Blastococcus saxobsidens sp. nov., and emended descriptions of the genus *Blastococcus* Ahrens and Moll 1970 and *Blastococcus aggregatus* Ahrens and Moll 1970

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Ten Gram-positive, aerobic bacterial strains with coccoid cells occurring singly, in pairs, tetrads and small aggregates were isolated from the surface of marble and calcareous stones. The peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The major menaquinone was MK-9(H₄). The cellular fatty acid pattern consisted mainly of iso-branched chain components. According to their phylogenetic position, the organisms are members of the family *Geodermatophilaceae*, clustering close to *Blastococcus aggregatus* DSM 4725^T. Based on a combination of phenotypic, chemotaxonomic and genotypic characteristics, strain BC444^T and seven relatives group apart from strains BC412 (= DSM 44517) and BC521 (= DSM 44518), which themselves form two individual lineages. DNA–DNA reassociation studies revealed that members of the three lineages were only remotely related to *B. aggregatus* DSM 4725^T; as the strain-rich cluster containing BC444^T shared low DNA similarity values with strains BC412 and BC521, it will be described as a novel species of *Blastococcus*, for which the name *Blastococcus saxobsidens* sp. nov. is proposed. The type strain is BC444^T (= DSM 44509^T = NRRL 24246^T).

Isolates resembling members of the family *Geodermatophilaceae* from altered stone surfaces from monuments and natural stones in the Mediterranean basin have recently been demonstrated to belong phylogenetically to the genera *Geodermatophilus* (Eppard *et al.*, 1996), *Modestobacter* (Mevs *et al.*, 2000) and *Blastococcus* (Ahrens & Moll, 1970; Urzì *et al.*, 2001). The result of a detailed taxonomic investigation of some of the isolates that cluster with *Blastococcus aggregatus* is reported.

Isolation and cultivation

Ten strains were isolated from various sites (Table 1) by dilution plating on Bunt & Rovira medium (Bunt & Rovira, 1955), modified by addition of 0.5 % glucose, 0.5 % NaCl and 0.03 % Na₂CO₃, pH 8.6 (medium BRII), and maintained in Luedemann medium (LM; Luedemann, 1968). Growth was tested on LM agar plates at 6, 10, 20, 32, 37 and 45 °C. NaCl tolerance was checked at concentrations of 0.5, 3, 5, 6 and 10 % (w/v). Growth at different pH values was tested at pH 4, 5, 6.8, 8.6, 10 and 12.

Sparse growth occurred after 2 weeks at 28 °C on LM, malt extract agar (Oxoid) (no growth for strain BC519) and on YCS-Glc agar [0.5 % yeast extract (YE), 1.5 % casein hydrolysates, 1 % starch, 1 % glucose, 1.5 % agar] and YG-Glyc agar (0.5 % YE, 1 % glucose, 10 % glycerol, 1.5 % agar; Luedemann & Fonseca, 1989) (no growth for strain BC521). Growth was not supported on potato dextrose agar (Oxoid). Growth was observed at 20 and 37 °C, with an optimum at 32 °C. Strains BC412 and BC518 also grew at

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Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BC512, BC444^T, BC517, BC521 and BC412 are AJ316570–AJ316574.

Detailed fatty acid compositions of *B. aggregatus* DSM 4725^T and nine novel strains are available as supplementary material in IJSEM Online.

Table 1. Isolation sites of BC strains

Strain(s)	Origin	Site
BC412, BC448	Marble	Archaeological site, Eleusys, Greece
BC444 ^T	Limestone	Church, Siggiewi, Malta
BC509, BC512, BC517, BC518, BC519, BC521, BC525	Calcarene	'Santa Maria La Scala' quarry, Noto, Italy

15 °C. No growth occurred below 10 °C or at 45 °C. Growth in LM occurred at pH 5–8.6 with an optimum at pH 6.8; except for strains BC412, BC512 and BC521, which grew at up to pH 8.6, all strains grew at up to pH 10.5. No growth was observed at or above 3 % NaCl, except for strain BC448, which grew at 5 % NaCl.

Colony and cell morphology

Cell and aggregate morphology were determined by bright-field microscopy and by phase-contrast microscopy (DML; Leica) (oil-immersion objective, magnification $\times 100$). For scanning electron microscopy analysis, bacterial samples were dehydrated with a graded series of acetone, critical-point-dried with CO₂ and sputter-coated with gold before examination in a Zeiss field emission scanning electron microscope DSM982 Gemini at 5 kV using the Everhart-Thornley SE-detector and the in-lens SE-detector in a 50:50 ratio. Morphology of colonies was studied with a stereomicroscope (Wild M10; Leica). Colonies of all strains were pink- to orange-pigmented, circular (diameter 2–3 mm) with a smooth or rough surface. Cell morphology was characterized by coccoid cells (0.5–1.7 μ m in diameter) occurring singly, in pairs or in tetrads, often tending to form aggregates (Fig. 1a, b). Under the conditions used, bud formation was observed only for strains BC412, BC509, BC517, BC518 and BC521, and small motile zoospores were produced by BC509, BC412 and BC521. Strains BC412 and BC521 formed elongated cells, produce germ tubes and long filaments.

Chemotaxonomy

The fatty acid profiles (Stead *et al.*, 1992; Schumann *et al.*, 1997) of *B. aggregatus* DSM 4725^T and the BC strains were characterized by the predominance of iso-C_{16:0} and iso-C_{16:1} acids. Other fatty acids, some occurring in major amounts (C_{17:1} 8c), differed among strains and are indicated in the species description. The fatty acid profiles are available as supplementary material in IJSEM Online. The major respiratory quinones, determined as described previously (Collins *et al.*, 1977; Groth *et al.*, 1996), were MK-9(H₄) and MK-9; homologues of the H₄ series (7, 8) as well as the MK-9 series (H₂, H₆) may occur in minor amounts. The polar lipid pattern (Minnikin *et al.*, 1979; Collins & Jones, 1980) of strain BC444^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-inositol and phosphatidylethanolamine. *B. aggregatus* DSM 4725^T and strain BC412 also contained two unknown

phospholipids. The cell walls of strains BC444^T, BC521 and BC412 and of *B. aggregatus* DSM 4725^T contained meso-diaminopimelic acid (meso-A₂pm), confirming the observation stated to be characteristic of members of the family *Geodermatophilaceae* (Normand *et al.*, 1996). The DNA G + C content (Mesbah *et al.*, 1989) of *B. aggregatus* DSM 4725^T was 73.9 mol%.

Phylogenetic analyses

Of the isolates for which partial 16S rRNA gene sequences (5550 nucleotides) have been published previously (Urzì *et al.*, 2001), six strains were selected for determination of almost complete sequences. These strains represented

Fig. 1. Electron micrographs of *B. aggregatus* DSM 4725^T (a) and strain BC444^T (b). Bars, 1 μ m.

Fig. 2. 16S rRNA gene sequence-based dendrogram, displaying the relatedness of *Blastococcus*-like organisms to type strains of species of *Geodermatophilus*, *Modestobacter* and *Blastococcus* (Eppard *et al.*, 1996). The sequences of members of *Micrococcineae* served as an outside reference. Bootstrap percentages (1000 replicates, >70% confidence) are indicated at respective nodes. Bar, 2% sequence difference.

three different lineages, branching adjacent to that of *B. aggregatus* DSM 4725^T. Sequencing methods and analyses of phylogenetic relatedness followed described procedures (Rainey *et al.*, 1996). The phylogenetic analysis supported analyses based on partial sequences: three lineages emerged, defined by strains BC444^T/BC448/BC512/BC517, sharing >99.9% similarity, BC412 and BC521; sequences of members of these lineages were 97.6–98.2% similar among themselves. *B. aggregatus* DSM 4725^T was about equidistantly related to the BC521 and BC412 lineages (99.2 and 98.6% similar) and slightly less closely related to strain BC444^T and related strains (98.0%). Based upon partial sequences, the BC444^T lineage contained several additional isolates that were >99.9% similar to strain BC444^T (Urzì *et al.*, 2001). Similarity values were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). A distance matrix dendrogram (DeSoete, 1983) is shown in Fig. 2.

The genomic homogeneity of some members of the BC444^T lineage was determined in comparison to some other BC isolates and *B. aggregatus* DSM 4725^T by RiboPrint analysis (Bruce, 1996; Stackebrandt *et al.*, 2002) using the restriction enzymes *Pvu*II and *Pst*I. The patterns were in accord with 16S rRNA gene sequence analysis in that members of the

BC444^T cluster were homogeneous, while members of the other lineages were defined by individual patterns (Fig. 3).

Discrimination at a lower taxonomic level by DNA–DNA hybridization (Escara & Hutton, 1980; Huß *et al.*, 1983; Jahnke, 1992) revealed that strain BC444^T shared only 39% similarity with strain BC412 and 50% similarity with strain BC521. All three isolates were clearly unrelated to *B. aggregatus* DSM 4725^T (25–37% similarity). Because of the low sequence similarity (98.2%), the binary DNA–DNA reassociation value was not determined for strains BC521 and BC412.

Physiology

Physiological tests were performed at 28 °C using Biolog GN, API ZYM and API 20 NE substrate panels. Catalase and oxidase activities were determined by the formation of bubbles in a 3% hydrogen peroxide solution; oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine was done on filter paper. Properties are compiled in Table 2. The metabolic reactions of members of the three novel *Blastococcus* lineages showed significant differences from that of *B. aggregatus* DSM 4725^T as well as among themselves.

Taxonomic conclusions

Urzì *et al.* (2001) studied 65 isolates from natural stones and monuments attributed to the family *Geodermatophilaceae* and showed that, on rock surfaces, members of the genera *Geodermatophilus*, *Modestobacter* and *Blastococcus* had different distributions. In fact, strains belonging phylogenetically to *Geodermatophilus* were very rare, while strains belonging to *Modestobacter* were very common and were distributed on stone surfaces located in different climates. Strains taxonomically affiliated to the only species of the genus *Blastococcus*, *B. aggregatus*, isolated from the Baltic Sea (Ahrens & Moll, 1970), were found on rock surfaces located in the Mediterranean basin and, in particular, they were isolated from monuments and rocks in Greece, Italy and Malta. The ten strains share a few characteristics with *B. aggregatus* (Table 2) such as the formation of pink/orange colonies, aerobic metabolism and the presence of MK-9(H₄) as the predominant menaquinone. On the other hand, differences were observed in

Fig. 3. RiboPrint patterns of *B. aggregatus* DSM 4725^T and novel *Blastococcus* isolates, generated with *Pvu*II (a) and *Pst*I (b).

Table 2. Differentiation between strains DSM 4725^T and other isolates using morphological and physiological parameters

Tests are scored as: +, >80 % positive, –, <10 % positive, v, 10–80 % positive; v^w, variable (mostly weak); w, weak reaction. Reaction of type strain BC444^T is given in parentheses. All strains formed aggregates and were positive or weak for the following reactions: esterase (C4), esterase (C8), leucine arylamidase, Tweens 40 and 80, D-ribose and methyl succinate. All strains were negative for nitrate reduction, indole, glucose fermentation, urease, β -glucosidase, assimilation of mannitol, N-acetylglucosamine, maltose, caproate, citrate and phenylacetate, lipase, α -galactosidase, β -galactosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase and trypsin (API 20NE and API ZYM). Reactions towards the other 67 substrates provided by the Biolog GP2 panel were negative.

Test	<i>B. aggregatus</i> DSM 4725 ^T	BC444 ^T and seven related strains	BC521	BC412
Cell shape*	C, R, vibrios	C	C/R	C/R
Bud formation	+	– (except one strain)	+	+
Germ tube	+	–	+	+
Motility	+	v	+	+
Oxygen requirement	Aerobic/microaerophilic	Aerobic	Aerobic	Aerobic
Colony colour†	P	P/O	P/O	P
API 20 NE				
Arginine dihydrolase	–	v (+)	–	–
β -Galactosidase	–	+	+	+
Assimilation of:				
Glucose	–	–	w	w
Arabinose	–	+	+	+
Adipate	–	w (–)	+	w
Mannose	–	v ^w (–)	–	–
Gluconate	–	w	–	–
Malate	–	v	–	–
API ZYM				
Valine arylamidase	–	v (–)	w	+
Alkaline phosphatase	–	+	–	w
Cystine arylamidase	–	v ^w (–)	w	–
Naphthol-AS-BI-phosphohydrolase	–	v ^w	–	w
α -Glucosidase	w	+	–	+
β -Glucosidase	–	w	–	w
β -Glucuronidase	–	v (+)	–	–
Biolog GP2				
Utilization of:				
D-Fructose	–	v (–)	+	+
β -Cyclodextrin	–	v (–)	+	+
D-Mannose	–	–	+	+
D-Malic acid	+	–	+	+
D-Psicose	–	v (–)	+	+
Pyruvic acid	–	–	+	+
Alaninamide	–	–	+	+
Adenosine	–	–	+	+
Thymidine	–	–	+	+
Dextrin	–	v (–)	–	+
Mannan	–	v (–)	–	+
D-Galactose	–	–	–	+
α -D-Glucose	–	v (–)	+	–
Maltotriose	–	–	–	+
D-Melezitose	–	–	–	+
Palatinose	–	–	–	+
Acetic acid	+	–	–	+
α -Ketoglutaric acid	+	–	+	–

Table 2. cont.

Test	<i>B. aggregatus</i> DSM 4725 ^T	BC444 ^T and seven related strains	BC521	BC412
α -Hydroxybutyric acid	—	—	—	+
Glycyl-L-glutamic acid	—	—	—	+
α -Ketovaleric acid	+	—	—	—
Inosine	—	v (—)	—	—
Thymidine-5'-monophosphate	—	—	—	+

*C, Coccus; R, rod.

†P, Pink; O, orange.

the composition of fatty acids and physiological properties. Compared with the stone surface isolates, *B. aggregatus* DSM 4725^T is characterized by a rather low metabolic profile. Based on phylogenetic, genomic and phenotypic grounds, strains of the BC444^T cluster also differ from strains BC521 and BC412 and can therefore be described as representatives of a novel species of the genus *Blastococcus*, for which the name *Blastococcus saxobidens* sp. nov. is proposed.

As a consequence of newly reported properties not included in the original description of strain DSM 4725^T (Ahrens & Moll, 1970), the description of the genus *Blastococcus* must be emended. At this stage, we refrain from describing strains BC521 and BC412, though, based upon genomic and phenetic evidence, these single-strain taxa would merit formal description. The rationale for not doing so is based on the finding that the novel species *B. saxobidens* shows a significant number of variable and weak physiological reactions. This indicates that the intraspecies metabolic diversity is higher than is expressed by the type strain.

Emended description of the genus *Blastococcus* Ahrens and Moll 1970

Blastococcus (Blasto.coc'cus. Gr. n. *blastos* sprout, bud; Gr. n. *coccus* a grain, berry; N.L. masc. n. *Blastococcus* the budding coccus).

A formal description has never been provided. The description is based on the morphological observations of Ahrens & Moll (1970), supplemented with data reported in this study.

Gram-positive. Coccoid units, often reproducing by budding and multiple fission, giving rise to a variety of cell forms and aggregates. Single cells may be motile rods and vibrioid or non-motile cocci that tend to form aggregates. Formation of buds is common but not universal. One to four membranous bodies per cell may be present, which are linked to the cytoplasmic membrane. Aerobic; some cells may be microaerophilic. Strains from surfaces of marble and limestone utilize a broader spectrum of organic compounds than the only known marine strain. Predominant fatty acids are iso-C_{16:0}, iso-C_{16:1}, C_{18:1}ω9c and, in

some strains, C_{17:1}ω8c, iso-C_{15:0} and C_{17:0}. Peptidoglycan contains *meso*-A₂pm as diagnostic diamino acid. Predominant menaquinone is MK-9(H₄); MK-9 may also occur in large amounts. Polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine; two unknown phospholipids may occur. DNA G+C content is 74 mol%. Phylogenetically, a member of the family *Geodermatophilaceae*.

The type species is *Blastococcus aggregatus*.

Emended description of *Blastococcus aggregatus* Ahrens and Moll 1970

The emendation is based on the description of Ahrens & Moll (1970), supplemented with data reported in this study.

Gram-positive, highly irregular vibrioid or rod-shaped cells occurring singly or in three-dimensional coccoid aggregates. The manifestation of one of these stages is influenced by environmental conditions. Low salt concentrations, temperatures of 5–10 °C and microaerophilic conditions favour the motile rod stage. Single cells are either motile vibrioid (0.3–1.5 × 0.4–3.0 µm) or rods or ellipsoid (1.2–1.5 × 1.5–3.0 µm). Rods are often separated by disc-like septa. These motile or non-motile rods carry one to six vibrioform buds, attached to the mother cell by a tapered pole. Increased temperatures and salt concentrations induce the formation of non-motile coccoid aggregates (1.2–2.5 µm in diameter), appearing as linear, band-like or column-like three-dimensional forms. Larger aggregates (1 mm diameter) are separated by rectangular and/or radial partitions. Cell types of different size occur mostly side by side. After 5 days on peptone/yeast extract agar at 20 °C, colonies are pink, turbid, round and convex with a shiny surface. In liquid media, thin turbidity and formation of pink sediment is observed. Catalase-positive, oxidase-negative. No growth in mineral medium; weak growth in the presence of ethanol, but not with glucose, acetate, citrate, methanol, ethylamine or paraffin. Good growth in mineral medium plus peptone (0.5 %) and yeast extract (0.1 %); in these media, addition of 1 % of either glucose, acetate, citrate, ethylamine or paraffin reduces growth. Growth is enhanced in peptone/yeast extract medium by

the addition of 1% NaCl. No acid or gas from glucose, galactose, fructose, xylose, sucrose, maltose, lactose or glycerin. Methyl-red and Voges-Proskauer reactions are negative. No hydrolysis of starch, chitin, alginate or cellulose. Reactions towards substrates provided by the API ZYM, API 20 NE and Biolog GP2 panels are indicated in Table 2. Major fatty acids are 14-methyl pentadecanoic acid (iso-C_{16:0}) and 14-methyl pentadecenoic acid (iso-C_{16:1}). Diamino acid of peptidoglycan, menaquinone and polar lipids are as described for the genus. Two unknown phospholipids may occur. Extinction maxima of carotenoids at 470 and 500 nm. The DNA G + C content is 73.9 mol%.

The type strain, DSM 4725^T (= ATCC 25902^T), was isolated from the Baltic Sea.

Description of *Blastococcus saxobsidens* sp. nov.

Blastococcus saxobsidens (sax.ob'si.dens. L. n. *saxum* rock; L. v. *obsideo* to occupy; N.L. part. adj. *saxobsidens* rock-occupying).

Aerobic, Gram-positive. Motile and non-motile cells (1.0–1.7 µm in diameter) are coccoid, occurring in tetrads with a tendency to remain aggregated. Sparse growth on LM, malt agar and YCS-Glyc agar. No growth on potato dextrose agar. Temperature range for growth is 20–37 °C, with an optimum at 32 °C; growth at pH 5–8.6, with an optimum at pH 6.8. No growth at 3% NaCl, except strain BC448, which grows at 5% NaCl. Colonies are pink/orange-pigmented, irregular, convex with a smooth to rough surface (2–3 mm in diameter). Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Carbon sources utilized for growth are D-glucose, D-fructose, L-arabinose, ribose, *myo*-inositol and lactose. No acid production from D-fructose, *myo*-inositol or lactose. Casein, gelatin and starch are not hydrolysed. DNA, tyrosine, xanthine and hypoxanthine are not decomposed. Tweens 20, 40, 60 and 80 are not hydrolysed. Major fatty acids are 14-methyl pentadecanoic acid (iso-C_{16:0}), *cis*-9-heptadecenoic acid (C_{17:1ω8c}) and 13-methyl tetradecanoic acid (iso-C_{15:0}). Diamino acid of peptidoglycan, major menaquinone and polar lipids are as described for the genus.

The type strain, BC444^T (= DSM 44509^T = NRRL 24246^T), was isolated from limestone sampled in Malta. Other strains, BC448, BC509, BC512, BC517, BC518, BC519 and BC525, were isolated from calcarenite and marble from Italy and Greece. Phenotypic differences from the type strain are shown in Table 2.

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