Comparative Study of "Micrococcus sp." Strains CCM 168 and CCM 1405 and Members of the Genus Salinicoccus

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Two culture collection strains, CCM 168 and CCM 1405, previously assigned to the genus Micrococcus were shown by molecular chemical characterization to belong to the genus Salinicoccus. A more detailed comparison of the physiological and biochemical properties of these strains and comparison with the type strain of Salinicoccus roseus indicated high degrees of relatedness among the three strains. DNA-DNA hybridization studies confirmed the high degrees of relatedness. All of the data demonstrate quite clearly that strains CCM 168 and CCM 1405 are members of the species S. roseus.

The red-pigmented, moderately halophilic strains CCM 168 and CCM 1405 were isolated originally from a salted hide and salted meat, respectively (14, 17). These organisms were initially considered strains of Micrococcus roseus, but Bohacek et al. (1) found that the guanine-plus-cytosine (G+C) content of their DNAs was 49.4 mol%, a value significantly lower than the G+C contents of members of the genus Micrococcus (8). Schleifer and Kandler (19) also reported that strains CCM 168 and CCM 1405 contained murein of the L-Lys-Gly-L-Ala type. Taken together, these data are not in agreement with the current concept of members of the genus Micrococcus (8). In view of the fact that the results of a study of the molecular chemical properties of a microorganism may support the phylogenetic position of a strain, thus permitting an organism to be assigned to or excluded from groupings determined by 16S rRNA analysis, a study of the respiratory lipoquinones, fatty acids, polar lipids, and murein structure of strains CCM 168 and CCM 1405 was undertaken. It has previously been shown by a combination of chemical and molecular genetic methods that Salinicoccus roseus is phylogenetically distantly related to the staphylococci (21) and that Marinococcus hispanicus is a member of the genus Salinicoccus on the basis of chemical data (22). The results presented here quite clearly demonstrated that isolates CCM 168 and CCM 1405 are also members of the genus Salinicoccus.

In the absence of comprehensive comparative data for CCM 168, CCM 1405, and members of the genus Salinicoccus, a more detailed physiological and biochemical characterization of these organisms was carried out. In addition, DNA-DNA hybridization experiments were carried out to examine the relatedness of strains CCM 168 and CCM 1405 to each other, as well as to members of the genus Salinicoccus and other gram-positive halophilic cocci. The results presented here indicate unequivocally that not only do strains CCM 168 and CCM 1405 belong to the genus Salinicoccus, but they may also be considered isolates of the species Salinicoccus roseus, whose description was based on characterization of a single strain (22).

MATERIALS AND METHODS

Organisms and growth conditions. Strain CCM 168, originally isolated from salted horse hide (14), and strain CCM 1405, isolated from salted meat (17), were obtained from the Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia. The following reference strains were used for comparative purposes: Micrococcus halobius ATCC 21727T (T = type strain), Sporosarcina halophila DSM 2266T, Marinococcus halophilus CCM 2706T, Marinococcus albus CCM 3517T, Marinococcus hispanicus J-82T (= ATCC 49259T = CCM 4148T = DSM 5352T), and Salinicoccus roseus 9T (= ATCC 49258T = CCM 3516T = DSM 5351T). All strains were cultivated in complex medium containing 8.1% (wt/vol) NaCl, 0.7% (wt/vol) MgCl2, 0.96% (wt/vol) MgSO4, 0.036% (wt/vol) CaCl2, 0.2% (wt/vol) KCl, 0.006% (wt/vol) NaHCO3, 0.0026% (wt/vol) NaBr, 0.5% (wt/vol) proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), 1.0% (wt/vol) yeast extract (Difco), and 0.1% (wt/vol) glucose (pH 7.5) (the pH was adjusted with 1 N KOH). When required, the medium was solidified with 2.0% (wt/vol) agar.

Phenotypic characterization. The methods used for phenotypic characterization, which have been described previously (18, 24), were standardized by using media containing 10% (wt/vol) mineral salts (pH 7.5) and a temperature of 37°C.

Extraction of lipid material. Liquid cultures of the strains were grown at 37°C with shaking, harvested by centrifugation, and freeze dried. Lipid material was extracted from 100 mg of freeze-dried cells by a two-stage method (20). A 3-ml portion of hexane-methanol (1:2, vol/vol) was added to the freeze-dried material, and the preparation was extracted for 30 min with stirring under nitrogen. The suspension was then cooled in an ice bath for 10 min with occasional shaking to encourage separation of the hexane and methanol phases. A 1-ml portion of ice-cold hexane was then added, the hexane and methanol layers were allowed to separate, and the hexane phase was removed with a Pasteur pipette. A 2-ml portion of cold hexane was added to the methanol layer, and then 2 ml of 0.3% (wt/vol) NaCl was added. After brief centrifugation of the suspension, the hexane phase was removed and combined with the first hexane phase for lipoquinone analysis. Extraction of the polar lipids from the remaining cell material was done by adding chloroform and methanol to the saline-methanol cell suspension to give a...
chloroform–methanol–0.3% NaCl ratio of 1:2:0.8 (vol/vol).
Following extraction by this method, cell material was
removed by centrifugation, the supernatant was decanted,
and the polar lipids were recovered from the supernatant by
adding chloroform and 0.3% NaCl to give a chloroform-
methanol–0.3% NaCl ratio of 1:1:0.9 (vol/vol).

Lipoquinone analysis. Lipoquinones were purified from
the hexane phase by thin-layer chromatography on silica gel thin
layers (Macherey Nagel article no. 818033, cut to 10 by 10
mm). UV-absorbing bands were eluted and separated by
high-performance liquid chromatography (LDC Analytical)
by using a reverse column (Spherisorb 5 μm; 150 by 4.6
mm). Methanol was used as a mobile phase, and lipoqui-
none standards available from the Deutsche Sammlung von Mikroorganismen.

Polar lipid analysis. Polar lipids were analyzed by two-
dimensional thin-layer chromatography on silica gel thin
layers (Macherey Nagel article no. 818033, cut to 10 by 10
cm) by using chloroform-methanol-water (65:25:4) in the first
dimension and chloroform-methanol-acetic acid-water (80: 12:15:4) in the second dimension. Polar lipids were charac-
terized with spray reagents specific for α-glycols (periodate-
Schiff), sugars (α-naphthol-H2SO4, anisaldehyde-H2SO4),
phosphate (Zindzadze), free amino groups (ninhydrin), and
quaternary nitrogen compounds (Dragendorff).

Fatty acid analysis. Fatty acids were released from 10 mg
of freeze-dried cells by heating the cells overnight at 50°C,
using the modified acid methanolysis procedure of Minnikin
et al. (12). Fatty acids were identified by gas-liquid chroma-
tography on a Hewlett Packard model 5896 gas chromato-
graphy.

DNA extraction. Cells grown to exponential phase were
harvested, and the DNA was extracted by the method of
Marmur (9). Purity was assessed by comparing the ratio of
extinction at 230 nm to extinction at 260 nm (7).

Determination of the DNA base composition. The G+C
content of the DNA in 0.1× standard saline citrate (SSC; 1×
SSC is 0.15 M NaCl plus 0.015 sodium citrate) was deter-
mined from the thermal denaturation profile (10) by using a
Perkin-Elmer model UV-Vis 551s spectrophotometer, a
wavelength of 260 nm, and temperature increases of 1.0°C/
min. The midpoint of the thermal denaturation profile (Tm)
was determined graphically (5), and the G+C content was
calculated as described by Owen and Hill (15). The midpoint
of the thermal denaturation profile of reference DNA from
Escherichia coli NCTC 9001 was assumed to be 74.6°C in
0.1× SSC (16).

Preparation of 3H-labeled DNA and DNA-DNA hybridiza-
tion experiments. DNA was radioactively labeled by the multi-
prime system with a commercial kit (RPN 1601Y; Amer-
sham International, Amersham, England), using [1',2',5-3H]dCTP (Amersham). The average specific activity obtained
with this procedure was 8.8 × 106 cpm/μg of DNA. The
labeled DNA was denatured before hybridization by being
heated at 100°C for 5 min and then placed on ice.

DNA-DNA similarity was studied by the competition
procedure described by Johnson (7). Competitor DNAs were
sonicated (Braun Melsungen, Melsungen, Germany) at 50 W
for two 15-s time intervals. Membrane filters (HAHY; Mil-
lipore Corp., Bedford, Mass.) containing reference DNA
c(25 μg cm−2) were placed in 5-ml screw-cap vials which
contained the labeled, sheared, denatured DNA and the
denatured and sheared competitor DNA. The ratio of the
concentration of competitor DNA to the concentration of
labeled DNA was at least 150:1. The final reaction concentra-
tions were 2× SSC and 30% formamide, and the final
volume was 140 μl. The hybridization experiments were
carried out under optimal conditions, with temperatures
ranging between 50 and 51°C, which is within the limits of
validity for the filter method (4). The vials were shaken
slightly for 18 h in a water bath (Grant Instruments, Cam-
bridge, England); these procedures were done in triplicate.
After hybridization the filters were measured with a liquid
scintillation counter (Beckman Instruments, Inc., Palo Alto,
Calif.), and the percentage of homology was calculated as
described by Johnson (7). At least two independent deter-
minations were carried out for each experiment, and the
mean values are reported here.

RESULTS AND DISCUSSION
Strains CCM 168 and CCM 1405 were gram-positive cocci,
0.8 to 1.5 μm in diameter, occurring singly, in pairs, or in

<table>
<thead>
<tr>
<th>TABLE 1. Fatty acid compositions of strains</th>
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<tr>
<td>% in:</td>
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<tr>
<td>CCM 168</td>
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<td>----------------</td>
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<tr>
<td>iso-14:0</td>
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<tr>
<td>14:0</td>
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<tr>
<td>iso-15:0</td>
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</tr>
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<td>iso-17:0</td>
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<tr>
<td>iso-20:0</td>
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<td>20:0</td>
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* ND, not detected.
TABLE 2. Phenotypic characteristics of strains CCM 168, CCM 1405, \textit{Salinicoccus roseus} CCM 3516\textsuperscript{T}, and \textit{Marinococcus hispanicus} CCM 4148\textsuperscript{T}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CCM 168</th>
<th>CCM 1405</th>
<th>\textit{Salinicoccus roseus} CCM 3516\textsuperscript{T}</th>
<th>\textit{Marinococcus hispanicus} CCM 4148\textsuperscript{T}</th>
</tr>
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<tbody>
<tr>
<td>Motility</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spore production</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Pink or red pigmented</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzidine test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND\textsuperscript{a}</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid produced from glucose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Casein</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Esculin</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Gelatin</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>Starch</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<td>−</td>
<td>−</td>
<td>+</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>Phosphatase</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Urease</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Phenylalanine deaminase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DNase</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dihydrolease</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Egg yolk reaction</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data from references 11 and 22.

\textsuperscript{b} ND, not determined.

irregular clumps. Neither spore formation nor motility was observed. Colonies were round, slightly convex, smooth, and pink to orange pigmented at salinities ranging from 5 to 15\% (wt/vol) NaCl. The pigment did not diffuse into the medium. After freeze drying pink, orange, and colorless colonies were observed when the organisms were resuscitated. Such variation in pigmentation is not unusual after freeze drying of pigmented cultures and has been observed previously in members of the genus \textit{Salinicoccus} (21). In nutrient broth the cultures were slightly turbid and formed a viscous sediment.

No growth occurred in media containing no salt. The minimum inorganic salt requirement was 0.9\% (wt/vol), and the maximum concentration permitting growth was 25\% (wt/vol); the optimum inorganic salt concentration was 10\% (wt/vol). The optimum growth temperature was 37°C.

The cell walls of strains CCM 168 and CCM 1405 contained murein of the L-Lys-Gly\textsubscript{2} type. The presence of a L-Lys-Gly\textsubscript{2}-L-Ala type reported previously in these two strains (19) could not be confirmed (26). The L-Lys-Gly\textsubscript{2} cell wall type corresponds to the A3\alpha type according to Schleifer and Kandler (19). A similar cell wall type is found in members of the genus \textit{Salinicoccus} and certain members of the genus \textit{Staphylococcus} (19, 22).

Examination of the respiratory lipoquinone contents of strains CCM 168 and CCM 1405 showed that menaquinones with six isoprene units were the major components present.

The fatty acids of strains CCM 168 and CCM 1405 were predominately straight- and branched-chain fatty acids (Table 1). Taken together, the chemotaxonomic data support the inclusion of these two strains in the genus \textit{Salinicoccus} (21-23). The biochemical characteristics of strains CCM 168 and CCM 1405, \textit{Salinicoccus roseus}, and \textit{Marinococcus hispanicus} are shown in Table 2. Some differences were observed in the production of amylase, DNase, phosphatase, and lipase (Tween 80) and the degradation of tyrosine.

The DNA base compositions of the strains used in this study and the results of the DNA-DNA hybridization experiments are presented in Table 3. The G+C content of strains CCM 168 and CCM 1405 was 49.3 mol\%, a value similar to values reported previously (1). This value is close to the value found for \textit{Salinicoccus roseus} (22) and to the upper limit of the range (45.6 to 49.3 mol\%) reported for \textit{Marinococcus hispanicus} (11). The results of the DNA-DNA hybridization experiments indicate that there is a specific relationship among CCM 168, CCM 1405, and the type and only other strain of \textit{Salinicoccus roseus}. The high degree of homology between the type strain of \textit{Salinicoccus roseus} and strain CCM 1405 indicates that these two strains are members of the same species. The hybridization data indicate that there is a more distant relationship between strain CCM 1405 and the type strain of \textit{Salinicoccus roseus} on the
one hand and strain CCM 168 on the other hand. On the basis of current recommendations for the delineation of species, however, it is probable that strain CCM 168 is also a member of the species *Salinicoccus roseus* but somewhat less closely related to the other two isolates (16, 25). Taken together, the data present clear evidence that strains CCM 168 and CCM 1405 should be classified in the species *Salinicoccus roseus*.

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