Nosocomiicoccus ampullae gen. nov., sp. nov., isolated from the surface of bottles of saline solution used in wound cleansing

Marta Alves,¹ Célia Nogueira,¹ Augusto de Magalhães-Sant'Ana,¹ Ana Paula Chung,² Paula V. Morais^{2,3} and Milton S. da Costa³

Correspondence

Paula V. Morais
pvmorais@ci.uc.pt

¹Institute of Microbiology, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal

²IMAR, University of Coimbra, 3004-517 Coimbra, Portugal

³Department of Biochemistry, University of Coimbra, Apartado 3126, 3001-401 Coimbra, Portugal

Strains TRF-1^T and TC-9 were isolated from transfer spikes of two separate bottles containing 0.9 % NaCl (physiological saline) solution used repeatedly to wash wounds in hospital wards, months apart. Phylogenetic analysis of 16S rRNA gene sequences revealed that strains TRF-1^T and TC-9 formed a distinct branch within the family *Bacillaceae* most closely related to the members of the genus *Jeotgalicoccus*. The two strains, with identical 16S rRNA gene sequences, showed sequence similarities of 89.8–93.9 % with species of *Jeotgalicoccus*, 91.1–91.8 % with species of *Salinicoccus* and 89.1–89.7 % with species of *Staphylococcus*. Strains TRF-1^T and TC-9 are Gram-positive, non-spore-forming and non-motile cocci, with an optimum growth temperature of about 37 °C. Strain TRF-1^T grew optimally in medium containing 3 % (w/v) NaCl (maximum about 14 % NaCl), while strain TC-9 grew optimally in medium with 1 % (w/v) NaCl. Both strains produce a brown pigment when grown in the presence of NaCl. The cell-wall peptidoglycan is of the A3 α type with a cross-linkage containing the peptide L-Lys–Gly₄–L-Ser–Gly. The major respiratory quinones are menaquinone 7 (MK-7) and menaquinone 8 (MK-8), the major fatty acids are straight-chain C_{14:0} and C_{16:0} (more than 85 % of the total) and the major polar lipid is an unknown aminophospholipid. The DNA G + C content is 33.5 mol%. On the basis of the phylogenetic analysis and physiological and biochemical characteristics, we are of the opinion that strains TRF-1^T and TC-9 represent a novel species of a new genus, for which we propose the name *Nosocomiicoccus ampullae* gen. nov., sp. nov. The type strain of *Nosocomiicoccus ampullae* is strain TRF-1^T (=LMG 24060^T =CIP 109506^T).

In some hospital wards, the same bottle of physiological saline is used repeatedly for topical washing of wounds and can become contaminated with micro-organisms. Microbial colonization of these bottles and the transfer spikes was examined in the course of a study to assess their colonization with bacteria after frequent usage. Two halotolerant and moderately halophilic cocci (strains TRF-1^T and TC-9) were recovered, among a large number of other isolates, that were most closely related phylogenetically to the species of the genus *Jeotgalicoccus*. The genus *Jeotgalicoccus* was proposed by Yoon *et al.* (2003) to accommodate strains of two species, *Jeotgalicoccus psychrophilus* and *Jeotgalicoccus halotolerans*, isolated from a

fermented Korean food called jeotgal, which is prepared with seawater. A third species isolated from the mouth of an elephant seal was named *Jeotgalicoccus pinnipedialis* (Hoyle *et al.*, 2004). The species of the genus *Jeotgalicoccus* are characterized by having menaquinone MK-7 as predominant isoprenoid quinone, a cell-wall peptidoglycan of the A3 α type based on L-Lys–L-Gly_{3–4}–L-Ala(Gly) and DNA G + C contents of 36–42 mol% (Yoon *et al.*, 2003). On the basis of the data presented here, we propose that strains TRF-1^T and TC-9 represent a species of a distinct, novel genus.

Strains TRF-1^T and TC-9 were isolated on two occasions from separate transfer spikes of plastic bottles of physiological saline manipulated by hospital personnel to wash wounds. The transfer spikes were sampled using sterile cotton swabs, which were applied directly on Columbia blood agar medium and incubated aerobically at 37 °C for 72 h. Isolates were purified by streaking on brain heart

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TRF-1^T and TC-9 are EU240886 and EU240887.

Maximum-likelihood and maximum-parsimony trees based on 16S rRNA gene sequences are available as supplementary material with the online version of this paper.

infusion agar (BHIA). All strains were preserved at -80°C in heart infusion broth (BD) containing 15 % (v/v) glycerol. *J. pinnipedialis* CCUG 42722^T was obtained from the Culture Collection, University of Gothenburg, Sweden, and *J. halotolerans* CIP 107958^T and *J. psychrophilus* CIP 107952^T were obtained from the Institut Pasteur, France.

Several media were assessed for growth of strains TRF-1^T and TC-9, namely BHIA (Cultimed), heart infusion agar (BD), Luria-Bertani agar (LBA; Difco), tryptic soy agar (Sigma), marine agar (Difco), Columbia agar base (bioMérieux) with 5 % (v/v) sheep blood, Chapman agar (Difco) and MacConkey agar (bioMérieux). The organisms were grown under an aerobic, 5 % CO_2 atmosphere and under anaerobic conditions (Genebox) on BHIA at 30 and 37°C for 7 days. The temperature range and optimum for growth were tested on BHIA incubated at 4, 22, 30, 37, 40, 45 and 50°C and growth was examined after 10 days. The ability to grow in the presence of NaCl was examined at 37°C on BHIA supplemented with NaCl at final concentrations of 2, 5, 10, 14, 15, 16, 20 and 21 % (w/v) and on LBA supplemented with 1, 3, 5 and 10 % NaCl (w/v). Cell morphology and motility were examined by phase-contrast microscopy after growth on BHIA at 37°C for 24 h. The Gram reaction and the presence of cytochrome oxidase and catalase were determined after 24 h of incubation as described by Smibert & Krieg (1981). Enzyme profiles were determined using the API ZYM, API STAPH, API NE and API CORYNE test strips (bioMérieux) according to the manufacturer's instructions. Single-carbon-source assimilation was determined using API 50 CH test strips (bioMérieux) with Degryse medium containing 0.05 % (w/v) yeast extract (Degryse *et al.*, 1978). The pH range for growth was examined at 30°C in BHIA medium with the pH adjusted by the addition of 50 mM MES (pH 5, 6 and 7), TAPS (pH 8 and 9) or CAPSO (pH 10) (Branco *et al.*, 2004).

Cultures used for polar lipid analysis were grown on BHIA with 1 % NaCl at 37°C for 48 h (except for *J. psychrophilus* CIP 107952^T, which was grown at 25°C). Cultures were harvested and lyophilized, lipids were extracted and one-dimensional TLC was performed as described previously (Collins *et al.*, 1980). Lipoquinones were extracted from freeze-dried cells, purified by TLC and identified by HPLC (Tindall, 1989). Cultures for fatty acid analysis were grown on BHIA with 1 % NaCl and strains TRF-1^T and TC-9 were also grown on LBA plates, in sealed plastic plates, for 48 h (Morais *et al.*, 2004); fatty acids were identified using the standard MIS Library Generation software (Microbial ID). Purification of cell wall and the acquisition of amino acids and peptides in cell-wall hydrolysates were performed by the method of Schleifer & Kandler (1972). The terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as described by Schleifer (1985).

The G+C content of the genome was determined by HPLC as described by Mesbah *et al.* (1989). The 16S rRNA gene was sequenced as described by Morais *et al.* (2004).

Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were constructed using maximum-parsimony, maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) algorithms. The tree topologies were evaluated by performing bootstrap analysis (Felsenstein, 1985) of 1000 datasets.

Strains TRF-1^T and TC-9 were Gram-positive, non-spore-forming and non-motile cocci. After 2 days incubation on BHIA, colonies were small and light yellow in colour. The new isolates produced very large cell aggregates in medium without additional NaCl. Both strains produced a brown pigment in older cultures in the presence of NaCl. Both organisms grew on all media examined at 37°C , with the exception of Chapman agar. Growth was best on BHIA and LBA. Strains TRF-1^T and TC-9 had a very narrow growth temperature range ($30\text{--}45^{\circ}\text{C}$) with an optimum at about 37°C (Table 1). Strains TRF-1^T and TC-9 grew aerobically and under microaerophilic conditions but did not grow anaerobically. In this study, we found that *J. halotolerans* CIP 107958^T and *J. psychrophilus* CIP 107952^T did not grow anaerobically, despite the previous report that they did (Yoon *et al.*, 2003). The organisms grew in media without additional salt, but growth was better in the presence of NaCl; strain TRF-1^T grew better in LBA containing 3 % (w/v) NaCl, but strain TC-9 grew better in the same medium containing 1 % (w/v) NaCl. Both organisms also grew in LBA containing 14 % (w/v) NaCl but, in BHIA, strain TRF-1^T grew with a maximum of 10 % NaCl.

Assimilation of single carbon sources using API 50 CH was not observed, and only L-arabinose gave a weak positive fermentation reaction using API NE (Table 1). Unlike the species of the genus *Jeotgalicoccus*, which possess very high levels of iso- and anteiso-branched fatty acids, we found only straight-chain fatty acids in the two new isolates; this unusual composition was not dependent on the growth medium or the concentration of salt in the medium (Table 2). The polar lipid pattern of strain TRF-1^T revealed the presence of an unidentified major aminophospholipid, not present in the species of the genus *Jeotgalicoccus*, and several unidentified minor phospholipids and aminolipids (Fig. 1). All species of the genus *Jeotgalicoccus* examined possessed diphosphatidylglycerol and phosphatidylglycerol along with several aminolipids. MK-7 and MK-8 were found in equimolar proportions in both strains.

The almost-complete 16S rRNA gene sequence was determined for strains TRF-1^T and TC-9 (1502 and 1490 bp, respectively). 16S rRNA gene sequences were aligned with representative members of the phylum *Firmicutes*, showing these organisms to be most closely related to the cluster comprising species of the genus *Jeotgalicoccus*, but the two strains formed a distinct line supported by bootstrap analysis at a confidence level of 71 % (Fig. 2). Furthermore, this tree topology was also found using the maximum-likelihood and maximum-

Table 1. Phenotypic characteristics that differentiate strain TRF-1^T (*Nosocomiicoccus ampullae* gen. nov., sp. nov.) from the type strains of described species of *Jeotgalicoccus*

Data for strain TRF-1^T and *J. pinnipedialis* CCUG 42722^T were obtained in this study. Fatty acid compositions of all strains were determined in this study (see Table 2); other data for *J. halotolerans* YKJ-101^T and *J. psychrophilus* YKJ-115^T were taken from Yoon *et al.* (2003). +, Positive; –, negative; w, weak.

Characteristic	Strain TRF-1 ^T	<i>J. pinnipedialis</i> CCUG 42722 ^T	<i>J. halotolerans</i> YKJ-101 ^T	<i>J. psychrophilus</i> YKJ-115 ^T
Source	Surface of used saline bottles	Mouth of an elephant seal	Fermented seafood	Fermented seafood
Growth temperature (°C)				
Range	30–45	25–42	4–42	4–34
Optimum	37	37	30–35	20–25
Growth at:				
0 % NaCl	+	+	+	–
14 % NaCl	(+)*	–	+	+
20 % NaCl	–	–	+	–
Pigment production >72 h	+	+	–	–
Predominant menaquinone (s)	MK-7 (49.5 %), MK-8 (44.5 %)	MK-7 (92.4 %)	MK-7 (86 %)	MK-7 (80 %)
Peptidoglycan type	L-Lys–Gly ₄ –L-Ser(Gly)	L-Lys–Gly _n –L-Ala	L-Lys–Gly _{3–4} –L-Ala(Gly)	L-Lys–Gly _{3–4} –L-Ala(Gly)
Major fatty acids (%)				
C _{14:0}	38.3	2.3	–	0.5
iso-C _{15:0}	–	31.4	21.9	26.3
anteiso-C _{15:0}	–	47.6	37.7	42.1
C _{16:0}	49.5	1.3	6.5	1.6
C _{20:0}	5.8	–	–	–
Acid production from:				
L-Arabinose	w	–	+	–
Ribose	–	–	+	–
Mannose	–	–	+	–
Mannitol	–	–	+	–
Sucrose	–	–	–	w
Hydrolysis of gelatin	+	+	–	–
Activity of:				
Acid phosphatase	–	w	–	+
Alkaline phosphatase	w	–	–	+
Ester lipase (C8)	+	+	–	–
Esterase (C4)	w	–	w	+
G + C content (mol%)	33.5	36	42	42

*Growth was positive on LBA but not on BHIA.

parsimony algorithms (Supplementary Figs S1 and S2, available in IJSEM Online). The novel strains clustered with the type strains of *J. psychrophilus*, *J. halotolerans* and *J. pinnipedialis* at 16S rRNA gene sequence similarities of 89.8, 90.9 and 93.9 %, respectively.

Nucleotide signatures were found in the 16S rRNA gene sequences of strains TRF-1^T and TC-9 that distinguished the sequences from those of species of the genera *Jeotgalicoccus* and *Salinicoccus* (Table 3). Moreover, the type strain of *J. pinnipedialis* has specific nucleotide signatures within its 16S rRNA gene that distinguish this organism from the other species of *Jeotgalicoccus*, indicating that the classification of *J. pinnipedialis* should be reassessed.

The new taxon represented by strains TRF-1^T and TC-9 can be distinguished from species of the genus *Jeotgalicoccus* primarily by the fatty acid composition. The type strains of *J. psychrophilus*, *J. halotolerans* and *J. pinnipedialis* possess large amounts of iso- and anteiso-branched fatty acids, while strains TRF-1^T and TC-9 do not. Moreover, the peptidoglycan of the species of the genus *Jeotgalicoccus* possesses an interpeptide bridge containing L-Lys–Gly_n–L-Ala(Gly), while the two novel strains possess an interpeptide bridge composed of L-Lys–Gly₄–L-Ser(Gly). The absence of diphosphatidylglycerol and phosphatidylglycerol in strain TRF-1^T differentiate this organism from the species of the genus *Jeotgalicoccus* and from the species of the genus *Salinicoccus* for which the polar lipids have been examined. The observation that

Table 2. Fatty acid compositions of strains TRF-1^T and TC-9 and type strains of the genus *Jeotgalicoccus*

Reference strains: 1, *J. pinnipedialis* CCUG 42722^T; 2, *J. halotolerans* CIP 107958^T; 3, *J. psychrophilus* CIP 107952^T. Fatty acid profiles of reference type strains were determined after growth on BHIA with 1 % NaCl. All data were obtained in this study. —, Not detected or present at less than 0.5 %.

Fatty acid	Strain TRF-1 ^T		Strain TC-9		1	2	3
	BHIA + 1 % NaCl	LBA + 3 % NaCl	BHIA + 1 % NaCl	LBA + 3 % NaCl			
iso-C _{13:0}	—	—	—	—	1.0	—	—
anteiso-C _{13:0}	—	—	—	—	0.5	—	—
iso-C _{14:0}	—	—	—	—	1.1	1.5	2.3
C _{14:0}	35.0	38.3	43.8	32.3	2.3	0.8	0.5
iso-C _{15:0}	1.4	—	—	—	31.4	21.9	26.3
anteiso-C _{15:0}	—	—	—	—	47.6	37.7	42.1
C _{16:1} ω7c alcohol	3.0	1.1	0.5	2.6	—	0.8	4.2
iso-C _{16:0}	—	—	—	—	1.3	6.5	1.6
C _{16:1} ω11c	—	—	—	—	—	—	2.2
C _{16:0}	50.2	49.5	47.9	52.8	2.3	3.5	0.6
iso-C _{17:1} ω10c	—	—	—	—	2.5	7.1	6.5
Summed feature 4*	—	0.7	—	—	—	—	1.8
iso-C _{17:0}	—	—	—	—	3.0	5.8	3.3
anteiso-C _{17:0}	—	—	—	—	4.7	9.2	5.5
C _{18:1} ω9c	0.8	0.7	—	1.0	—	—	—
C _{18:0}	2.5	3.0	2.6	3.1	—	—	—
iso-C _{19:0}	—	—	—	—	0.5	1.3	1.0
C _{20:0}	4.1	5.8	2.6	6.7	—	—	—

*Summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B, which could not be separated by GLC with the MIDI system.

strains TRF-1^T and TC-9 have MK-7 and MK-8 in similar molar ratios (49.5:44.5), unlike the species of *Jeotgalicoccus*, where MK-7 is the major respiratory quinone, may also indicate that the novel organisms belong to a new genus. Phylogenetic analyses of the 16S rRNA gene indicated that the strains formed a branch that

was almost equidistant from the type strains of the genus *Salinicoccus* (91.1–91.8 %), *J. halotolerans* (89.8 %) and *J. psychrophilus* (90.9 %), but slightly more closely related to the type strain of *J. pinnipedialis* (93.9 %). Since phenotypic characterization did not allow us to make a decision on the classification of *J. pinnipedialis*, it is our opinion that the organism should be retained as a member of the genus *Jeotgalicoccus*, while the novel strains should be assigned to a new genus, based on the phenotypic and phylogenetic evidence gathered here. We therefore propose that strains TRF-1^T and TC-9 represent a novel species of a new genus, for which we offer the name *Nosocomiicoccus ampullae* gen. nov., sp. nov.

Description of *Nosocomiicoccus* gen. nov. Morais, Chung and da Costa

Nosocomiicoccus (No'so.co'mi.i.coc'cus. L. n. *nosocomium* hospital; N.L. masc. n. *coccus* a coccus from Gr. n. *kokkos* a grain, berry; N.L. masc. n. *Nosocomiicoccus* a coccus isolated in a hospital).

Gram-positive, non-motile, non-spore-forming, coccus-shaped cells. Chemo-organotrophic, mesophilic, slightly halophilic and aerobic. Optimum pH for growth is around neutral. Catalase- and oxidase-positive. Cell-wall peptidoglycan contains L-lysine at position 3 and A3α-type cross-linkage peptide. Predominant menaquinones are MK-7

Fig. 1. One-dimensional TLC of polar lipids of *J. halotolerans* CIP 107958^T (lane 1), *J. psychrophilus* CIP 107952^T (2), *J. pinnipedialis* CCUG 42722^T (3) and strain TRF-1^T (4). DPG, Diphosphatidylglycerol; NPL, unidentified aminophospholipid; PG, phosphatidylglycerol; PL, unidentified phospholipid.

Fig. 2. 16S rRNA gene sequence-based phylogeny showing the relationships of strains TRF-1^T and TC-9 and closely related taxa. The phylogenetic dendrogram was constructed from the distance matrix using the neighbour-joining method. Numbers at branching points represent bootstrap percentages from 1000 data samplings. Bar, 1 inferred substitution per 100 nucleotide positions.

and MK-8. The major polar lipid is an unknown aminophospholipid. Fatty acids are predominantly straight-chain saturated and unsaturated. The genus belongs to the phylum *Firmicutes*. The type species is *Nosocomiicoccus ampullae*.

Description of *Nosocomiicoccus ampullae* sp. nov. Morais, Chung and da Costa

Nosocomiicoccus ampullae (am.pul'lae. L. gen. n. *ampullae* of a bottle, of a flask, referring to the isolation of the first strains from bottles containing physiological saline).

Colonies are small, smooth, glistening, convex, circular and light yellow in colour after 2 days incubation on BHIA and produce a diffusible pigment in cultures older than 72 h in

the presence of NaCl. Growth occurs in media without added NaCl and with NaCl up to 10 % (w/v). Cell aggregates are formed in liquid medium during growth without NaCl. Optimal growth temperature is 30–37 °C. Optimal pH for growth is 7.5–8.0. Does not grow anaerobically. Urease-negative. Gelatin is hydrolysed. Weak acid production is obtained from L-arabinose using the API NE test strip, but no other substrates are fermented or assimilated using API 50 CH. Nitrate is not reduced to nitrite. Major fatty acids are C_{16:0} and C_{14:0}. The DNA G + C content of the type strain is 33.5 mol% (determined by HPLC).

The type strain, TRF-1^T (=LMG 24060^T =CIP 109506^T), was isolated from the transfer spike of a bottle containing physiological saline. Strain TC-9, isolated from a similar source, is another strain of the species.

Table 3. 16S rRNA gene sequence signatures of strains TRF-1^T and TC-9 and phylogenetically related species of the genera *Jeotgalicoccus* and *Salinicoccus*

Positions are given relative to the sequence of *Escherichia coli*. Signatures for *Jeotgalicoccus* relate to the sequences of *J. halotolerans* YKJ-101^T and *J. psychrophilus* YKJ-115^T, whereas those for *Salinicoccus* relate to all type strains of the genus.

Positions	TRF-1 ^T and TC-9	<i>J. pinnipedialis</i> A/G14/99/10 ^T	<i>Jeotgalicoccus</i>	<i>Salinicoccus</i>
86–90	CGGGA	GGGUU	GCUC	GCUC(C/U) ^{a*}
193–194	AU	GG	GG	(G/C) ^b G
418–419	UC	AU	GU	GU
1270–1273	AGU	GGC	GGC	GG(C/U) ^c

*Nucleotide substitutions detected in some strains as follows: a, U in *S. jeotgali* S2R53-5^T and *S. siamensis* PN1-2^T; b, C in *S. kunmingensis* YIM Y15^T; c, U in *S. siamensis* PN1-2^T.

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