Polyphasic study of *Zymomonas mobilis* strains revealing the existence of a novel subspecies *Z. mobilis* subsp. *francensis* subsp. nov., isolated from French cider

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Zymomonas mobilis strains recently isolated from French 'framboisé' ciders were compared with collection strains of the two defined subspecies, *Z. mobilis* subsp. *mobilis* and *Z. mobilis* subsp. *pomaceae*, using a polyphasic approach. Six strains isolated from six different regions of France were compared with three strains of *Z. mobilis* subsp. *mobilis*, including the type strain LMG 404^T, and four strains of *Z. mobilis* subsp. *pomaceae*, including the type strain LMG 448^T, using phenotypic and genotypic methods. For phenotypic characterization, both physiological tests and SDS-PAGE protein profiles revealed significant differences between the two known subspecies and the French isolates; three distinct groups were observed. These findings were further confirmed by random amplified polymorphic DNA and repetitive extragenic palindromic-PCR genotyping methods in which the French isolates were clearly distinguished from the other two subspecies. Sequence analysis of a fragment ranging from 604 to 617 nucleotides corresponding to the 16S–23S rRNA gene intergenic spacer region (ISR), a 592 nucleotide HSP60 gene fragment and a 1044 nucleotide *gyrB* gene fragment confirmed the presence of three distinct groups. The French strains exhibited almost 94 % similarity to the ISR, 90 % to HSP60 and 86 % to *gyrB* sequences of the three collection strains of *Z. mobilis* subsp. *mobilis* and 87, 84 and 80 % sequence similarity, respectively, was observed with the four *Z. mobilis* subsp. *pomaceae* strains. Based on both the phenotypic and genotypic results, the French strains are proposed to represent a novel subspecies, *Zymomonas mobilis* subsp. *francensis* subsp. nov. Strain AN0101^T (=LMG 22974^T = CIP 108684^T) was designated as the type strain.

Zymomonas mobilis, a bacterium isolated from various alcoholic beverages, can be considered as either a necessary

flora in natural fermentations (agave, palm sap) and in bioreactors (ethanol, acetaldehyde production) or as a spoilage bacterium (ciders, beers and perries) (Swings & De Ley, 1977; Buchholz *et al.*, 1987; Miyake & Shibamoto, 1993; O'Mullan *et al.*, 1995; Jespersen & Jakobsen, 1996; Coton & Coton, 2003; Herrero *et al.*, 2003). In 1977, based on phenotypic and genetic data, Swings and De Ley concluded that *Z. mobilis* was the only species in the genus and that two subspecies existed: *Z. mobilis* subsp. *mobilis* and *Z. mobilis* subsp. *pomaceae* (Swings & De Ley, 1977).

In a recent study, an amplified rDNA restriction analysis (ARDRA) method was developed to identify strains of *Z. mobilis* isolated from French 'framboisé' spoiled ciders at the subspecies level (Coton & Coton, 2003; Coton *et al.*, 2005). Consistent differences were observed in the 16S rRNA gene sequences of these French isolates in comparison with the two previously described subspecies, *Z. mobilis* subsp. *mobilis* and *Z. mobilis* subsp. *pomaceae* (previously isolated

Published online ahead of print on 9 September 2005 as DOI 10.1099/ijs.0.63732-0.

Abbreviations: HSP, heat-shock protein; ISR, intergenic spacer region; RAPD, random amplified polymorphic DNA; REP-PCR, repetitive extragenic palindromic-PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S–23S ISR sequences of *Zymomonas mobilis* strains LMG 404^T, LMG 445, AN0101^T, AN0102 and LMG 448^T are AY940084–AY940087, respectively. Those of the partial HSP60 gene sequences of strains LMG 404^T, LMG 445, LMG 448^T, LMG 5993 and AN0101^T are AY940079–AY940083, respectively, and those of the *gyrB* gene sequences for strains LMG 404^T, LMG 445, LMG 448^T and AN0101^T are AY940075–AY940078, respectively.

Figures showing the results of SDS-PAGE analysis and phylogenetic trees generated from the ISR, HSP60 and *gyrB* gene sequences are available as supplementary material in IJSEM Online.

from English spoiled ciders). These genetic differences raised questions about the taxonomy of this species and the French strains were considered to be part of a new genomovar within *Z. mobilis* subsp. *pomaceae*. In this paper, the taxonomical position of these French isolates was studied using a polyphasic approach.

Cultures of *Z. mobilis* were obtained from the DSMZ, *Z. mobilis* subsp. *mobilis* DSM 424^T and DSM 3580, or from the Belgian Coordinated Cultures of Microorganisms (BCCM), *Z. mobilis* subsp. *mobilis* LMG 404^T, LMG 445 and LMG 429 and *Z. mobilis* subsp. *pomaceae* LMG 448^T, LMG 450, LMG 451 and LMG 5993. Strain AN0101^T (= LMG 22974^T = CIP 108684^T) was designated as the type strain of the novel subspecies *Z. mobilis* subsp. *francensis* described in this study. Other strains (AN0102, AN0108, AN0202, AN0205 and AN0206) were part of the ADRIA NORMANDIE collection isolated from 'framboisé' spoiled ciders from different regions of France over the past 4 years. All strains were grown in either liquid medium (20 g glucose l⁻¹ and 5 g yeast extract l⁻¹) overnight or anaerobically on zymomonads-pimaricine (ZP) agar (Coton & Coton, 2003) for 5 days at 30 °C.

Morphological, physiological and biochemical characterizations of the French cider strains were performed as described by Swings & De Ley (1977) unless otherwise stated. All strains had Gram-negative, rod-shaped cells arranged singly or in pairs. Isolated colonies appeared white- to cream-coloured, brilliant, regularly edged and had a characteristic central peak. Motility was observed using phase-contrast microscopy for all French isolates after 5 days incubation under anaerobic conditions at 30 °C. One to two polar flagella were observed using the staining method described by Heimbrook *et al.* (1989). Cells were catalase-positive and oxidase-negative. Strains were tested for carbohydrate fermentation abilities using the API 50 CHE and API 20E systems (bioMérieux). All strains were negative for urease, gelatinase, H₂S, lysine decarboxylase and ornithine decarboxylase. The Voges-Proskauer reaction (acetoin formation) was weak or negative and nitrates were not reduced. Two strains were arginine dihydrolase positive. All strains metabolized glucose, fructose and sucrose; assimilation of other substrates (galactose, D-mannose, mannitol, D-raffinose, gluconate and 5-ketogluconate) was weak and strain-dependent. Growth was also observed in liquid medium supplemented with 5 % ethanol and from pH 3.5 to 7.5. All strains were able to grow to stationary phase in the presence of 20 and 25 % (w/v) glucose after 2 and 6–14 days (depending on the studied strain), respectively. No growth was observed at 40 % (w/v) glucose after 20 days or in 1 % (w/v) peptone broth after 72 h.

Under the growth conditions described by Swings & De Ley (1977) for differentiation of subspecies (36 °C, 0.5 % NaCl and 0.2 % bile salts) as well as in a medium containing 100 g sucrose l⁻¹ as a sole carbon source and 5 g yeast extract l⁻¹ (Skotnicki *et al.*, 1981), phenotypic differences were observed. All tests showed a clear distinction between *Z. mobilis* subsp. *pomaceae* strains and *Z. mobilis* subsp. *mobilis*

strains. However, in the presence of NaCl, bile salts and at 36 °C, the French isolates showed very similar growth patterns to *Z. mobilis* subsp. *pomaceae* strains (no or slight growth). In the presence of 100 g sucrose l⁻¹, the strains behaved like *Z. mobilis* subsp. *mobilis* strains (normal growth). Thus, these physiological tests enabled three groups to be distinguished. Similar phenotypic characteristics were observed for the French strains and the two known subspecies, showing that they are strongly related phenotypically to both subspecies and not only to *Z. mobilis* subsp. *pomaceae* as was previously observed based on phenotypic data (Coton & Coton, 2003; Coton *et al.*, 2005).

To study the phenotypic differences observed between the three groups further, SDS-PAGE was performed in triplicate using whole-cell protein extracts obtained from overnight cultures. Cultures were grown to stationary phase, 10 ml was centrifuged at 5000 r.p.m. and the cell pellet was resuspended in 25 mM Tris buffer. An equivalent volume of glass beads was added to each sample and the mixture was vortexed in a Retsch MM20 bead beater (Retsch) for 3 min at maximum speed followed by 3 min on ice. This step was repeated twice. The mixture was then centrifuged at 12 000 r.p.m. for 10 min and the supernatant was transferred to a new tube. The Bradford assay was used to determine protein concentrations and 12 % SDS-PAGE gels were used for migration (80 V for 1.5 h) of the protein extracts using a Mini Protean system (Bio-Rad) and stained with Coomassie blue. Comparison of the gel lanes showed that three distinct groups were present, corresponding to the two known subspecies and to the French isolates (see Supplementary Fig. S1 in IJSEM Online). As whole-cell extracts were used, a large number of bands was observed corresponding to soluble proteins present in the bacteria; however, certain zones were clearly characteristic. For *Z. mobilis* subsp. *mobilis*, as well as for the French isolates, very few bands were observed just above 20 kDa, whereas this zone contained two distinct bands for the *Z. mobilis* subsp. *pomaceae* group. Furthermore, just below 20 kDa, the *Z. mobilis* subsp. *pomaceae* group was lacking two major bands that were present distinctly in the other two groups. For the French strains, a distinct banding pattern was observed around 25 kDa in comparison with the two other subspecies. Other distinct differences in protein profiles were also observed amongst the three groups near 37 and 50 kDa. This phenotypic characterization confirmed the findings that all the French strains are phenotypically related to each other, but also clearly demonstrated significant differences between the three groups. These results were confirmed for all other SDS-PAGE gels and all strains gave the same banding patterns on each gel that was run (data not shown).

For genetic characterization, genomic DNA was purified using the GenElute bacterial genomic DNA extraction kit (Sigma) according to the manufacturer's instructions. The concentration of purified DNA was determined using a spectrophotometer (SmartSpec 3000; Bio-Rad). Both random amplified polymorphic DNA (RAPD) analysis

Fig. 1. RAPD fingerprints produced with primer 1 for the studied strains. The tree was constructed using the Pearson correlation and UPGMA.

and repetitive extragenic palindromic-PCR (REP-PCR) were performed on the extracted DNA samples. All PCR products were separated on a 2% (w/v) agarose gel and visualized by ethidium bromide staining using GelDoc 2000 (Bio-Rad). Analysis of the banding patterns was performed using the BIONUMERICS program (Applied Maths).

To study strain biodiversity, genomic fingerprints of the French isolates and collection strains were obtained by RAPD analysis using the Ready-to-go RAPD analysis kit (Amersham) according to the manufacturer's instructions with 50 ng purified genomic DNA and primer 1 (Fig. 1). Analysis of the banding patterns showed that the strains clustered into four well-defined groups that clearly separated *Z. mobilis* subsp. *mobilis* strains into two subgroups that were different from both of the *Z. mobilis* subsp. *pomaceae* strains and from the French strains. Few common bands were observed between these clusters. Within the fourth group, comprising the French strains, nine common bands were observed between strains isolated from different cider-producing regions. However, some bands allowed for subtyping of these strains. This result indicates that the French strains are closely related, but demonstrate strain biodiversity. For the *Z. mobilis* subsp. *mobilis* strains, the two subgroups consisted of strains LMG 429 and LMG 404^T in the first group and strain LMG 445 in the second. The presence of two subgroups is in accordance with observations made by Swings & De Ley (1977) based on genome DNA relatedness. They stated that two subgroups of almost identical *Z. mobilis* subsp. *mobilis* strains coexisted and that the first group corresponded to 28 Zaïrese palm wine strains, including LMG 445, and that the second group

corresponded to three Zaïrese palm wine strains, four infected British beer strains (including LMG 429) and one strain from Mexican pulque (LMG 404^T).

To study genomic differences further, REP-PCR was performed according to Versalovic *et al.* (1991) using either the BOX1A primer or the primer pair ERIC1R/ERIC2 in the presence of approximately 50 ng purified genomic DNA. Banding patterns were analysed and revealed the presence of four distinct clusters (Fig. 2). Two clusters corresponded to *Z. mobilis* subsp. *mobilis* strains, one to *Z. mobilis* subsp. *pomaceae* strains and the fourth to the French isolates. For the two *Z. mobilis* subsp. *mobilis* clusters, they again corresponded to LMG 429/LMG 404^T and to LMG 445.

In general, within each of the four clusters, all banding patterns were similar and therefore did not allow for strain-specific fingerprinting. On the other hand, this technique allowed for analysis below the species level and clearly showed that the French strains belong to a distinct group. As with the RAPD analysis, only a few common bands were observed between groups, although within each cluster up to 15 common bands were present.

Comparison of REP-PCR and RAPD showed that RAPD was a more suitable molecular tool to study biodiversity at the strain level; however, both techniques were able to generate genomic fingerprints below the species level, making them suitable to study taxonomy and to obtain rapid identification of isolates.

To determine the phylogenetic relatedness of the strains studied, the 16S–23S rRNA gene intergenic spacer region

Fig. 2. REP-PCR fingerprints produced with the ERIC1/ERIC2 primer pair (a) or the BOX primer (b) for the studied strains. The tree was constructed using the Pearson correlation and UPGMA.

(ISR), as well as housekeeping gene sequences [60 kDa heat-shock protein (HSP60) and subunit B protein of DNA gyrase (*gyrB*)] were compared as recommended by Stackebrandt *et al.* (2002). Amplification and sequencing of the ISR was carried out using the primers Z16pr1436 (5'-GCGACTGG-GGTGAAGTCG-3'), targeting the 3' end of the 16S rRNA gene, and a modified universal primer named BLR187 (5'-TACTTAGATGTTTCAGTTTCGC-3'), targeting the 5' region of the 23S rRNA gene of eubacteria. Approximately 50 ng genomic DNA was used with the following amplification conditions: 95 °C for 5 min, 30 cycles of 95 °C for 45 s, 48 °C for 45 s and 72 °C for 1 min, with a final extension of 72 °C for 5 min. For the *gyrB* gene, universal primer pairs for amplification (UP-1/UP-2r) and direct sequencing (UP-1S/UP-2Sr) (Yamamoto & Harayama, 1995) were used with the following amplification conditions: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 5 min. For HSP60, degenerate primers were created, HSP-for (5'-GACAAGTTCGAAAAYATGGG-3') and HSP-rev (5'-GGCTTYGGYG-ATCGYCGYAA-3'), and used in PCR with the following amplification conditions: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, with a final extension 72 °C for 5 min. Samples of 18 µl of each PCR were run on 0.8 % (w/v) agarose gels and visualized as described above. PCR products were purified using the GenElute PCR purification kit (Sigma) according to the manufacturer's instructions and air-dried. Samples were then sent for sequencing to MWG Biotech. Sequence comparisons and analysis were performed with the BLAST (Altschul *et al.*, 1990), CLUSTAL_X (Thompson *et al.*, 1997) and BIONUMERICS (Applied Maths) programs.

ISR sequences, situated between the 16S and 23S rRNA genes and ranging from 604 to 617 bp, were determined for all of the strains studied and representative sequences were deposited in GenBank. Comparisons of the aligned sequences using a global cluster analysis, UPGMA and 1000 bootstraps showed the presence of three distinct groups corresponding to the two known subspecies and the French isolates (see Supplementary Fig. S2 in IJSEM Online). For the French strain sequences, one mismatch was observed for two of the six strains, AN0102 and AN0202, while all *Z. mobilis* subsp. *pomaceae* strains were 100 % identical. On the other hand, within the *Z. mobilis* subsp. *mobilis* cluster, more sequence diversity was observed with up to seven mismatches being observed between the four strains studied. Sequences for the French strains showed >94 % similarity to the *Z. mobilis* subsp. *mobilis*, while only ~87 % similarity was observed to those of *Z. mobilis* subsp. *pomaceae* showing that the French isolates are more closely related to the *Z. mobilis* subsp. *mobilis* cluster at a genetic level.

Partial HSP60 gene sequences (592 bp) were obtained for all strains studied and representative sequences were deposited in GenBank. Sequence alignments showed that three distinct clusters were present (see Supplementary Fig. S3 in IJSEM Online). Complete sequence identity was found between all

French strains. Within the *Z. mobilis* subsp. *pomaceae* subspecies, two mismatches were observed between the LMG 448^T sequence and the three other sequences. Three closely related sequence subgroups were also observed for the *Z. mobilis* subsp. *mobilis* cluster, merging at >99 %. Overall, strong genetic discrimination was observed for these three groups, with French strains showing almost 90 % similarity with the *Z. mobilis* subsp. *mobilis* group and almost 84 % with the *Z. mobilis* subsp. *pomaceae* cluster.

For *gyrB*, 1044 bp sequences were obtained for all of the studied strains and representative sequences were deposited in GenBank. The resulting phylogenetic tree confirmed the above findings that the French strains are more closely related from a genomic point of view to *Z. mobilis* subsp. *mobilis* (86 % similarity versus 80 % with *Z. mobilis* subsp. *pomaceae* strains) (see Supplementary Fig. S4 in IJSEM Online). Again, strong genetic discrimination was observed using this housekeeping gene which allowed differentiation of the French isolates from the two previously described subspecies.

The use of the subspecies differentiation tests of Swings & De Ley (1977) showed that the French isolates seemed to belong to *Z. mobilis* subsp. *pomaceae*. However, the addition of a growth test using sucrose as a sole carbon source showed that the French strains, which are able to utilize this sugar, are part of a distinct phenotypic group, as *Z. mobilis* subsp. *pomaceae* strains are unable to grow on this substrate (Skotnicki *et al.*, 1981). SDS-PAGE protein profiles further confirmed this finding. In genotyping experiments, distinct genomic fingerprints were obtained and these confirmed that the French isolates were clearly different from the two subspecies described by Swings & De Ley (1977). RAPD analysis enabled strain biodiversity to be observed amongst the French isolates. Previous sequence analysis based on 16S rRNA genes showed more than 98 % similarity between the three groups, indicating that they all belonged to the same species, *Z. mobilis* (Coton & Coton, 2003; Coton *et al.*, 2005). In this study, sequencing of more variable targets allowed for the taxonomical positioning of the French isolates. Comparison of phylogenetic trees constructed from the 16S–23S ISR, *gyrB* and HSP60 sequences showed that the trees were in close agreement and also revealed three distinct clusters. The sequencing analysis presented in this paper clearly allows for differentiation below the species level. As previously described (Dahllöf *et al.*, 2000; Zhu *et al.*, 2003), the use of housekeeping genes to study taxonomy of closely related micro-organisms is more informative than 16S rRNA gene analysis. In this study, the sequencing of the *gyrB* and HSP60 genes proved to be a useful tool for the identification of *Z. mobilis* isolates below the species level. The results obtained in this polyphasic study proved that the isolates of *Z. mobilis* from French cider were phenotypically and genotypically different from the previously described subspecies; therefore, the French isolates are proposed as members of a novel subspecies, *Z. mobilis* subsp. *francensis* subsp. nov.

Emended description of *Z. mobilis* subsp. *mobilis* (Lindner 1928) De Ley and Swings 1976

The description is as given by De Ley and Swings (1976) with the following modifications. Sucrose is fermented. Growth at 36 °C and in the presence of 0.5 % NaCl and 0.2 % bile salts.

Emended description of *Z. mobilis* subsp. *pomaceae* (Millis 1956) De Ley and Swings 1976

The description is as given by De Ley and Swings (1976) with the following modifications. Sucrose is not fermented. No growth at 36 °C, in the presence of 0.5 % NaCl and 0.2 % bile salts.

Description of *Zymomonas mobilis* subsp. *francensis* subsp. nov.

Zymomonas mobilis subsp. *francensis* (fran.cen'sis. N.L. fem. adj. *francensis* of, or belonging to, France).

All strains meet the species description. Motile. Sucrose is fermented. No growth or slight growth at 36 °C and in the presence of 0.5 % NaCl; no growth in the presence of 0.2 % bile salts. All strains grow in 25 % glucose, but not in 40 %. Urea is not hydrolysed and H₂S is not produced. Acetoin is formed weakly by some strains. Lysine- and ornithine decarboxylase-negative. Some strains are arginine dihydrolase-positive. Some strains are able to metabolize galactose (weak), D-mannose, mannitol, D-raffinose, gluconate and 5-ketogluconate.

The type strain, AN0101^T (=LMG 22974^T=CIP 108684^T), was isolated from French 'framboisé' cider.

Acknowledgements

We are grateful to Jean Euzéby for support with the nomenclature. This study was supported by funding from the Conseil Régional de Basse-Normandie in the framework of Agrobio-Industries and by the European Regional Development Fund (ERDF).

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