Biodegradation of olive washing wastewater pollutants by highly efficient phenol-degrading strains selected from adapted bacterial community.

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Abstract:

The bacterial community of an olive washing water (OWW) storage basin was characterized, by both cultivation and cultivation-independent methods. Analysis of PCR-TGGE fingerprints of different samples, taken along the olive harvesting season, revealed important variations of the bacterial community structure showing a rapid succession of prevalent bacterial populations. In order to select high phenol-degrading strains, for possible reduction of OWW pollutants in view of its disposal and reuse, 18 strains isolated from OWW were cultivated in media containing increasing amounts of polyphenols. Strains PM3 and PM15, affiliated to *Raoultella terrigena* and *Pantoea agglomerans* by 16S rRNA gene sequencing, were selected for their best performance and used for the OWW biological treatment under batch conditions in shake flasks culture. The contents of OWW main pollutants, phenols, COD, BOD_5 and colour, were reduced by 93, 89, 91 and 62%, respectively.

Keywords*:* Olive washing wastewater; PCR-TGGE characterization; phenol-degrading bacteria; biological treatment.

1. Introduction.

Although olive industry is traditionally a major food sector for Mediterranean countries, the production of olive oil is fast spreading in various areas of Australia, Chile and USA (D'Annibale et al., 2006; Cerrone et al., 2011), while other important countries, such as China and India, are now starting or promoting its production. Olive oil industry generates a huge amount of wastewater (ca. 5.4 x 10^6 m^3 yr^{-1}) and other wastes (Khatib et al., 2009).

Oil extraction could be carried out using either a three- or a two-phase system. In the first case, a solid waste (olive husks) and a wastewater (olive mill wastewater, OMWW) are released, while only a semisolid waste is generated (olive wet husks) by the two-phase process. For both systems, preliminary olive washing with potable water is necessary generating another type of effluent with different composition and characteristics (olive washing wastewater, OWW).

In three-phase mills, OWW is disposed with OMWW, while two-phase mills have the problem of OWW separated disposal (Cerrone et al., 2011). The two-phase process is generally preferred in order to save potable water and the use of these plants is now increased and it is already the main system (> 90%) in Spain, the world's principal olive oil producer (Rodriguez-Lucena et al., 2010). For these reasons, OWW disposal is becoming a consistent worldwide environmental issue (Guardia-Rubio et al., 2008); the effluent can not be disposed as such but needs specific treatments (Roig et al., 2006).

OMWW is a highly polluted effluent, being its main pollutants COD and phenols, ranging from 30 to 150 g L^{-1} and from 1.5 to > 8 g L^{-1}, respectively; its treatment and/or reuse have been studied intensively (Khoufi et al., 2009). However, an ultimate solution is yet to be found and, till now, no efficient disposal technology is

67 available even if some biological agents, such as white rot fungi, are very promising
68 (D'Annibale et al., 2004; Lakhtar et al., 2010).
69 OWW composition is similar to that of OMWW but with much lower (from 1/10
70 up to 1/50) concentration of pollutants such as COD and phenols (Pozo et al., 2007;
71 Cerrone et al., 2011). To the best of our knowledge, the only works attempting OWW
72 biological treatment are that of Pozo and co-workers (2007) using a submerged bacterial
73 biofilter and that of Cerrone et al. (2011) using the white-rot fungus *Trametes versicolor*
74 in bubble-column bioreactor through a continuous process. No process using selected
75 bacteria has been previously studied.
76 In this work, after characterization of OWW bacterial communities by
77 cultivation and cultivation-independent (PCR-TGGE) methods, isolation and selection
78 of phenol degrading strains has been carried out in order to obtain inocula for possible
79 effluent treatment. Moreover, batch process of OWW depollution has been investigated
80 using the selected strains. Time course of OWW pollutants removal during the
81 treatment is reported also.
82

83 ## 2. Materials and Methods

84 2.1 OWW characterization

85 The OWW samples were collected from the storage basin located outside the olive oil
86 factory (Nuestra Señora de los Desamparados, Puente Genil, Córdoba, Spain). The
87 basin (*ca.* 45m^3), is 3 meters deep with no agitation. Samples, taken at 1.5-2 meters
88 depth, were collected both in the middle (OWW-1, January 13th, 2009) and at the end
89 (OWW-2, March 4th, 2009) of olive harvesting season.
90 OWW samples were submitted to the following analyses (Table 1): colour, pH;
91 conductivity, COD, BOD_5, total phenol content, total and dissolved solids, organic and
92 inorganic matter content, platable heterotrophic bacteria counts, total N and P content.

Methodologies used for determinations were as described by the Standard Methods for the Examination of Water and Wastewater (APHA, 4500-N C and 4500-P C), except for the total phenol determination that was carried out spectrophotometrically (760 nm) by the Folin-Ciocalteu method as previously reported (D´Annibale et al., 2006).

2.2 Microbial Community Analysis by PCR-TGGE

2.2.1 DNA extraction

Total DNA was extracted from samples as follows: fifteen ml of OWW were filtered through 0.45 μm nitrocellulose sterile filter membranes (Millipore, USA). Each membrane was suspended in ca. 2 ml of sterile water and fragmented with a sterile pipette tip; tubes were then vigorously stirred using a vortexer (IKA, Germany) in order to re-suspend the material retained by filters. The suspension was transferred to a microcentrifuge tube and used for DNA extraction by the commercial kit MasterPureTM Complete DNA (Epicentre® Biotechnologies, USA).

2.2.2 PCR specific amplification of partial 16S rRNA genes

A two-step PCR (nested PCR) approach was selected for specific amplification of the V3 hypervariable region of the Bacteria 16S rRNA gene, as previously described (Molina-Muñoz et al., 2009).

Extracted DNA (2-5 ng) was used as a template for a first PCR carried out using the universal primers fD1 and rD1 (Weisburg et al., 1991). Subsequently, the nested PCR was performed using the universal primers GC-P1 and P2, amplifying the V3 hypervariable region of the 16S rRNA gene (Muyzer et al., 1993).

2.2.3 TGGE fingerprinting and analysis

Runs were done on a TGGE Maxi system (Whatman-Biometra, GmbH, Germany). Denaturing gels (6% PAGE with 20% deionized formamide, 2% glycerol and 8 M urea) were run, with 2 x TAE buffer, at 125 V for 18 h. Optimal temperature gradient for efficient bands separation was 43-63 °C. Gel bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific Rockford, IL, USA), following the manufacturer's indications.

TGGE band patterns were normalized, compared and clustered using the Gel Compar II image analysis software (version 5.102, Applied Maths, Belgium).

2.2.4 DNA sequencing of TGGE-isolated bands, phylogenetic and molecular evolutionary analyses

Portions of prominent TGGE bands were picked up with sterile pipette tips from stained gels, placed in 10 µl of filtered (0.22 µm) and autoclaved distilled water, and directly used for reamplification with the appropriate primers.

Sequences were compared to those filed in the Greengenes and GeneBank databases by the BLASTn program (Altschul et al., 1997), using the tools provided online at http://greengenes.lbl.gov and http://www.ncbi.nlm.nih. The closest taxonomic affiliation of each sequence was given by the Greengenes taxonomy tool.

2.3. Strain isolation, identification and phylogenetic affiliation.

For strain isolation, samples aliquots (0.1 ml) were serially diluted and spread on Triptycase Soy Agar (TSA) plates (Difco, UK). Plates were incubated aerobically at 28ºC for 24-48 h and then checked visually. Pure cultures of bacteria grown on TSA were obtained by streak plate method. In order to avoid duplicates of same species, isolates showing same morphological characteristics (colony shape, colour, morphology and aspect; cell morphology, dimensions and Gram staining) were discharged. The tests

allowed selection of 12 and 6 different isolates from OWW-1 and OWW-2, respectively.

For strain identification, a fresh cultured (24 h) colony of each isolate was lysed as described by Sánchez-Peinado et al. (2008). PCR reaction was kept as described by (Molina-Muñoz et al., 2009) using the universal primers from Sigma-Aldrich (USA). The PCR amplicons were directly sequenced by the DNA Sequencing Service of Instituto de Parasitologia y Bioquimica Lopez-Neyra (CSIC, Granada) using an ABI PRISM 3130XL Genetic Analyzer (Life Technologies, CA, USA).

DNA sequences were analyzed on-line by the European Bioinformatics Institute biocomputing tools (http://www.ebi.ac.uk). The BLASTn program (Altschul et al., 1997) was used for preliminary sequence similarity analysis. The ClustalX version 2.0.3 software (Jeanmougin et al., 1998) was used for sequences aligning. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Kumar et al., 2001). A p-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm.

2.4. Selection of phenol-degrading bacteria

All strains isolated from OWW were grown on synthetic media, composed of (mg l-1): sucrose (500), $NaNO_3$ (20), K_3PO4 (10), and traces of mineral elements (Kotturi et al., 1991). The media were added with increasing amounts of a 1:1:1 mixture (wt/wt) of caffeic acid, p-OH-benzoic acid and coumaric acid as follows (mg l-1): SM0, 0; SM1, 50; SM2, 100; SM3, 150. Media (50 ml in 250 ml Erlenmeyer flasks) were inoculated (5.5 10^6 cell ml-1) with cells grown in TSB at 28 ºC for 24 h in shake cultures (100 rpm). Selection tests were done in triplicate grown in the same media at 28 ºC for 72 h at 100 rpm. Strains showing best growth on media containing the highest phenol content were used for subsequent experiments.

Platable cell were counted in TSA medium. Inoculated plates were incubated at 28º C for 48 h prior to CFU counts. All the experiments were done in triplicate.

2.5. OWW treatment with selected bacteria in batch process

Bacteria selected, as best phenol degraders, were grown in 250-ml Erlenmeyer flasks containing 50 ml of OWW-2 (unsterilized) and cultured for 144 h at 28ºC and shake at 100 rpm. Flasks were inoculated with cell suspensions containing a mixture (1:1, v/v) of the selected strains, to reach a final concentration of 5.5 10^6 CFU ml-1. A control bioprocess was carried out under the same conditions, using 50 ml of un-inoculated OWW-2.

Samples from both inoculated and un-inoculated flasks were taken every 24 h and submitted to the following analyses: colour, pH, COD, BOD_5, total phenols, N and P contents, and platable heterotrophic bacteria, performed as described above (see 2.1).

2.6. Statistical analysis.

Analysis of variance (ANOVA) and multiple-range test (Student´s T test) were done using STATGRAPHICS 5.0 (STSC, Rockville, MD, USA). A significance level of 95% ($p<0.05$) was selected.

3. Results and Discussion

3.1 Characterization of OWW samples

As for the majority of wastes and wastewater from agro-industries, OWW composition depends upon a wide number of factors. In our case, OWW-2 showed significantly different increased levels of pollutants (soluble solids, total phenols, sugar) than OWW-1. This, probably due to sample concentration by evaporation and oxidation processes, was particularly true for the colour (Table 1). However, pollutant load of

both samples, in particular COD, colour and total phenols, were quite higher than those reported in previous studies (Pozo et al., 2007; Cerrone et al., 2011). Moreover, platable counts in OWW-2 were quite lower than those of OWW-1 (Table 1), indicating selection of adapted microbial populations under the increased contents of pollutants (phenols in particular). It is worth noting that OWW-1 was taken in the middle of the production season with daily input of fresh OWW containing bacteria from the harvested olives. OWW-2 was taken when the production season was already over with no input of fresh OWW. Therefore, the microbial community of OWW-1 mainly represented the microbiota found on the olives, while OWW-2 represented the surviving adapted bacteria in the OWW storage basin.

3.2 Analysis of the structure of bacterial communities by TGGE fingerprinting. Phylogenetic study of the DNA sequences of the prevalent TGGE bands.

The PCR-TGGE analysis demonstrated clear and significant differences of the bacterial community structure between OWW-1 and OWW-2 (Fig.1A). The Pearson coefficient-based analysis, calculated a similarity below 60% between the fingerprints of the two samples analyzed, indicating that the microbial community was significantly influenced by the season and by the time of permanence in the storage basin. Cluster analysis based on the Dice coefficient yielded equivalent results to the Pearson-based clustering (Fig.1B).

3.2.1 Taxonomical affiliation and phylogenetic study

Cluster analysis with Gel compar II detected a total of 52 bands classes in the TGGE fingerprints of bacteria among the two samples analyzed. The majority of the detected band classes were exclusive of each seasonal stage. These data showed that the bacterial communities in OWW consisted of populations displaying different degrees of susceptibility to the seasonality.

Nineteen bands selected from the TGGE fingerprinting were successfully amplified and sequenced, representing 36.6% of the recognized band classes. Phylogenetic analysis (Table 2) derived from the TGGE isolated bands confirmed the dominance of Firmicutes amongst Bacteria involved in the colonization of the storage basin of OWW. In particular, sequence analysis revealed that populations phylogenetically close to the order *Clostridiales* were dominant.

Interestingly, the results of the community analysis described here showed the longitudinal succession of different microbial communities in the OWW and reflects the particular environment being established in the storage basin. Indeed, the majority of populations identified by TGGE sequence analysis are related to anaerobic or facultative bacteria, many of them close to genera characterized by their ability to degrade polymeric compounds, such as *Pelosinus*, *Hyphomicrobium*, *Desulfotomaculum*, or *Clostridium* (Gliesche and Fesefeldt, 1998; Fichtel et al., 2012; Moe et al., 2012).

3.3 Isolation, identification and phylogenetic affiliation of cultivated strains isolated from OWW.

The phylogenetic tree (Fig. 2) shows in both samples the dominance of Gammaproteobacteria (41.7% and 50.0%, respectively) and Firmicutes (58.3 % and 17.0%, respectively) among the identified bacterial isolates. In sample OWW-1, all the isolated strains could be affiliated to the Firmicutes and Gammaproteobacteria. However, within these two main groups various subclusters were detected, with the presence of strains affiliated to the genera *Bacillus*, *Sporosarcina*, *Rahnella*, *Bacterium* and *Raoultella* (Fig. 2.A). In sample OWW-2, members of Actinobacteria were also isolated but less sub-clustering was detected (Fig. 2.B), with strains possibly affiliated to the genera *Micrococcus*, *Bacillus*, *Bacterium* and *Raoultella*.

Six of the strains (PM7, PM5, PM3, PM10 from OWW-1, and PM18 and PM15 from OWW-2) were phylogenetically close to members of the family *Enterobacteriaceae*. Studies by Wust et al. (2011) showed that exoenzymes produced by microorganisms of the *Enterobacteriaceae* and *Clostridium* stimulate the degradation of complex organic matter. Members of *Enterobacteriaceae*, *Bacillaceae* and *Micrococcaceae*, are universally found in soils of temperate regions. Among them, various species had been described for their ability to degrade aromatics (PHA, dioxins and clorinated phenols) (Zhao et al., 2012).

3.4 Selection of phenol degrading bacteria and OWW treatment

In order to select best phenol degrading bacteria, all strains isolated from OWW were cultivated in synthetic media containing increasing amounts of various polyphenols (Fig. 3). The bacteria ability to cope with the toxic effects of phenols is reflected on their growth rates and is proportional to the applied phenol concentration (Juárez-Jiménez et al., 2012).

In SM0 (no phenols), all bacterial strains grew showing an increased number of cells ranging from *ca.* 1.5 to *ca.* 3.0 logarithmic units (Fig. 3A). In SM1 (50 mg l-1 of phenols), toxic effects were recorded for the majority of strains, while some other maintained the same growth rates recorded in SM0 (Fig. 3B). Actually, there was a clear separation of the strain in two groups, evidencing phenol-resistant and phenol-sensitive bacteria. This phenomenon was even more evident in SM2 (100 mg l-1 of phenols). All phenol-resistant strains belonged to the *Enterobacteriacee* family (Fig.3C).

In SM3 (150 mg l-1 of phenols), growth inhibition was recorded for a larger group of bacteria and further strain sorting was obtained, being growth of isolates PM3 and PM15 (affiliated to *Raultella terrigena* and *Pantoea Agglomerans*, respectively)

significantly higher than that of all the other isolates. According to these results, strains PM3 and PM15 were further selected as powerful inocula to improve the treatment of OWW and fulfil the degradation of its pollutant load in shake cultures under batch conditions (Fig.3D).

Experiments were carried out on non-sterile OWW-2, in order to simulate possible conditions of field-application. Actually, sterilization of OWW would be not feasible under the economic point of view and quite problematic at the technological level.

Figure 4 shows the time course of COD, BOD_5, colour and phenols concentration in OWW-2 inoculated with the selected strains and incubated for 144 h in shake cultures under batch conditions. Time course of same parameters on the un-inoculated OWW-2 (control) and bacterial growth are also reported.

The inoculation with the selected strains reduced the contents of phenols, COD, BOD_5 and colour by 93, 89, 91 and 63%, respectively. The degradation process was rather fast; exponential pollutant degradation started after *ca.* 24 h of incubation to reach its maximum values around the 96 h. Fast removal of pollutants is very important in view of possible applications.

Even if most of the microflora revealed by the TGGE was affiliated to anaerobic bacteria, in this study, aerobic microorganisms for possible biological treatment were selected. Actually, it is well known that, in general, phenolic compounds are very toxic for various anaerobic microorganisms (Pozo et al., 2007), hence limiting the use of anaerobic degradation technologies for the treatment of these wastewaters. Besides, aerated systems are proven as efficient and low-cost biological technologies for other decontamination processes, involving phenol-containing effluents including OMWW, obtaining faster process efficiency (Ehaliotis et al., 1999; Bertin et al., 2001; Di Gioia et al., 2002). Our experimentation at laboratory scale has provided data that support the

297 possibility of applying *Raoultella terrigena* strain PM3 and *Pantoea agglomerans* strain
298 PM15 as inocula with a high potential for biotechnological applications targeting the
299 remediation of these pollutants. The degrading performance reported in this work for
300 PM3 and PM15 strains appeared to be quite superior to that described in earlier studies,
301 both in terms of global pollutant reduction and shorter process duration (Pozo et al.,
302 2007; Cerrone et al., 2011). Remarkably, the levels of all the pollutants measured in the
303 effluents after the treatment proposed in this study comply with the threshold values
304 given by the EC legislation for direct effluent disposal in water bodies without further
305 treatments. However, batch processes are not completely suitable for field applications
306 and their management at the olive mill level could be rather difficult. Thus, further
307 experiments are needed in order to fully explore the potential of strains PM3 and PM15
308 and the feasibility of more efficient bioprocess technologies (i.e. the use of continuous
309 cultures) for the OWW bioremediation (Cerrone et al., 2011).
310

311 ## 4. Conclusions

312 Significant changes of microbial community structure occurred in an OWW storage
313 basin along the olive harvesting season, suggesting selection of specialized populations
314 involved in the biodegradation of OWW pollutants. Two strains, *Raoultella terrigena*
315 PM3 and *Pantoea Agglomerans* PM15, isolated from OWW, strongly reduced COD,
316 colour, phenols and odour of OWW when grown in shake cultures, generating an
317 effluent complying with the standards of the EU legislation, allowing its reuse in ferti-
318 irrigations and/or its direct disposal. Since so far no efficient process for OWW
319 biological treatment has been developed, the selected strains appeared to be very
320 promising as new bioremediation tools.
321

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Figure Captions:

Fig. 1. Community structure of Bacteria in the OWW storage basin analyzed by TGGE profiling. Pearson coefficient-based (A) and Dice coefficient-based (B) analysis of the band patterns generated from samples. "□":TGGE bands which were re-amplified and sequenced in order to perform the phylogenetic study.

Fig. 2. Phylogenetic Neighbour-Joining tree of the 16S-rRNA gene sequences (650nt) from the bacterial isolates. Sequences retrieved from the EMBL database are indicated with their corresponding accession numbers. Bootstrap values below 50 are not shown. (A) OWW-1; (B) OWW-2.

Fig. 3. Time course of growth of the bacterial strains isolated from OWW on media containing different amounts of polyphenols (A: 0, B: 50, C: 100, D: 150 mg l-1). (▬)PM1, (■)PM2, (▭)PM3, (▮)PM4, (▫)PM5, (◆)PM6, (▬)PM7, (◇)PM8, (▭)PM9, (▫) PM10, (+)PM11, (*)PM12, (▬)PM13, (▬)PM14, (✥)PM15, (✦)PM16, (✢)PM17, (■)PM18.

Fig. 4. Incubation of shake flasks containing inoculated (continuous lines) or un-inoculated (dotted lines) OWW-2. (**A**) Time course of COD (▲), BOD_5 (♦) and colour (●) concentration; (**B**) Time course of phenol concentration (■) and total bacterial growth (♦).

438

439 **Table 1**. Physico-chemical characterization of olive washing water (OWW) samples.

Parameter	**OWW-1**	**OWW-2**
pH	5.87	6.40
ρ (g ml-1)	1009	1012
c (mS cm-1)	2.36	2.77
Colour (colorimetric units)	44.8	74.6
T (ºC)	11.3 ºC	23.5ºC
TS (mg l-1)	4756 ± 56^{a}	4834 ± 54^{a}
IS (mg l-1)	1751 ± 48^{a}	1523 ± 53^{a}
SO (mg l-1)	2984 ± 49^{a}	3284 ± 39^{b}
MI (mg l-1)	1854 ± 35^{a}	1938 ± 78^{a}
MO (mg l-1)	2857 ± 92^{a}	2886 ± 82^{a}
COD (mgO_2 l-1)	4137 ± 238^{a}	4575 ± 332^{a}
BOD_5 (mgO_2 l-1)	410 ± 86^{a}	470 ± 83^{a}
Tph (mg l-1)	469 ± 35^{a}	630 ± 45^{b}
P (mg l-1)	51.6 ± 14.0^{a}	62.0 ± 16.0^{a}
N (mg l-1)	60.9 ± 34.0^{a}	63.9 ± 41.0^{a}
S (%)	0.29 ± 0.04^{a}	$0.43 \pm 0{,}07^{b}$
Counts (CFU ml-1)	$1.40\ 10^{8} \pm 0.21^{a}$	$0.51\ 10^{8} \pm 0.01^{b}$

440

441 LEGEND. OWW-1: sample n° 1 (January 13, 2009); OWW-2: sample n°2 (March 4, 2009); COD:
442 chemical oxygen demand; ρ: density; C: conductivity; Col: colour; T: temperature; TS: total solids; IS:
443 insoluble solids; SO: soluble solids; MI: inorganic matter; MO: organic matter; BOD_5: biological oxygen
444 demand at 5 days; Tph: total phenols; P: total phosphorus; N: total nitrogen; S: sugars; Count: Total
445 platable counts of heterotrophic bacteria. Values in rows marked with same superscript letter are not
446 statically different (Student's t-test $p<0{,}05$).

447

Table 2. Closest taxonomic affiliation of sequences derived from TGGE isolated bands. (A) OWW-1, (B) OWW-2.

A

TGGE Band #	Closest taxonomic affiliation (Phylum/Class/Order/Family)	Most similar described organisms & database accession #	Overlap (nt)
1	Unclassified	Uncultured bacterium clone Rock2-4 from riverine rock (HM572444.1)	62/66
		Propionivibrio dicarboxylicus DSM 5885 (NR_026477.1)	61/66
2	*Firmicutes/Clostridia/Clostridiales/*	Uncultured bacterium clone NED3H5 from rabbit cecum (EF445206.1)	82/82
		Cellulosilyticum lentocellum DSM 5427 (NR_026101.1)	76/82
3	*Firmicutes/Clostridia/Clostridiales/Ruminococcaceae*	Uncultured bacterium clone A1_611 from fecal sample (EU761905.1)	67/69
		Anaerofilum pentosovorans DSM 7168 (NR_029313.1)	66/69
4	*Firmicutes/Clostridia/Clostridiales/*	*Clostridium proteolyticum* DSM 3090 (NR_029250.1)	64/69
5	*Firmicutes/Clostridia/Clostridiales/*	Uncultured bacterium isolate DGGE gel band 48 from MBR-treated municipal wastewater (GQ325302.1)	67/69
		Clostridium proteolyticum DSM 3090 (NR_029250.1)	66/69
6	Unclassified	Uncultured bacterium clone 51-4-E9 from human faeces (JQ307282.1)	65/69
		Hyphomicrobium sp.16-60 (HM124367.1)	62/66
		Massilia sp. str. LP01 (HM053474.1)	63/69
7	*Firmicutes/Clostridia/Clostridiales/Ruminococcaceae*	Uncultured bacterium clone J2_3_3314 from anaerobic sludge digester (JQ170346.1)	66/69
		Acetanaerobacterium elongatum Z7 (AY487928.1)	65/69
8	*Firmicutes/Bacilli/Lactobacillales/Carnobacteriaceae/*	Uncultured bacterium clone GDIC2IK01CV7NK from methanogenic enrichments of carrot waste inoculated with rumen fluid and sediments (JF601114.1)	67/70
		Trichococcus palustris DSM 9172 (NR_025435.1)	66/69

9	*Firmicutes/Clostridia/Clostridiales*	*Clostridium proteolyticum* DSM 3090 (NR_029250.1)	65/69
10	*Proteobacteria/Betaproteobacteria/Burkholderiales/Oxalobacteraceae*	Uncultured bacterium clone T1_5_152 from anaerobic sludge digester (JQ169260.1)	61/65
		Antarctic bacterium strain CC9 (EU636039.1)	60/65
11	*Firmicutes/Clostridia/Clostridiales/Ruminococcaceae*	*Acetanaerobacterium elongatum* Z7 (AY487928.1)	63/66
12	Unclassified	Uncultured organism clone SRM_OTU21940 from reindeer rumen (JN803749.1)	63/66
		Massilia sp. LP01 (HM053474.1)	60/66
		Herbaspirillum sp. CCBAU 10823 (HM107176.1)	60/66
13	Unclassified	Uncultured organism clone SRM_OTU21940 from reindeer rumen (JN803749.1)	64/66
		Peredibacter starrii DSM 17039 (NR_024943.1)	61/66
		Desulfotomaculum sp. cs1-2 (EU251186.1)	60/64

B

TGGE Band #	Closest taxonomic affiliation (Phylum/Class/Order/Family)	Most similar described organisms & database accession #	Overlap (nt)
1	*Firmicutes*	*Pelosinus fermentans* DSM 17108 (JF750002.1)	62/64
		Clostridium puniceum DSM 2619 (NR_026105.1)	63/66
2	*Firmicutes/Clostridia/Clostridiales/Clostridiaceae/*	Uncultured bacterium clone CF6327 from faeces (GU606315.1)	63/66
		Clostridium bovipellis B30 (EF512134.1)	62/66
3	*Firmicutes/Clostridia/Clostridiales*	Uncultured bacterium clone SHPD-19 from underground water (JQ757022.1)	133/133
		Psychrosinus fermentans FCF9 (DQ767881.1) *	131/133
		Pelosinus sp. BXM (HM768898.1)	126/133
		Sporotalea propionica TM1 (FN689723.1)	126/133
4	*Firmicutes/Clostridia/Clostridiales/Lachnospiraceae*	Uncultured *Firmicutes* bacterium clone Ola1.D12.invm13r from coastal water (AB691190.1)	105/105
		Robinsoniella peorensis 108 (JN642223.1)	104/105
		Hespellia porcina NRRL B-23458 (NR_025206.1)	103/106
5	Unclassified	Uncultured bacterium clone GB7N87003GWHWO from unvegetated soil environments on Anchorage Island (HM725836.1)	62/66
		Hyphomicrobium sp. 16-60 (HM124367.1)	61/66
		Clostridium bovipellis B30 (EF512134.1)	61/66
6	*Proteobacteria/Alphaproteobacteria/Rhizobiales/Hyphomicrobiaceae*	Uncultured bacterium clone 100-BAC057 from Grasse River sediment (JQ968741.1)	62/66
		Hyphomicrobium sp. 16-60 (HM124367.1)	61/66

* not validated species