Desulfoprunum benzoelyticum gen. nov., sp. nov., a Gram-stain-negative, benzoate-degrading, sulfate-reducing bacterium isolated from a wastewater treatment plant

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A strictly anaerobic, mesophilic, sulfate-reducing bacterium, strain KoBa311^T, isolated from the wastewater treatment plant at Konstanz, Germany, was characterized phenotypically and phylogenetically. Cells were Gram-stain-negative, non-motile, oval to short rods, 3–5 μm long and 0.8–1.0 μm wide with rounded ends, dividing by binary fission and occurring singly or in pairs. The strain grew optimally in freshwater medium and the optimum temperature was 30 °C. Strain KoBa311^T showed optimum growth at pH 7.3 7.6. Organic electron donors were oxidized completely to carbon dioxide concomitant with sulfate reduction to sulfide. At excess substrate supply, substrates were oxidized incompletely and acetate (mainly) and/or propionate accumulated. The strain utilized short-chain fatty acids, alcohols (except methanol) and benzoate. Sulfate and DMSO were used as terminal electron acceptors for growth. The genomic DNA G+C content was 52.3 mol% and the respiratory quinone was menaquinone MK-5 (V-H_2). The major fatty acids were $C_{16:0}$, $C_{16:1}\omega7c/\omega6c$ and $C_{18:1}\omega7c$. Phylogenetic analysis based on 16S rRNA gene sequences placed strain KoBa311^T within the family *Desulfobulbaceae* in the class *Deltaproteobacteria*. Its closest related bacterial species on the basis of the distance matrix were *Desulfobacterium catecholicum* DSM 3882^T (93.0 % similarity), *Desulfocapsa thiozymogenes* (93.1 %), *Desulforhopalus singaporensis* (92.9 %), *Desulfopila aestuarii* (92.4 %), *Desulfopila inferna* JS_SRB250Lac^T (92.3 %) and *Desulfofustis glycolicus* (92.3 %). On the basis of phylogenetic, physiological and chemotaxonomic characteristics, strain KoBa311^T was distinct from any related type species. Therefore, strain KoBa311^T is considered to represent a novel species of a new genus, for which the name *Desulfoprunum benzoelyticum* gen. nov., sp. nov. is proposed. The type strain of *Desulfoprunum benzoelyticum* is KoBa311^T (=DSM 28570^T= KCTC 15441^T).

Anaerobic processes find wide applications in the anaerobic treatment of domestic and industrial wastewaters. A major development has been to apply anaerobic processes for the treatment of aromatic compounds in industrial wastewaters (Li *et al.*, 1995). In the past decade, studies have demonstrated that simple aromatic compounds such as phenol and benzoate can be effectively degraded by anaerobic processes (Li *et al.*, 1995; Fang *et al.*, 1996). Benzoate is one of the central intermediates in the degradation of many naturally or chemically synthesized aromatic compounds. In addition, several syntrophically fermenting bacteria degrade benzoate, such as *Syntrophus buswellii* (Mountfort *et al.*, 1984) and *Syntrophus gentianae* HQGÖ1^T (Szewzyk & Schink, 1989). However, most wastewaters contain aromatic compounds along with large amounts of sulfate. Therefore, sulfate reduction becomes a crucial process under anoxic conditions and contributes to biogeochemical sulfur cycling in different environments. Sulfate-reducing bacteria (SRB) comprise a diverse group of species (Castro *et al.*, 2000; Kuever *et al.*, 2006). More than 10 sulfate-reducing cultures that can grow with various aromatic substrates have been reported since 1980 (Beller *et al.*, 1996), and these exhibit a broad metabolic diversity (see detailed review by Plugge *et al.*, 2011). Some species of SRB oxidize benzoate with sulfate as electron acceptor (Widdel, 1987). Also, a thermophilic, benzoate-degrading, sulfate-reducing bacterium, *Desulfotomaculum thermobenzoicum*, has been reported (Tasaki *et al.*, 1991). In the present study,

Abbreviations: CFA, cellular fatty acid; SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Desulfoprunum benzoelyticum* KoBa311^T is KJ766003.

we report the isolation of strain KoBa311^T, a benzoate-degrading, sulfate-reducing bacterium from a wastewater treatment plant.

Enrichment cultures with *o*-phthalate as substrate were inoculated with activated sewage sludge from the municipal wastewater treatment plant at Konstanz, Germany. Although strain KoBa311^T was isolated from an enrichment with *o*-phthalate, it did not grow with this substrate: perhaps benzoate is a side product of phthalate degradation in the enrichment culture and supports growth of strain KoBa311^T by cross feeding. Therefore, the strain described in this study was grown with benzoate as sole carbon and energy source. Isolation, cultivation and growth experiments were performed in anoxic, bicarbonate-buffered, sulfide-reduced freshwater mineral medium containing (g l^{-1}, except where indicated): NaCl, 1; $MgCl_2 . 6H_2O$, 0.4; KH_2PO_4, 0.2; NH_4Cl, 0.25; KCl, 0.5; $CaCl_2 . 2H_2O$, 0.15; $NaHCO_3$, 2.5; $Na_2S . 9H_2O$, 1 mM; Na_2SO_4, 20 mM (Widdel & Bak, 1992). The medium (excluding $Na_2S . 9H_2O$ and $NaHCO_3$) was autoclaved at 121 °C for 25 min and cooled under an oxygen-free mixture of N_2/CO_2 (80 : 20) gas phase. Sterile stocks of 1 ml trace element solution SL-10 (Widdel *et al.*, 1983), 1 ml selenate tungstate (Tschech & Pfennig, 1984) and 1 ml seven-vitamin solution (Pfennig, 1978) were added from concentrated stocks. The initial pH of the medium was adjusted to 7.3 ± 0.1 with sterile 1 M NaOH or 1 M HCl. Cultivation and transfer of the strain were performed under N_2/CO_2 (80 : 20) atmosphere. The strain was cultivated in the dark at 30 °C. Pure cultures were obtained by repeated application of deep-agar (1 %) shake dilutions as described by Widdel & Bak (1992). The medium was supplemented with 1 mM sodium benzoate plus sulfate (20 mM). The agar shake tubes were incubated in inverted positions for 2–3 weeks until visible colonies appeared. The strain was routinely checked for purity under the light microscope (Zeiss west Germany). Stock cultures were transferred every 4–5 weeks and stored in liquid medium at 4 °C. Each electron donor was added at a final concentration of 5 mM unless indicated otherwise. The isolate was examined by phase-contrast microscopy (Axiophot Zeiss) and photographs were taken using the agar slide technique (Pfennig & Wagener, 1986). Gram staining was determined using a staining kit (Difco Laboratories) according to the manufacturer's instructions, and by the KOH test (Gregersen, 1978).

The effects of temperature, pH and salinity were studied to optimize the growth conditions. The temperature range for growth was performed by growing the strain at 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C. The initial pH range for growth was determined over the pH range 4–9 at intervals of 0.5 pH units. The salt (NaCl) optimization of strain was determined in the absence of NaCl and with 0.01, 0.02, 0.05, 0.10, 0.5, 1.0, 1.5 and 2 % (w/v) NaCl. The ability to utilize various electron acceptors, namely elemental sulfur, ferric hydroxide (10 mM), thiosulfate (10 mM), sulfite (10 mM), sulfate (20 mM), DMSO (10 mM) and nitrate (5 mM), was studied with succinate (8 mM) as electron donor. The medium with sulfate served as a positive control and medium without sulfate as a negative control. The following substrates were tested for utilization, based on the list of compounds metabolized by the different genera of SRB (Widdel & Bak, 1992) with sulfate as electron acceptor: acetate, propionate, butyrate, valerate, pyruvate, lactate, succinate, malate, fumarate, glucose, benzoate, nicotinate, betaine, choline chloride, formate, methanol, ethanol, propanol, butanol, alanine, glutamate, 2-oxoglutarate, oxaloacetate, citrate (all 10 mM), *o*-phthalate (1 mM), iso-phthalate (1 mM), tere-phthalate (1 mM), 3-hydroxybenzoate (1 mM), 4-hydroxybenzoate (1 mM) and 3,4-dihydroxybenzoate (1 mM). Utilization of each electron acceptor or donor was analysed via turbidity and sulfide production. Organic acids were quantified with an HPLC system (LC-prominence; Shimadzu) equipped with an Aminex HPX-87H ion-exclusion column (Bio-Rad) and analysed at 60 °C, with 10 mM H_3PO_4 as the mobile phase and UV detection at 200 nm. The mobile phase was used at a flow rate of 1 ml min^{-1}. Growth experiments were carried out in duplicate and terminated after 3 weeks of incubation.

Genomic DNA G+C content analysis (Cashion *et al.*, 1977) was performed by HPLC at the Identification Service of the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany). Fatty acid methyl esters of cellular fatty acids (CFAs) (Kämpfer & Kroppenstedt, 1996) and respiratory quinones (Tindall, 1990) were also identified by DSMZ.

The genomic DNA of the isolate was extracted with a genomic DNA extraction kit (Cat. No. 19060; Qiagen) according to the manufacturer's instructions, and amplification of the 16S rRNA gene was performed by PCR as described by Junghare *et al.* (2012) with bacterial universal primers 27F and 1492R (Lane, 1991). Micro-organisms and environmental sequences closely related to strain KoBa311^T

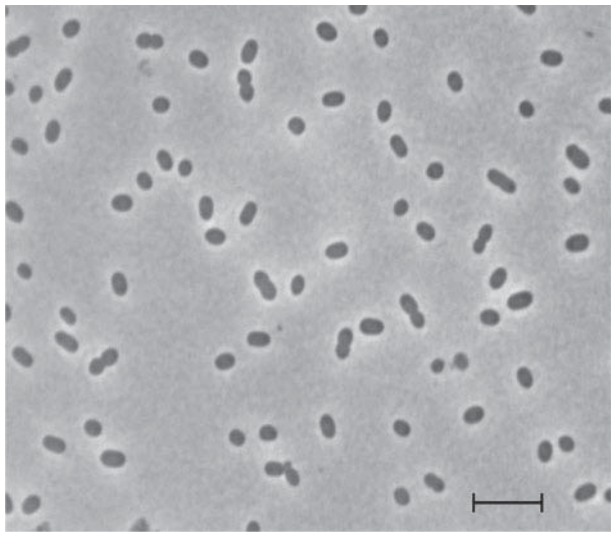

Fig. 1. Phase-contrast micrograph of cells of strain KoBa311^T grown with benzoate plus sulfate. Bar, 10 μm.

Table 1. Stoichiometry of benzoate and succinate degradation by strain KoBa311^T with sulfate as the electron acceptor

Substrate	Substrate utilized (mM)	Sulfide produced (mM)	Volatile fatty acids produced (mM)	
			Acetate	Propionate
Benzoate	0.98	3.1	0.03	0
Succinate	3.7	4.9	0.02	0.01
	3.8 (5.8)*	5.7	4.08	0.01

*Excess substrate concentration.

were determined by BLAST search against the non-redundant GenBank database (Altschul *et al.*, 1990) and the EzTaxon-e tool (Kim *et al.*, 2012). The 16S rRNA gene sequences of strain KoBa311^T (1358 bp) and closely related taxa obtained from the GenBank database were aligned using the SINA sequence alignment program (http://www.arb-silva.de/aligner/; Pruesse *et al.*, 2012; Quast *et al.*, 2013). The ARB program package was used for phylogenetic tree reconstruction. Phylogenetic trees were reconstructed based on 1116 nt unambiguously aligned sequence positions and a 50 % conservation filter using the RAxML method (Stamatakis *et al.*, 2008). The results achieved using the maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods also supported the same branching pattern and phylogenetic placement of strain KoBa311^T (data not shown). Confidence in the resultant tree topology was evaluated by resampling 100 bootstrap trees (Tamura *et al.*, 2011) using the RAxML algorithm (Stamatakis *et al.*, 2008).

Cells of strain KoBa311^T were oval to short rods, 3–5 μm long and 0.8–1.0 μm wide, with rounded ends, occurring singly or in pairs (Fig. 1). The cells divided by binary fission and were non-motile. Cells were Gram-stain-negative as determined both by Gram staining and by the KOH test. No spore formation was observed.

Strain KoBa311^T reduced sulfate to sulfide, with benzoate and succinate as electron donors, and produced sulfide close to the theoretical molar ratio of 1 : 3.75 (benzoate/sulfide) and 1 : 1.75 (succinate/sulfide), as shown in Table 1. Thus, strain KoBa311^T showed complete oxidation of electron donors. However, succinate at excess concentration led to accumulation of acetate as an intermediate product in the medium. This could be due to inhibition of acetate utilization by accumulating sulfide.

The strain grew even in the absence of added NaCl. Optimum growth was at 0.01–0.10 % (w/v) NaCl in the medium. Growth was inhibited at higher NaCl concentrations. Optimum temperature for growth was 30 °C; little growth was observed at 20, 25 and 37 °C. The optimum initial pH was 7.3–7.6.

Strain KoBa311^T used sulfate and DMSO as electron acceptors. Slow growth was observed with thiosulfate. However, the strain could not grow with elemental sulfur, $Fe(OH)_3$ or sulfite. Nitrate was not reduced to nitrite. Electron donors utilized by the strain included pyruvate, butanol, H_2, ethanol, benzoate, propionate, propanol, fumarate, malate, succinate, acetate (slow growth), formate, lactate, butyrate and oxaloacetate. It could not grow with 2-oxoglutarate, citrate, valerate, nicotinate, choline chloride, 3,4-dihydroxybenzoate, methanol, 3-hydroxybenzoate, glucose, glutamate, alanine or betaine. The strain could not ferment substrates in the absence of an electron acceptor.

Table 2. CFA composition of strain KoBa311^T and related type strains

Strains: 1, KoBa311^T; 2, *Desulfopila aestuarii* MSL86(T) (Suzuki *et al.*, 2007); 3, *Desulfopila inferna* JS SRB250Lac(T) (Gittel *et al.*, 2010). Values are percentages by weight of total fatty acids. , Not present.

Fatty acid	KoBa311(T)	*Desulfopila aestuarii* MSL86(T)	*Desulfopila inferna* JS SRB250Lac(T)
$C_{14:0}$	0.87	1.4	1.1
$C_{14:0}$ 3 OH		1.8	
$C_{15:0}$	0.87		1.6
$C_{15:1}\omega6c$	0.29	1.1	
$C_{15:1}\omega9c$		11.7	
$C_{16:0}$	20.57	33.6	23.3
$C_{16:0}$ 3 OH	0.36	1.6	
$C_{16:1}\omega5c$	5.03	17.1	11.4
$C_{16:1}\omega7c$			18.3
$C_{16:1}\omega9c$			0.7
$C_{16:1}\omega7c/\omega6c$	34.97	6.0	
$C_{17:0}$	1.51	3.4	8.3
C_{17} cyclo			15.3
$C_{17:1}\omega6c$	3.90	13.7	
$C_{17:1}\omega8c$	0.55		
$C_{18:0}$	2.63	2.5	11.5
$C_{18:1}\omega5c$	0.78	2.7	
$C_{18:1}\omega7c$	23.45	1.7	8.6
$C_{18:1}\omega9c$		1.3	
$C_{18:1}\omega7c$ 11 methyl	0.72		
$C_{19:0}$ 10 methyl	0.53		
$C_{20:1}\omega7c$	1.41		

The predominant CFAs were $C_{16:0}$ (20.6 %), $C_{16:1}\omega7c/\omega6c$ (35.0 %) and $C_{18:1}\omega7c$ (23.5 %). Branched-chain fatty acids comprised about 72.6 % of the total. However, only 24.1 % of the CFAs were even-numbered straight-chain fatty acids. No cyclo fatty acids were found. The detailed fatty acids profiles of strain KoBa311^T and two other members of the family *Desulfobulbaceae*, *Desulfopila aestuarii* MSL86(T) (Suzuki *et al.*, 2007) and *Desulfopila inferna* JS SRB250Lac(T) (Gittel *et al.*, 2010), are shown in Table 2. The total contribution of about 20.6 % by $C_{16:0}$ was equal among the members of the family *Desulfobulbaceae*. Strain KoBa311^T had about 23.5 % of $C_{18:1}\omega7c$ and this component is not observed in the above two type strains of the family *Desulfobulbaceae* (Table 2). The major respiratory quinone was menaquinone MK-5 (V-H_2). The G+C content of the genomic DNA of strain KoBa311^T was 52.3 mol%.

The almost-complete 16S rRNA gene sequence of strain KoBa311^T (1458 bp) was obtained. 16S rRNA gene sequence analysis revealed that strain KoBa311^T is a member of the family *Desulfobulbaceae* (Kuever *et al.*, 2005). However, it showed only 93.0, 93.1, 92.9, 92.3, 92.4 and 92.3 % similarity to the most closely related type species, *Desulfobacterium catecholicum* DSM 3882^T (Szewzyk & Pfennig 1987), *Desulfocapsa thiozymogenes* Bra2(T) (Janssen *et al.*, 1996),

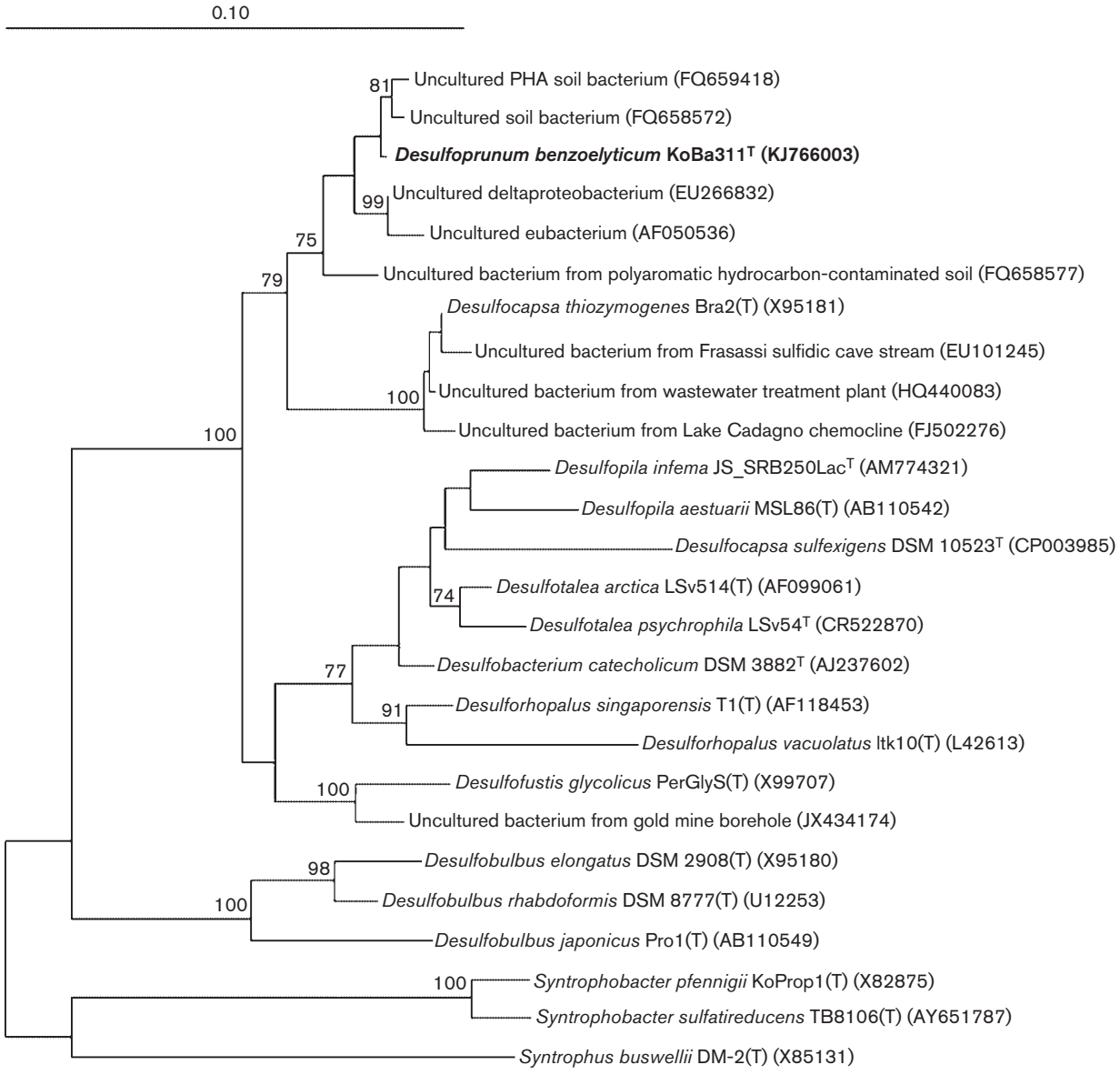

Fig. 2. Maximum-likelihood tree showing the phylogenetic placement of the 16S rRNA gene sequences of strain KoBa311^T and related taxa as generated using the RAxML algorithm (Stamatakis *et al.*, 2008). Members of *Syntrophobacteraceae* were used as an outgroup. Numbers at nodes represent bootstrap percentages. Bar, 10 % estimated sequence divergence.

Table 3. Differential physiological and chemotaxonomic characteristics between strain KoBa311^T and related species within the family *Desulfobulbaceae*

Strains: 1, KoBa311^T (this study); 2, *Desulfocapsa thiozymogenes* Bra2(T) (Janssen *et al.*, 1996); 3, *Desulfobacterium catecholicum* DSM 3882^T (Szewzyk & Pfennig, 1987); 4, *Desulfopila aestuarii* MSL86(T) (Suzuki *et al.*, 2007); 5, *Desulforhopalus singaporensis* T1(T) (Lie *et al.*, 1999); 6, *Desulfofustis glycolicus* PerGlyS(T) (Friedrich *et al.*, 1996); 7, *Desulfopila inferna* JS SRB250Lac(T) (Gittel *et al.*, 2010). +, Positive; , negative; ND, no data available; NA, not applicable.

Characteristic	1	2	3	4	5	6	7
Isolation source	Wastewater treatment plant	Freshwater lake sediment	Anoxic mud from a bay	Estuarine sediment	Sulfide rich black marine mud	Marine sediment	Tidal sediment
Cell shape	Rod	Rod	Oval to lemon	Rod	Rod	Rod	Rod
Motility	Non motile	Motile	Motile	Non motile	Non motile	Motile	Non motile
Optimum growth							
NaCl (%, w/v)	0.01 0.1	1.5	0.1*	1	ND	2	2 3
Temperature (°C)	30	20 30	28	35	31	28	28
pH	7.3 7.5	7.3 7.5	6.9 7.1	7.5 7.6	7.4	7.3	ND
Metabolism	Complete oxidation	Incomplete oxidation	Complete oxidation	Complete oxidation	Complete oxidation	Incomplete oxidation	Complete oxidation
Electron donors							
Acetate	+		+	+			
Propionate	+		+		ND		
Butyrate	+		+		+		+
Fumarate	+		+	+	+	+	+
Malate	+		+		ND	+	
Succinate	+		+	?	+	+	+
Lactate	+		ND	ND	+	+	+
Oxaloacetate	+		ND	ND	ND	ND	ND
Pyruvate	+		ND	ND	+	ND	+
Methanol		ND	+		ND		+
Ethanol	+	+	ND	ND	+		+
Propanol	+	+	+	+	ND		+
Butanol	+	+	+	+	+		
Glycerol	ND	ND	ND	+	ND	ND	
Glycine	ND		ND		ND		ND
Alanine	ND		ND		ND	ND	+
Glutamate			+		ND	ND	ND
H_2	+	ND	+	ND		+	+
Formate (acetate)	+	ND	ND	ND			ND
α Ketoglutarate		ND	ND	ND	ND	ND	ND
Citrate		ND	ND	ND	ND	ND	ND
Valerate		ND	ND	ND	ND		ND
Glucose			ND	ND	ND		ND
Benzoate	+	ND	ND	ND	ND		
Nicotinate	ND	ND	ND	ND	ND	ND	ND

Table 3. cont

Characteristic	1	2	3	4	5	6	7
Choline chloride	−	ND	ND	ND	ND	−	−
3,4-Dihydrobenzoate	−	ND	ND	ND	ND	ND	ND
3-Hydroxybenzoate	−	ND	ND	ND	ND	ND	ND
Betaine	−	ND	ND	ND	ND	−	−
Electron acceptors							
Sulfate	+	ND	ND	ND	+	+	+
Sulfite	−	+	+	+	+	+	+
Thiosulfate	−	+	+	+	+	+	+
S^0	−	NA	NA	NA	NA	NA	NA
DMSO	+	ND	ND	ND	ND	ND	ND
Nitrate	−	ND	ND	ND	+	−	−
DNA G+C content (mol%)	52.3	50.7	52.4	54.4	50.6	56.6	50.3
Respiratory quinone	MK-5 ($V\text{-}H_2$)	ND	ND	MK-8 (H_4)	ND	MK-5 (H_2)	ND
16S rRNA gene sequence similarity to strain KoBa311^T (%)	NA	93.1	93.0	92.4	92.9	92.3	92.3

*Strain NZva20^T grows best in freshwater medium containing 0.1 % (w/v) NaCl and does not grow in medium with NaCl concentrations exceeding 0.5 % (w/v) NaCl.

Desulforhopalus singaporensis T1(T) (Lie *et al.*, 1999), *Desulfofustis glycolicus* PerGlyS(T) (Friedrich *et al.*, 1996), *Desulfopila aestuarii* MSL86(T) (Suzuki *et al.*, 2007) and *Desulfopila inferna* JS SRB250Lac^T (Gittel *et al.*, 2010), respectively. Phylogenetic analysis using the maximum-parsimony algorithm revealed that strain KoBa311^T clustered with uncultured bacterial clone sequences obtained from polyaromatic hydrocarbon-contaminated soil, which represent a separate lineage (Fig. 2) and also form a clade separate from the existing type species in the family *Desulfobulbaceae*. The differential morphological and biochemical characteristics of strain KoBa311^T and other type species are listed and compared in Table 3. The physiological characteristics of strain KoBa311^T are quite distinct from these type species. Therefore, strain KoBa311^T needs to be accommodated in a new genus in the family *Desulfobulbaceae*. Based on these data, we suggest that strain KoBa311^T represents a novel species of a new genus, for which the name *Desulfoprunum benzolyeticum* gen. nov., sp. nov. is proposed.

Description of *Desulfoprunum* gen. nov.

(De.sul.fo.pru′num. L. pref. *de-* off, from; L. n. *sulfur* sulfur, L. neut. n. *prunum* plum; N.L. neut. n. *Desulfoprunum* a plum-shaped sulfate-reducing bacterium).

Cells are Gram-stain-negative, strictly anaerobic, non-motile, oval to short rods. The predominant respiratory quinone is menaquinone MK-5 ($V\text{-}H_2$). Major fatty acids are $C_{16:0}$, $C_{16:1}\omega7c/\omega6c$ and $C_{18:1}\omega7c$. Mostly utilizes short-chain fatty acids, alcohols and benzoate as carbon and energy source. Growth is mesophilic; grows optimally in freshwater medium. A member of the family *Desulfobulbaceae* of the class *Deltaproteobacteria*; the type species is *Desulfoprunum benzoelyticum* sp. nov.

Description of *Desulfoprunum benzoelyticum* sp. nov.

Desulfoprunum benzoelyticum [ben.zo.e.ly′ti.cum. N.L. n. *benzoe* (from arabic *luban dschawi*) benzoic resin; N.L. adj. *lyticum* (from Gr. adj. *lytikos*) dissolving; N.L. neut. adj. *benzoelyticum* degrading benzoate].

Has the following characteristics in addition to those given for the genus. Cells are non-sporulating and 3–5 μm long and 0.8–1.0 μm wide when grown with benzoate or succinate. Growth occurs at 20–30 °C, with an optimum at 30 °C. Grows at pH 7–8 (optimum growth at pH 7.3 ± 0.2). Optimal growth occurs with 0.01–0.1 % (w/v) NaCl; higher concentrations inhibit growth. Sulfate is reduced to sulfide with complete oxidation of the electron donors. Besides sulfate, grows with DMSO as terminal electron acceptor and thiosulfate (slow growth). Elemental sulfur, ferric hydroxide and sulfite are not utilized as electron acceptor, and nitrate is not reduced to nitrite. The following compounds are utilized as electron donors: H_2, pyruvate, butanol, propanol, ethanol, benzoate, propionate, formate, fumarate, malate, succinate,

acetate, lactate, butyrate and oxaloacetate. The following compounds are not utilized: 2-oxoglutarate, citrate, valerate, nicotinate, choline chloride, 3,4-dihydroxybenzoate, methanol, 3-hydroxybenzoate, glucose, glutamate, alanine and betaine. Does not ferment any substrate in the absence of electron acceptors.

The type strain is KoBa311^T (=DSM 28570^T=KCTC 15441^T), which was isolated from the wastewater treatment plant at Konstanz, Germany. The DNA G+C content of the type strain is 52.3 mol%.

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