strain CR-95^T at an 85.3 % similarity level.

The G + C content of strain CR-119^T is 41.0 mol% and that of strain CR-95^T is 42.5 mol%; these values concur with

Table 1. Characteristics that distinguish between *Bacillus axarquiensis* CR-119^T and *Bacillus malacitensis* CR-95^T and phenotypically and phylogenetically related *Bacillus* species

Strains: 1, *B. axarquiensis* CR-119^T; 2, *B. malacitensis* CR-95^T; 3, *B. amyloliquefaciens* DSM 7^T; 4, *B. atrophaeus* DSM 7264^T; 5, *B. mojavensis* DSM 9205^T; 6, *B. subtilis* subsp. *subtilis* CECT 39^T; 7, *B. vallismortis* DSM 11031^T; 8, *B. velezensis* CECT 5686^T. Data from this study and from Claus & Berkeley (1986), Logan & Berkeley (1984), Nakamura (1989), Roberts *et al.* (1994, 1996) and Ruiz-García *et al.* (2005).

Characteristic	1	2	3	4	5	6	7	8
Pigment	Cream	Cream	Cream	Brown	Cream	Cream	Cream	Cream
Oxidase	—	+	+	—	+	+	+	+
Acids from:								
L-Arabinose	+	+	+	—	+	+	+	+
Ribose	+	+	+	—	+	+	—	+
D-Xylose	+	+	—	—	+	—	—	+
Sorbitol	+	+	+	+	+	+	—	+
Methyl α -D-glucoside	+	—	+	—	—	+	—	+
Amygdalin	+	—	+	+	+	+	—	+
Lactose	—	—	+	—	—	—	—	+
Melibiose	—	—	+	—	—	+	—	—
Raffinose	+	—	+	—	+	+	—	+
Trehalose	+	+	+	—	+	+	+	+
Starch	+	+	+	—	+	+	—	+
Glycogen	+	+	—	—	—	—	—	+
Gentiobiose	+	—	—	—	—	—	—	—
D-Turanose	—	—	—	—	—	+	—	—
Citrate utilization	+	—	+	+	+	+	+	—
Dihydroxyacetone production	+	—	—	—	—	—	—	+
ONPG hydrolysis	+	+	—	+	+	+	+	+
Growth with lysozyme (0.001 %)	+	+	—	—	—	—	—	—
Lecithinase	+	+	—	+	+	—	+	—
Arginine dihydrolase	+	—	—	—	—	+	—	—
Lysine decarboxylase	+	—	—	—	—	—	—	—
Ornithine decarboxylase	+	—	—	—	—	—	—	—
Susceptibility to:								
Ceftazidime (30 μ g)	+	—	—	—	—	—	—	—
Colistin (10 μ g)	+	+	—	—	—	—	—	+
DNA G+C content (mol%)	41	42.5	43	42	43	43	43	46.4

those shown by the genus *Bacillus* (Claus & Berkeley, 1986). The main quinone of both strains is menaquinone with seven isoprene units (MK-7) (98.5 and 97.1 %, respectively), which is also a characteristic common to *Bacillus* species (Claus & Berkeley, 1986).

The results of DNA–DNA hybridization are set out in Supplementary Table A. Strains CR-119^T and CR-95^T were not related to the other type strains of phylogenetically related species, showing less than 46.9 % hybridization with them.

Following the protocols of Stackebrandt *et al.* (2002), we determined almost complete 16S rRNA gene sequences of strains CR-119^T (1454 bp) and CR-95^T (1402 bp), corresponding to positions 46 and 1445 of the *E. coli* 16S rRNA gene. The two sequences share 97 % similarity and are on the same phylogenetic branch as *B. mojavensis*, *B. subtilis* subsp. *spizizenii*, *B. vallismortis*, *B. subtilis* subsp. *subtilis* and *Bacillus velezensis*. The highest similarity values of strain

CR-95^T and CR-119^T with these species are 99.5 %, with *B. mojavensis*, and 97.4 %, with *B. mojavensis* and *B. subtilis* subsp. *spizizenii*, respectively. The phylogenetic tree, constructed using the neighbour-joining method, is depicted in Fig. 1. The maximum-parsimony algorithm gave a similar result (data not shown).

The relationship between strains CR-119^T and CR-95^T and other *Bacillus* species was also determined by the RAPD rapid DNA typing method, a useful technique for determining inter- and intraspecies relatedness (Stackebrandt *et al.*, 2002). The fingerprints obtained with primers OPA3 and OPA12 are shown in Fig. 2. Dendrograms for the fingerprints are available as Supplementary Figs B and C. The results are similar with both primers and show the differences between strains CR-119^T and CR-95^T and other selected *Bacillus* species.

Table 1 shows the features that distinguish strains CR-119^T and CR-95^T from other related taxa. In both strains, as in

Fig. 1. Phylogenetic relationships between *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov. and among other *Bacillus* species. The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 50% are shown (1000 replications). Bar, 1% estimated sequence divergence.

many *Bacillus* species, terminally methyl-branched iso- and anteiso-fatty acids with 15 to 17 carbons predominate (Claus & Berkeley, 1986). Strain CR-119^T also contains a

significant quantity (11.5%) of 16:0 fatty acid (Table 2). Neither CR-119^T nor CR-95^T contains unsaturated fatty acids. Therefore, on the basis of our phenotypic, genotypic, chemotaxonomic and phylogenetic observations, we propose the acceptance of two novel *Bacillus* species for strains CR-119^T and CR-95^T: *Bacillus axarquiensis* and *Bacillus malacitensis*.

Description of *Bacillus axarquiensis* sp. nov.

Bacillus axarquiensis (a.xar.qui.en'sis. N.L. adj. masc. *axarquiensis* pertaining to Axarquia, the Arabic name for the region surrounding the city of Málaga in southern Spain).

Cells are Gram-positive, aerobic, round-ended rods ($2.0 \times 0.5 \mu\text{m}$), occurring singly or in pairs and occasionally in short chains or filaments. They are motile by peritrichous flagella. The endospores are mainly ellipsoidal and lie in subterminal positions in non-swollen sporangia. They do not contain parasporal crystals or accumulate poly- β -hydroxybutyrate (PHB). When grown on TSA the colonies are cream-coloured, slightly irregular in shape and bulge upward. When the medium is supplemented with salt the colonies become mucous. In liquid medium a thin film is formed at the surface whilst the rest of the medium is uniformly cloudy. The bacterium grows within a temperature range of 15 to 45 °C and pH values of between 5 and 10. It is halotolerant, being capable of growth in salt concentrations (mixture of sea salts) of 0 to 12% w/v. Optimum growth is at 32 °C, pH 7.2 and 0.5% w/v sea salts. It produces surfactants. It is catalase-positive and oxidase-negative. It reduces nitrate aerobically. Starch, Tween 20, Tween 80, DNA, gelatin, casein and lecithin are hydrolysed. Citrate is used as sole carbon and energy source. Haemolysis, Voges-Proskauer test, dihydroxyacetone production, H₂S from cysteine, O-nitrophenyl β -D-galactopyranoside (ONPG) hydrolysis, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are positive. It grows in

Fig. 2. Fingerprints obtained by RAPD analysis with primers OPA3 (a) and OPL12 (b). Lanes: M, molecular mass marker (λ EcoRI/HindIII ladder); 1, *B. atrophaeus* DSM 7264^T; 2, *Brevibacillus brevis* CECT 5^T; 3, *B. licheniformis* CECT 29^T; 4, *B. mojavensis* DSM 9205^T; 5, *B. pumilus* CECT 20^T; 6, *B. sonorensis* DSM 13779^T; 7, *B. subtilis* subsp. *subtilis* CECT 39^T; 8, *B. vallismortis* DSM 11031^T; 9, strain CR-95^T; 10, strain CR-119^T.

Table 2. Fatty-acid compositions of *B. axarquiensis* CR-119^T, *B. malacitensis* CR-95^T and related *Bacillus* species

Strains: 1, *B. axarquiensis* CR-119^T; 2, *B. malacitensis* CR-95^T; 3, *B. amyloliquefaciens* DSM 7^T; 4, *B. atrophaeus* NRRL NRS-213^T; 5, *B. mojavensis* NRRL B-14698^T; 6, *B. subtilis* CECT 39^T; 7, *B. vallismortis* NRRL B-14890^T; 8, *B. velezensis* CECT 5686^T. Values are percentages of total fatty acids; —, not detected. Data from this study and from Roberts *et al.* (1994, 1996) and Ruiz-García *et al.* (2005).

Fatty acid	1	2	3	4	5	6	7	8
14:0 iso	1.89	0.87	0.98	1.44	0.98	1.13	1.07	1.08
15:0 iso	16.76	18.32	22.33	15.02	22.33	29.27	24.60	29.86
15:0 anteiso	32.44	37.54	42.51	51.36	42.51	40.19	37.5	32.70
16:1 <i>cis</i> 9	—	—	0.69	1.16	0.69	0.23	0.45	—
16:0 iso	5.57	3.24	2.56	3.10	2.56	2.36	4.06	1.31
16:1 <i>cis</i> 5	—	—	1.74	1.72	1.74	1.52	0.64	—
16:0	11.46	5.4	2.05	1.99	2.05	3.14	2.71	13.41
17:1 <i>cis</i> 7 iso	—	—	3.45	1.99	3.45	1.72	1.55	—
17:0 iso	13.43	15.99	8.92	4.97	8.92	9.59	14.43	7.67
17:0 anteiso	10.16	15.80	12.53	14.83	12.53	9.38	12.07	4.27
18:0	3.53	2.9	—	—	—	—	—	—

the presence of lysozyme and in media without yeast extract. Tyrosine and urea are not hydrolysed. Tryptophan and phenylalanine deaminase, pigment after growth on tyrosine medium, H₂S from sodium thiosulphate, gas from carbohydrates, indole, methyl red and growth on MacConkey agar are negative. Acids are produced from the following sugars: glycerol, L-arabinose, ribose, D-xylose, fructose, glucose, mannose, inositol, mannitol, sorbitol, methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, raffinose, starch, glycogen, xylitol and gentiobiose. Acids are not produced from erythritol, D-arabinose, L-xylose, adonitol, methyl β -D-xyloside, galactose, sorbose, rhamnose, dulcitol, methyl α -D-mannoside, N-acetylglucosamine, lactose, melibiose, inulin, melezitose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. It is susceptible to amoxicillin (2 μ g), amoxicillin/clavulanic acid (30 μ g), cephalothin (30 μ g), ceftazidime (30 μ g), chloramphenicol (30 μ g), colistin (10 μ g), doxycycline (30 μ g), erythromycin (15 μ g), kanamycin (30 μ g), nalidixic acid (30 μ g), nitrofurantoin (300 μ g), norfloxacin (5 μ g), novobiocin (30 μ g), rifampicin (30 μ g), trimethoprim/sulfamethoxazol (1.25–23.7 μ g) and vancomycin (30 μ g). It is resistant to aztreonam (30 μ g). Major fatty acids (%) are 15:0 anteiso (32.4), 15:0 iso (16.8), 17:0 iso (13.4), 16:0 (11.5) and 17:0 anteiso (10.2). It has menaquinone (MK-7) as principal quinone compounds. Its DNA G+C content is 42.5 mol% (*T_m* method).

The type strain is strain CR-119^T (=CECT 5688^T=LMG 22476^T), isolated from brackish sediments taken from the mouth of the river Vélez in the province of Málaga in southern Spain.

Description of *Bacillus malacitensis* sp. nov.

Bacillus malacitensis (ma.la.ci.ten'sis. L. adj. masc. *malacitensis* pertaining to Flavia Malacita, the Roman name for Málaga in southern Spain).

Cells are Gram-positive, aerobic, round-ended rods (2.0 $\times$ 0.5 μ m), occurring singly or in pairs and occasionally in short chains or filaments. They are motile by peritrichous flagella. The endospores are mainly ellipsoidal and lie in subterminal positions in non-swollen sporangia. They do not contain parasporal crystals or accumulate PHB. When grown on TSA the colonies are cream-coloured, slightly irregular in shape and bulge upward. When the medium is supplemented with salt the colonies become mucous. In liquid medium a thin film is formed at the surface whilst the rest of the medium is uniformly cloudy. The bacterium grows within the temperature range of 15 to 45 °C and pH values of between 5 and 10. It is halotolerant, being capable of growing in salt concentrations (mixture of sea salts) of 0 to 12 % w/v. Optimum growth is at 32 °C, pH 7.2 and 0.5 % sea salts. It produces surfactants. Catalase and oxidase are positive. It reduces nitrate and nitrite. Starch, DNA, Tween 20, Tween 80, gelatin, casein and lecithin are hydrolysed. Haemolysis and ONPG hydrolysis are positive. Tyrosine and urea are not hydrolysed. Reactions for pigment production on tyrosine medium, gas from carbohydrates, H₂S from sodium thiosulphate, arginine dihydrolase and lysine and ornithine decarboxylase are negative. Dihydroxyacetone production, indole and methyl-red are negative. Phenylalanine and tryptophan deaminase are not produced. Growth on MacConkey agar and growth with citrate as sole carbon and energy source are not viable. It grows in the presence of lysozyme and in media without yeast extract. Voges-Proskauer is positive. It produces H₂S from L-cysteine. Acids are produced from the following sugars: glycerol, L-arabinose, ribose, D-xylose, glucose, fructose, mannose, inositol, mannitol, sorbitol, arbutin, aesculin, salicin, cellobiose, maltose, sucrose, trehalose, starch and glycogen. It does not produce acids from erythritol, D-arabinose, L-xylose, adonitol, methyl β -D-xyloside, galactose, sorbose, rhamnose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, lactose, melibiose, inulin, melezitose, raffinose, xylitol,

gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. It is susceptible to amoxicillin (2 µg), amoxicillin/clavulanic acid (30 µg), cephalothin (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), trimethoprim/sulfametoxazol (1·25/23·7 µg) and vancomycin (30 µg). It is resistant to aztreonam (30 µg) and ceftazidime (30 µg). The major fatty acids (%) are 15:0 anteiso (37·5), 17:0 iso (15·9) and 17:0 anteiso (15·8). It has menaquinone MK-7 as principal quinone compounds. Its DNA G + C content is 41·0 mol% (T_m method).

The type strain is strain CR-95^T (=CECT 5687^T=LMG 22477^T), isolated from brackish sediments taken from the mouth of the river Vélez in the province of Málaga in southern Spain.

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