Vagococcus entomophilus sp. nov., from the digestive tract of a wasp (Vespula vulgaris)

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Three unknown Gram-stain-positive, catalase-negative, facultatively anaerobic and coccus-shaped strains of bacteria were isolated from the digestive tracts of wasps (Vespula vulgaris). Analysis of 16S rRNA gene sequences revealed that these strains had identical sequences and showed that Vagococcus salmoninarum, with 96.2 % sequence similarity, was the closest phylogenetic neighbour. Further analyses based on hsp60 and pheS gene sequences of representatives of the family Enterococcaceae and genotypic and phenotypic characterization using (GTG)5-PCR fingerprintings, EcoRI ribotyping, DNA G+C content, whole-cell protein profiling, cellular fatty acid profiles analysis and extensive biotyping confirmed that the investigated strains were representatives of a novel bacterial species within the genus Vagococcus for which the name Vagococcus entomophilus sp. nov. is proposed. The type strain is VOSTP2T (DSM 24756T = CCM 7946T).

Representatives of the genus Vagococcus were separated from related genera within the order Lactobacillales in 1990 and are classified as members of the family Enterococcaceae (Collins et al., 1989; Collins, 2009). Vagococci represent bacteria that have been isolated from different environments. Different species have been found in ground beef (Shewmaker et al., 2004), an acidogenic fermentation bioreactor (Wang et al., 2011), a swine-manure storage pit (Lawson et al., 1999), a dead seal and a harbour porpoise (Hoyles et al., 2000), human clinical sources and pigs (Teixeira et al., 1997), common otter (Lutra lutra) (Lawson et al., 1999), the spoilage microbiota of cooked shrimp (Jaffrès et al., 2010) and diseased salmonid fish (Wallbanks et al., 1990). The ecological importance of vagococci has not been explored. Some strains may act as potential pathogens (Ruiz-Zarzuela et al., 2005). However, there are some studies indicating that Vagococcus fluvialis could be a probiotic bacterium for economically important marine fish (Román et al., 2012; Sorroza et al., 2012).

In this study, we describe three novel strains of bacteria isolated from a wasp (Vespula vulgaris) and representing a novel species of the genus Vagococcus; the strains were distinct from all other species of bacteria isolated from the digestive tracts of insects.

Individual wasps (Vespula vulgaris) had been caught in the locality of Modráň (Prague, Czech Republic) in 2010 and transported to the microbiological laboratory immediately.
after capture. The body surfaces of wasps were treated with 70% ethanol to remove potential contamination. After decapsulation, digestive tracts were removed aseptically and immediately transported to Hungate tubes containing sterile anaerobic tryptone–phytone–yeast extract (TPY) broth (Scardovi, 1986) and glass beads. Samples were then weighed, homogenized in a common lab vortex mixer and serially diluted. The range of dilutions from $10^{-7}$ to $10^{-2}$ w/v was used for the experiments. The 0.5 ml aliquots of the extracts were plated on modified TPY (MTPY) agar (Rada & Petr, 2000) and incubated under anaerobic conditions (Anaerobic jars, Oxoid) at 37 °C for 72 h. Individual bacterial colonies were picked up, transferred into tubes containing anaerobic TPY broth and cultivated at 37 °C for 24 h. Investigated strains designated as VOSTP2T, VOSTP5 and VOSTP6 were isolated from three different wasps.

Reference type strains of species of the genus Vagococcus V. salmoninarum CCM 4305T; V. penaei CCM 8416T; V. fluvialis CCM 4304T; V. carniphilus CCM 8414T; V. lutrae CMM 4937T; V. fessus CCM 8413T; V. acidifermentans CCM 8417T and V. elongatus CCM 8415T were obtained from the Czech Collection of Microorganisms (CCM, Masaryk University, Brno, www.sci.muni.cz/ccm).

Chromosomal DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen). Then, almost-complete 16S rRNA gene sequences (1490 bp) were then amplified by PCR using the forward primer fD1 (5’-CCGAATTCCGTGAC-AACAGAGTGTGATCCTGGCTCAG-3’) and reverse primer rP2 (5’-CCTCGGCTATCCAGCGTATCTTTGTTACGACTT-3’) (Weisburg et al., 1991). The PCR was performed under the following conditions: 92 °C for 5 min; 35 cycles of 92 °C for 1 min, 52.5 °C for 90 s and 72 °C for 2 min; 72 °C for 5 min. Checked and purified DNA fragments were sequenced using an automatic genetic analyser ABI PRISM 3100xl (Applied Biosystems).

Sequences of 16S rRNA genes were then edited and compared with the sequences from the most closely related species as described previously (Killer et al., 2011). Strains VOSTP2T, VOSTP5 and VOSTP6 (lengths of sequences 1430 bp, 1410 bp and 1409 bp, respectively) revealed identical 16S rRNA gene sequences. The 16S rRNA sequences of the most closely related strains and other phylogenetic markers were searched using the BLAST algorithm freely available on the website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence similarities of phylogenetic markers with those of the most closely related strains were calculated using the jPHYDIT program (Jeon et al., 2005). Only the sequences of the type strains of most closely related species have been used for calculation. V. salmoninarum CCG 33394T (GenBank accession number Y18097) was the closest phylogenetic relative with a validity published name on the basis of the 16S rRNA gene, with the similarity value of 96.2%. However, the closest relative based on 16S rRNA gene sequence similarity is the unpublished Enterococcaceae bacterium AaMG2 (GenBank accession number GQ915078) isolated from the digestive tract of mosquito (Aedes albopictus, Diptera: Culicidae) with a similarity value of 99.8%. Multilocus Sequence Analysis (MLSA) is a suitable tool to confirm the status of novel bacterial species (Ventura et al., 2006). Partial sequences of hsp60, pheS, rpoA and atpA genes were amplified by PCR methods according to the protocols of Goh et al. (2000), Naser et al. (2005b) and Naser et al. (2005a), respectively. The most closely related sequences observed on the basis of individual comparisons of hsp60 and pheS genes were those of Vagococcus carniphilus (80.8% sequence similarity; GenBank accession number JX576771) and Vagococcus salmoninarum (80.0% sequence similarity; GenBank accession number JQ363693), respectively. Individual comparisons of rpoA and atpA genes revealed closely related sequences from species of the genus Enterococcus with sequence similarities ≤ 82.3%. Nevertheless, the GenBank database does not contain sufficient data related to the sequences of these genes in members of the family Enterococcaceae. Strains VOSTP2T, VOSTP5 and VOSTP6 had identical hsp60 and pheS gene sequences (not shown).

The internal transcribed spacer (ITS) was also sequenced according to the protocol of Dobson et al. (2002). It was revealed that the 16S–23S rRNA intergenic spacer of Enterococcus saccharolyticus KCTC 3643T was the sequence most similar to the sequences of the ITS region amplified from bacterial strains from the digestive tract of wasps, with sequence similarity of 77.2%. Obtained results indicate that the novel bacterial strains represent a novel species belonging to the family Enterococcaceae. Phylogenetic analysis based on 16S rRNA and housekeeping gene sequences were performed in order to clarify the phylogenetic position of the investigated strains. Sequences of 16S rRNA genes and other housekeeping gene sequences were identical in all three isolates from wasps. For this reason, the type strain VOSTP2T was the predominant strain characterized.

The clonal status of strains VOSTP2T, VOSTP5 and VOSTP6 and differentiation from other representatives of species of the genus Vagococcus were characterized using (GTG)6-PCR fingerprinting, automated ribotyping and whole-cell protein profiling and compared with the type strains representing all hitherto described species of vagococci in order to clarify their taxonomic position within the genus Vagococcus. Repetitive-sequence-based PCR fingerprinting with the (GTG)3 primer was performed according to the protocol of Švec et al. (2008). Automated ribotyping with the EcoRI restriction enzyme was performed in the same strains using the Riboprinter microbial characterization system (DuPont Qualicon) in accordance with the standard protocol provided by the manufacturer. Whole-cell protein analysis was performed according to the protocol described by Pot et al. (1994). The isolates were grown on BHI agar at optimal temperature for 24 h. Harvested cells were disrupted by sonication using a Labsonic M ultrasonic homogenizer (Sartorius). Protein extracts were separated using SDS-PAGE. Fingerprint profiles resulting from individual methods were digitized, processed and analysed using BioNumerics v. 6.6 software.
Phylogenetic trees were reconstructed using MEGA 5.05, Gblocks and MrBayes (Ronquist et al., 2012) programs as described previously (Killer et al., 2013). The phylogenetic tree based on 16S rRNA gene sequences of representatives of the family Enterococcaceae showed that members of the genera within the family Enterococcaceae, Vagococcus, Enterococcus, Melissococcus, Catelicoccus, Tetragenococcus, Bavariococcus and Pilibacter were clustered into distinct phylogenetic groups. VOSTP2\(^T\), VOSTP5 and VOSTP6 were situated on a separate phylogenetic branch within the group of vagococci (Fig. 1). Phylogenetic trees based on available hsp60 and pheS (Fig. S2 and Fig. S3) sequences of representatives of the family Enterococcaceae were reconstructed to confirm the assumption that strain VOSTP2\(^T\) belongs to the genus Vagococcus. Sequences of these genes are not available in the GenBank database for all species of vagococci. Therefore, fragments of the genes were amplified and sequenced in type strains of the genus Vagococcus species as described above. Also, the pheS phylogenetic tree confirmed the phylogenetic delineation of vagococci (except V. salmoninarum) and enterococci. As shown in the phylogenetic tree, the novel strain was grouped within a cluster of vagococci together with V. acidifermens KCTC 13418\(^T\) (Fig. S3). Species of enterococci and vagococci were also separated into two phylogenetic groups based on partial hsp60 gene sequences. However, some species of vagococci formed separate phylogenetic branches. Within the phylogenetic tree, the novel strain was located on a separate branch between the phylogenetic groups of enterococci and vagococci (Fig. S2). Phylogenetic studies based on rpoA and atpA gene sequences and the ITS region have not been performed due to the lack of sufficient data on sequences of representatives of the family Enterococcaceae.

A modified enzymic degradation method (Killer et al., 2011) was used for determination of the DNA G+C contents in the VOSTP2\(^T\) strain and other type strains of known species of the genus Vagococcus. The DNA G+C mol% content was determined according to the method of Mesbah et al. (1989) with some minor modifications of the analytical set-up. The separation of four deoxynucleosides (Sigma–Aldrich) was achieved with an analytical HPLC system Dionex Summit (Dionex), consisting of a P680 quaternary gradient pump, diode array detector UVD340U, and a column thermostat, interfaced with the Waters 717 autosampler (Waters), using a Phenomenex Fusion C18 column (250 × 4.6 mm internal diameter, 5 μm particle size, Phenomenex). The column temperature was 22°C. Nucleosides were eluted under gradient conditions using A: 20 mM ammonium acetate (pH 4.5) and B: acetonitrile. The linear gradient started at 4% of A and changed linearly to 26% B. The linear gradient started at 4% of B and changed linearly to 26% B in 12 min. The flow rate was set to 0.8 ml min\(^{-1}\) and the injection volume was 20 μl. The value of 39.7 mol% (mean of three experiments, sd=0.07) was determined for strain VOSTP2\(^T\). DNA G+C contents in the range from 32.6 to 45.2 mol% have been found in representatives of the genus Vagococcus (Table 1). Some of the values detected by the modified enzymic degradation method are lower compared with those that had been determined by authors who described some of the novel species of the genus Vagococcus. V. fessus and V. elongatus are examples of species for which lower values of C+G content were determined by thermal denaturation spectrophotometric methods (Hoyles et al., 2000; Lawson et al., 2007).

Phenotype characterization using Rapid ID32A Strep and API ZYM systems (bioMérieux) has been proved to be a useful tool for distinguishing species of the genus Vagococcus (Collins, 2009). These test kits were used to differentiate strains VOSTP2\(^T\), VOSTP5 and VOSTP6 from the remaining type strains of all hitherto described species of vagococci. Production of 15 enzymes displayed differences among species of the genus Vagococcus (Table 1). Subsequently, the characteristics covered by the Biolog Identification System (GP2 MicroPlate) and API 50 CH test strips (bioMérieux) were determined in order to obtain extensive phenotypic data on the isolates VOSTP2\(^T\), VOSTP5 and VOSTP6 and type strains of all hitherto described species of vagococci. Species of vagococci differ in the utilization of 35 substrates (Table S1). The novel strains were not able to utilize maltotriose or glycerol, unlike the type strains of species of the genus Vagococcus with validly published names. On the other hand, the novel strains are able to utilize inulin, unlike members of other species of the genus Vagococcus.

Trypticase soy yeast extract medium (TSYE; Sigma–Aldrich) suitable for cultivation of vagococci and enterococci was used to evaluate the ability to grow in aerobic, microaerophilic and anaerobic environments, at different temperatures and pH values by methods described previously (Killer et al., 2013). Best growth of the strains VOSTP2\(^T\), VOSTP5 and VOSTP6 was observed on TSYE medium under aerobic conditions. However, poor growth was observed also under microaerophilic and anaerobic conditions. Growth occurred at temperatures between 10

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and 40 °C. The temperature ranges for growth of the type strains of other species of the genus Vagococcus in TSYE broth are shown in Table 1. The minimum initial pH for growth under aerobic conditions in TSYE broth was 5.0 and the maximum was 10.0.

Motility was tested on stab-inoculated semi-solid medium according to the method of Švec et al. (2012) and showed that VOSTP2T is non-motile.

The detailed morphology of the cells was studied by scanning electron microscopy (Killer et al., 2009). The ovoid cell morphology of strain VOSTP2T is shown in Fig. 2.

Lactic, acetic and propionic acids at concentrations of 16.5 mmol l⁻¹ (71 % of the total short-chain fatty acids produced), 5.6 mmol l⁻¹ (24 %) and 1.1 mmol l⁻¹ (5 %), respectively, were quantified using the isozatophoresis analytical method (Killer et al., 2011) after cultivation at 37 °C for 24 h. The aerobic TSYE broth supplemented with 5 g glucose l⁻¹ was used for determination of end products of hexose catabolism in strain VOSTP2T. Obtained results showed that this strain is a representative of the homofermentative lactic acid bacteria.

Production of lactic acid optical isomers was tested by the d/l-lactic acid kit (Megazyme) according to the manufacturer’s instructions. VOSTP2T produced only L-lactic acid, which corresponded with results obtained for the other species of the genus Vagococcus (Collins, 2009).

For the quantitative analysis of cellular fatty acids, strain VOSTP2T was grown at 30 °C for 72 h in the trypticase soy broth (Sigma–Aldrich) under aerobic conditions. Growth was stopped in the stationary phase of the growth curve. The profile of cellular fatty acids was determined as described previously by Jaffrès et al. (2010) with some modifications described by Killer et al. (2009). The major fatty acids detected in VOSTP2T strain were 10-methyl C₁₆:₀ C₁₄:₀ and C₁₆:₀ respectively. Compared with other species of vagococci (Wang et al., 2011), the tested strain has a higher proportion of 10-methyl C₁₆:₀ and a lower proportion of C₁₈:₀ 9c fatty acids (Table S2).

VOSTP2T was also tested for susceptibility to 33 different antibiotics and two chemotherapeutics using a disc diffusion method (Vlková et al., 2006). Sensitivity to most tested cephalosporines, macrolides, penicillin-derived antibiotics, vancomycin, rifampicin, clindamycin, chloramphenicol, tetracycline and gentamicin was observed. On the other hand, the tested strain was resistant to three different cephalosporines, meropenem, aminoglycosides, fluoroquinolones, sulphonamides, mupirocine and metronidazole (Table S3). In contrast to these results, V. fluvialis and V. salmoninarum were resistant to clindamycin but susceptible to cefotaxime and trimethoprim–sulphamethoxazole based on results from Teixeira et al. (1997).

Structure and amino acid composition of the cell-wall peptidoglycan and the profile of polar lipids were evaluated by the

**Fig. 1.** Phylogenetic tree showing that the novel strains VOSTP2T and VOSTP6 belong to the genus Vagococcus. It was reconstructed based on 16S rRNA (length of 1303 nt) gene sequences using the maximum-likelihood algorithm within the MEGA version 5.05 software after removing hypervariable positions using the program Gblocks. Sequence data were aligned using the CLUSTAL W algorithm. The Jukes–Cantor model was used for reconstruction of the phylogenetic tree. Bootstrap values, expressed as percentages of 1000 datasets, are given at nodes. Numbers in parentheses correspond to the GenBank accession numbers. The tree was reconstructed as unrooted. Bar, 0.008 substitutions per nucleotide position.
Identification Service (DSMZ, Braunschweig, Germany) by
the methods described previously (Killer et al., 2010). It was
concluded that VOSTP2T displayed peptidoglycan type A1γ;
meso-diaminopimelolic acid; type A31 according to DSMZ
www.dsmz.de/catalogues/catalogue-microorganisms/specific-
catalogues/peptidoglycans.html. The cell-wall peptidoglycan
has been determined only for V. fluvialis among the species of
the genus Vagococcus. Collins et al. (1989) determined the
peptidoglycan type to be L-lysine-D-aspartic acid in the
species. Polar lipids detected in cells of VOSTP2T strain
consisted of phosphatidylglycerol, two phosphoglycolipids,
six phospholipids, three unidentified aminolipids, six glyco-
lipids and three unknown polar lipids, respectively (Fig. S4).
Fischer & Arneth-Seifert (1998) found unusual membrane
polar lipids such as cardiolipin [bis(phosphatidyl)glycerol],
d-alanylcarnitincarnilin, lysocardilin, ε-D-glycinecardiolipin,
D-alanylsphingosyglycerol and D-alanylsphingosyglycerol-
cer in V. fluvialis. However, the compositions of the polar
lipids in other species of the genus Vagococcus have not been
determined yet. Therefore, it was impossible to attempt the
identification of distinctive polar lipids.

The results of a wide range of genotypic, phenotypic and
phylogenetic analyses demonstrated that the bacterial strains
VOSTP2T, VOSTP5 and VOSTP6 represent a novel species for
which the name Vagococcus entomophilus sp. nov. is proposed.

**Description of Vagococcus entomophilus sp. nov.**

*Vagococcus entomophilus* [(en.to.mo’phi.lus. Gr. n. entom
insect; N.L. adj. philus -a -um (from Gr. adj. philos -é

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<td>A4γ; 1-Lys–D-Asp</td>
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| Naphthol-AS-BI-
phosphorydrolase | + | + | + | + | + | W | W | W | – |
| Pyroglyutamic acid arylamidase | – | + | + | W | + | + | – | – | – |
| Production of acetoin (Voges–Proskauer test) | – | – | + | – | – | – | – | – | – |

*Values for DNA C+G content are mean of three experiments: SDs=0.02–0.07.
†Data from previous studies (Teixeira et al., 1997; Hoyles et al., 2000 and Jaffre’s et al., 2010).
Cells growing in liquid aerobic TSYE medium are Gram-stain-positive, non-spore-forming, non-motile, ovoid-shaped cocci, measuring 0.7–0.9×0.5–1.1 μm. They are arranged mainly in pairs or singly (Fig. 2). Colonies on TSYE agar under aerobic conditions after 48 h incubation at 37 °C are generally triangular or disc-shaped with regular edges, white, smooth and reach 0.82–2.75 mm in diameter. Cells are facultatively anaerobic, obligately homofermentative and produce L-lactic acid. Growth occurs at temperatures between 10 and 40 °C and at a pH range of 5–10. Has the ability to use the following carbon sources via respiration: inulin, N-acetyl-D-glucosamine, amygdalin, arbutin, cellobiose, D-fructose, gentiobiose, α-D-glucose, D-mannose, methyl-β-D-glucoside, salicin, sucrose, trehalose, adenosine and uridine. Negative for utilization of α-cyclodextrin, β-cyclodextrin, glycogen, mannan, Tween 40, Tween 80, L-arabinose, D-arabitol, L-fucose, D-galactose, D-galacturonic acid, D-glucuronic acid, myo-inositol, α-lactose, lactulose, maltose, maltotriose, D-mannitol, melezitose, melibiose, methyl-α-D-galactoside, methyl-β-D-galactoside, 3-methyl glucose, methyl-α-D-glucoside, methyl-α-D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, D-ribose, sedoheptulose, D-sorbitol, stachyose, D-tagatose, turanose, xylitol, D-xylene, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, β-ketoglutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycy l L-glutamic acid, L-prolylglycamin acid, L-serine, putrescine, 2,3-butanediol, glycerol, 2′-deoxyadenosine, inosine, thymidine, adenosine-5′-monophosphate, thymidine-5′-monophosphate, uridine-5′-monophosphate, D-fructose-6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Variable results between strains for dextrin utilization; the type strain is not able to utilize dextrin. Produces α-chymotrypsin, α-glucosidase, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, arginine arylamidase, phenylalanine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Does not produce α-galactosidase, β-galactosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, α-mannosidase, N-acetyl-β-glucosaminidase, cystine arylamidase, arginine dihydrolase, pyroglycamin acid arylamidase, β-galactosidase-6-phosphate, α-fucosidase, proline arylamidase, leucyl glycine arylamidase, glutamyl glutamic acid arylamidase, lipase (C14), valine arylamidase, trypsin, urease, catalase, oxidase, indole from L-tryptophan and protease (gelatin hydrolysis). Negative for acetoin production. Susceptible to some cephalosporines, macrolides and some penicillin-derived antibiotics. Resistant to aminoglycosides, fluoroquinolones, sulphonamides, mupirocin and metronidazole (Table S3). The major fatty acids detected were 10-methyl C16:0, C14:0 and C16:0 in that order (Table S2). The peptidoglycan type is A2L meso-diaminopimelic acid. Principal polar lipids are phosphatidylglycerol, two phosphoglycolipids, five phospholipids, three unidentified aminolipids and six glycolipids.

The type strain, VOSTP2T (=DSM 24756T =CCM 7946T) was isolated from the digestive tract of wasp (Vespu laria vulgaris) which had been caught in the locality of Modrany (Prague, Czech Republic) in 2010. The DNA G+C content for VOSTP2T is 39.7 mol%. Values of DNA G+C content of other strains is in the range from 39.3 to 40.1 mol%.

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