Discovery and characterization of a new bacterial candidate division by an anaerobic sludge digester metagenomic approach

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Summary

We have constructed a large fosmid library from a mesophilic anaerobic digester and explored its 16S rDNA diversity using a high-density filter DNA–DNA hybridization procedure. We identified a group of 16S rDNA sequences forming a new bacterial lineage named WWE3 (Waste Water of Evry 3). Only one sequence from the public databases shares a sequence identity above 80% with the WWE3 group which hence cannot be affiliated to any known or candidate prokaryotic division. Despite representing a non-negligible fraction (5% of the 16S rDNA sequences) of the bacterial population of this digester, the WWE3 bacteria could not have been retrieved using the conventional 16S rDNA amplification procedure due to their unusual 16S rDNA gene sequence. WWE3 bacteria were detected by polymerase chain reaction (PCR) in various environments (anaerobic digesters, swine lagoon slurries and freshwater biofilms) using newly designed specific PCR primer sets. Fluorescence in situ hybridization (FISH) analysis of sludge samples showed that WWE3 microorganisms are oval-shaped and located deep inside sludge flocs. Detailed phylogenetic analysis showed that WWE3 bacteria form a distinct monophyletic group deeply branching apart from all known bacterial divisions. A new bacterial candidate division status is proposed for this group.

Introduction

A limiting step in understanding any microbial ecosystem resides in our ability to inventory the microorganisms inhabiting the ecosystem, and to assess their metabolic potential, the interactions between them and their biotope. A partial answer to this challenge was (i) culture-independent studies based on the development of molecular microbial diversity analyses using the 16S rDNA gene as a phylogenetic marker (Olsen et al., 1985; Woese, 1987; Ludwig and Schleifer, 1999), and (ii) the development of metagenomic studies of complex ecosystems. Large-scale sequencing efforts in various ecosystems such as a community from acid mine drainage (Tyson et al., 2004; Baker et al., 2006), the Sargasso Sea (Venter et al., 2004) and the Global Ocean Sampling expedition (Rusch et al., 2007) have considerably enriched our understanding of uncultured microbial communities. These studies made it possible to link phylogeny and function, revealing a surprising abundance of different types of genes, and enabled the reconstruction of genomes of organisms that have not been cultured to date (Erkel et al., 2006).

Anaerobic sludge digesters are complex ecosystems in which a consortium of microorganisms degrades organic matter into methane and carbon dioxide under anaerobic conditions. The organisms involved are still awaiting species diversity and metabolic characterization. Recent studies of the diversity of wastewater microbial communities based on the analysis of 16S rDNA sequences have extended our knowledge about the diversity of this ecosystem (Godon et al., 1997; Chouari et al., 2003; 2005a,b). Moreover, the recent discovery of the new WWE1 (Waste Water of Evry 1) candidate division by
molecular inventories of the anaerobic digester of Evry (Chouari et al., 2005b) has shown that additional bacterial and archaeal populations remain to be described. In the present study, we report that in the course of analyzing a metagenomic fosmid library constructed from an anaerobic digester, we detected an unusual group of 16S rDNA bacterial gene sequences. These sequences, which presented many mismatches with the 16S rDNA universal primers, cannot be obtained by the classical polymerase chain reaction (PCR)-based amplification methods. Specific 16S rDNA PCR primers were developed for this group of sequences, named WWE3. Phylogenetic analyses show that this group constitutes a new bacterial candidate division. Fluorescence in situ hybridization (FISH) experiments helped to provide information about morphology and localization of the WWE3 bacteria within a microbial anaerobic sludge sample. Furthermore, the absence of the H17 helix in the WWE3 16S rDNA secondary structures is unprecedented and seems to be a characteristic of the bacterial candidate division WWE3 and its closest relatives.

Results

Metagenomic clone library construction and screening, fosmid sequencing and primer design

In order to analyse the microbial diversity and the metabolic potential of a mesophilic anaerobic digester, a large fosmid library was constructed using DNA extracted from the sludge digester of the WWTP of Evry, France. A part of the library (27 648 fosmid clones) was screened by hybridization with 16S rDNA gene targeted-hybridization probes. The 16S rDNA genes of 570 positive clones were directly sequenced using internal primers. While for 541 of these positive clones, the 16S rDNA gene sequences were obtained and affiliated to known bacterial or archaeal phyla, we were unable to obtain a 16S rDNA sequence for 29 clones. Analysis of HindIII fingerprints of these clones showed that their profiles were very similar. Southern blot hybridization using 16S rDNA-targeting probes revealed that 27 out of the 29 clones showed a common 1.6 kb HindIII positive fragment while the remaining two clones possess a positive 1.65 kb fragment. Shotgun sequencing of one of these 29 fosmid clones (DIGA11YD11) revealed that it does contain a complete 16S rDNA gene sequence which affiliates (88% identity) with a single sequence (AY953190) in public databases. The 16S rDNA sequences of the remaining 28 fosmids were determined by direct sequencing with specific primers derived from the DIGA11YD11 16S rDNA (Table 1). All these 16S rDNA gene sequences share more than 99% identity. Two of them (fosmids DIGA75YB16 and DIGA43YA13; corresponding to those presenting the 1.65 kb positive HindIII hybridization fragment) have a 65 bp insertion (type I insertion). The 29 16S rDNA gene sequences present at least two mismatches with the commonly used 16S rDNA PCR and sequencing primers used in the study (Table 2). The presence of these mismatches could explain the failure to obtain their 16S rDNA sequence as well their absence in public databases.

The extent and diversity of WWE3 representatives

In order to investigate the presence and the diversity of the WWE3 phylogenetic group, specific PCR primers targeting different regions of the DIGA11YD11 16S rDNA
Phylogenetic analysis

In order to solve the WWE3 phylogenetic position, phylogenetic analysis were conducted using 16S rDNA sequences representing the major bacterial divisions, along with WWE3 sequences. Results show that, independently of the method used for tree construction (distance, parsimony and maximum likelihood using ARB, PAUP and PhyML software) and beyond the number of sequences included in the analysis, WWE3 sequences form a monophyletic group, branching distinctly apart from all bacterial and archaeal divisions, with OP11 and WS6 candidate divisions as their closest relatives (data not shown).

In order to refine the WWE3 position, phylogenetic analyses were carried out using representatives of the nine WWE3 OTUs and representatives of OP11, WS6, OD1 and TM7 bacterial candidate divisions. Beyond the method used for tree construction, WWE3 sequences always form a monophyletic group within the OP11-WS6-OD1 cluster (Fig. 1).

The WWE3 division encompasses the swine lagoon clone AY953190 and the partial 16S rDNA gene sequence (713 bp) of the ‘uncultured archaeon’ clone AJ556482 (Wu et al., 2006) (this result was supported by a bootstrap value of 100%, data not shown). Based on conducted phylogenetic analysis, we proposed the affiliation of the partial 16S rDNA gene sequence of clone AJ556482 to the WWE3 candidate division. AY953190 and AJ556482 shared an average of 88.0% and 82.4% sequence identity, respectively, with WWE3 representatives. The sequences AB193897 and KY193166 showed 76% average identity with WWE3 sequences and corresponded to the nearest relatives of this division (Fig. 1).

Clonal library analysis

A total of 21 16S rDNA libraries were constructed from 12 DNA samples extracted from eight anaerobic biofilm samples, using different primer sets (sets 1, 2, 5, 6 and 7, Table 2). A total of 1639 sequences were imported into the ARB database (Ludwig et al., 2004) and analysed. These sequences were grouped into nine operational taxonomic units (OTU), using a 97% identity cut-off (Stackebrandt and Goebel, 1994) (Fig. 1). Among these OTUs, we noticed that OTU-1 encompasses 91% of the sequences obtained in the study and that representatives of this OTU were recovered exclusively from anaerobic sludge digesters, with all the PCR primer sets used. A 65 bp insertion (type I) was found within 28% of the OTU-1-related 16S rDNA sequences.

An additional 61 bp insertion (type II) was found in the OTU-4 sequences. The two insertions (I and II) are located within the same region but exhibit completely different sequences. The overall intradivergence between WWE3 16S rDNA gene sequences reached 20% and is in the same order of magnitude as that for other uncultured bacterial candidate divisions [e.g. 29% for the OP11 candidate division, 27% for the OD1 candidate division and 15% for the SR1 candidate division (Harris et al., 2004)].

Clone library analysis

A total of 21 16S rDNA libraries were constructed from 12 DNA samples extracted from eight anaerobic digesters (Table 3), one swine lagoon and three freshwater biofilm samples, using different primer sets (sets 1, 2, 5, 6 and 7, Table 2). A total of 64 different DNA samples (Table 3 and Experimental procedures) were tested using the four DIGA11YD11-specific primer sets 1–4 (Table 1). Polymerase chain reaction amplification products were obtained from 20 anaerobic digesters located in different countries in Europe and America as shown in Table 3. Cloning and sequencing of part of these PCR products confirmed their affiliation to the WWE3 group (see bottom).

To explore the extent of diversity of this novel group of 16S rDNA sequences, we proceeded in two steps: (i) 16S rDNA libraries were constructed from DNA extracted from seven anaerobic digesters, using the DIGA11YD11-specific primers or a combination of specific and universal primers, (ii) sequence analysis of these 16S rDNA confirmed their inclusion in the WWE3 phylogenetic group and allowed us to design degenerate primers with a broader specificity. The presence of WWE3 bacteria was further investigated and confirmed in other anaerobic or anoxic environments such as swine lagoon slurries (6/10 samples) and freshwater biofilms (6/6 samples). The presence of WWE3 bacteria was tested by PCR on DNA sampled from three digesters over a 6-year period (2000–2006). During this period, WWE3 bacteria were detected in 16 out of 23 Evry sludge samples, three out of six from Corbeil and in all three sludge samples from Creil, showing that the WWE3 population size is subject to large variations in these anaerobic digesters.
In order to investigate the specific WWE3 branching, another phylogenetic analysis was undergone using the radA gene found in the DIGA11YD11 insert. As previously shown, recA and radA (subfamily member of the recA group) can be used as valid gene markers for bacterial and archaeal phylogeny (Eisen, 1995; Sandler et al., 1999). Results showed that WWE3-radA gene clusters with the bacterial radA genes (Fig. S1), but did not belong to any recognized bacterial division.

### Table 3. Characteristics of the anaerobic digesters that were tested for the presence of WWE3 bacteria.

| Country     | Digesters          | WWE3a | Scaleb | Processc | Effluentd |
|-------------|--------------------|-------|--------|----------|-----------|
| Canada      | Montreal           | –     | I      | CST      | W         |
|             | Montreal           | –     | L      | UASB     | Phenolic compounds |
| Chile       | El Trebal          | –     | I      | CST      | W         |
| Czech Republic | Brno              | –     | I      | CST      | W + 30% dairy, food industry |
|             | Zabreh             | –     | I      | CST      | W + 30% dairy, food industry |
| France      | Aix en Provence I, II | –     | I      | CST      | W         |
|             | Asnières sur Oise  | +     | I      | CST      | W         |
|             | Carré de la réunion | +     | NA     | NA       | NA        |
|             | Cholet*            | +     | I      | CST      | W + 9% slaughterhouse |
|             | Clos de Hilde      | –     | I      | CST      | W         |
|             | Conneré            | +     | I      | FBR      | Cassoulet and sauerkraut |
|             | Corbeil*           | +     | I      | CST      | W         |
|             | Creil*             | +     | I      | CST      | W         |
|             | Evy*               | +     | I      | CST      | W         |
|             | Haguenau           | –     | I      | CST      | W + 30% mechanical, food industry |
|             | La Roche sur Foron | –     | I      | CST      | W + 50% food industry |
|             | Les Mureaux        | –     | P      | CST      | W         |
|             | Marseille          | –     | I      | CST      | W         |
|             | Marseille          | –     | I      | CST      | W         |
|             | Montandon          | –     | P      | SBR      | Pig slurry |
|             | Narbonne           | –     | L      | SBR      | Pig slurry |
|             | Narbonne           | –     | L      | SBR      | Pig slurry |
|             | Narbonne           | +     | L      | UASB     | Lignin    |
|             | Narbonne           | –     | P      | FBR      | Vinasses  |
|             | Narbonne           | –     | L      | SBR      | Vinasses  |
|             | Rochefort          | –     | I      | CST      | W + 12% industry, heavy metals |
|             | St.Laurent de Cognac | –     | I      | FB       | Acidogenic vinasses |
|             | St.Laurent de Cognac | –     | I      | FB       | Acidogenic vinasses |
|             | St.Laurent de Cognac | +     | I      | CST      | Lees vinasses |
| Germany     | Goslar             | +     | I      | CST      | W         |
|             | Manheim*           | +     | I      | CST      | W + 50% paper industry |
|             | Mulheim            | –     | I      | CST      | W + green wastes |
|             | Rostock            | +     | I      | CST      | W + 28% food industry |
| Ireland     | Cork               | +     | I      | UASB     | Citric acid from beet molasses production |
| Italy       | Casolino I, II     | +     | I      | CST      | W + 15% industry, heavy metals |
| Mexico      | Culiacan           | –     | I      | CST      | W         |
|             | Mexico city        | +     | L      | UASB     | Rum vinasses |
|             | Mexico city        | –     | L      | FBR      | Rum vinasses |
|             | Mexico city        | –     | L      | UASB     | Yeast factory |
|             | Puebla             | –     | I      | CST      | W + 20% textile, colouring industry |
| Spain       | Blanes             | –     | I      | CST      | W         |
|             | Hoya de Lorca      | –     | I      | CST      | W + 70% industrial heavy metals, oil, greases |
|             | Palencia*          | +     | I      | CST      | W + food industry |
|             | Roquetas de mar    | –     | I      | CST      | W + 15% paper industry |
|             | Vic*               | +     | I      | CST      | W + 40% industry, heavy metals |
| Switzerland | Bliten             | +     | I      | CST      | W + 40% paper, food, textile industry |
| UK          | Stressholme        | –     | I      | CST      | W         |

a. Polymerase chain reaction detection of WWE3 bacteria was performed using the DIGA11YD11-specific primer sets 1, 2, 3 and 4.
b. I: industrial scale; L: laboratory scale; P: pilot scale.
c. CST, continuously stirred tank, FB, fixed biofilm, FBR, fluidized bed reactor, UASB, up-flow anaerobic sludge blanket.
d. W, wastewater.
e. Used for 16S rDNA library construction.

All the digesters operated at mesophilic temperature, usually 37°C, except Zabreh and one of the digesters of Aix-en-Provence which operates at thermophilic conditions. The Casolino I sample was obtained when the digester was operating at 37°C and sample II after the switch of the temperature to 31°C.

NA, not available.
Fig. 1. Maximum-likelihood phylogenetic tree showing the relationship of the environmental WWE3 sequences to representatives of the OP11, WS6, OD1 and TM7 divisions. Sequences were aligned with the ARB database and software package. Aligned sequences were analysed by three methods (BioNJ, maximum likelihood and maximum parsimony) provided by PAUP 4.0b10 as described in the text. A total of 1176 homologous positions was used for tree construction. The numbers at the nodes indicate the percentage of recovery of relevant branch points in 100 bootstrap re-samplings. The Anabaena circinalis 16S rDNA sequence was used as the outgroup to define the root of the tree. The scale bar represents the 10% estimated difference in nucleotide sequence positions.
Ribosomal RNA secondary structures

Secondary structures of the DIGA11YD11 16S ribosomal RNA (rRNA) as well as one representative of each of the nine WWE3 OTUs were calculated. Except for a limited number of supplementary nucleotides, the overall secondary structure of WWE3 16S rRNA was almost homologous to the archetypal 16S rRNA structure (Gutell et al., 1994), characteristic of architecture conservation through evolutionary changes (Fig. 2). When comparing the DIGA11YD11 16S rRNA structure with the archetypal Escherichia coli K12 16S rRNA structure (Cole et al., 2005), we observed that a large number of covariant modifications affect both bases of a number of stem base pairs rather than single bases alone. Multiple nucleotide loop variations were also observed. Several regions of the structure are clearly less subject to changes than others, mainly H16/H18, H23/H24, H27 and H34 compared with H6 and H10. We observed the absence of the entire H17 helix for all the WWE3 16S rRNA structures. The function of H17, which interacts with the S4 and S16 ribosomal proteins (Brodersen et al., 2002; Schuwirth et al., 2005), is not clear. Sequence analysis showed that only two 16S rDNA sequences (AY953190 and AB193897) from the RDP release 9.36 lack this H17 helix (data not shown).

The lack of H17 was reported in another sequence, AY193166 (absent from the RDP II database), and classified as a member of the WS6 division by Harris and colleagues (2004). Thus, the absence of this helix appears as a characteristic of all members of WWE3 candidate division and some unclassified closest relatives. The H10 region is characterized for some members of the WWE3 division belonging to OTU-1 and OTU-4 by insertions of type I and II (65 and 61 bp respectively) while the H6 subdomain is extremely variable between WWE3 representatives but is conserved in all structures as a coaxial stacked helix.

Fosmid annotation

The complete DIGA11YD11 clone sequence was obtained using standard shotgun strategy. The 39 kb DIGA11YD11 fosmid presents a low percentage of G+C (36.13%). Thirty-one predicted protein-coding sequences and five RNA-coding genes were annotated. Some of the predicted genes seem to be directly related to DNA or RNA metabolism and also to known enzymatic functions involved in glucose metabolism and membrane transport; the others correspond to hypothetical proteins (Table 4). No conclusions regarding specific metabolism of WWE3 organisms indicating their possible role in the anaerobic sludge digestion could be inferred from the annotation of this fosmid.

FISH experiments

Fluorescence in situ hybridization analysis using the DIGA11YD11-21-Cy3-labelled probe was performed on Evry digester sludge samples. Microscopic observations using a confocal laser scanning microscope revealed that DIGA11YD11-21-Cy3-positive cells are oval-shaped and are usually observed to form aggregates located inside sludge flocs (Fig. 3). No hybridization signals were recorded when nonsense DIGA11YD11-21-Cy3-labelled probe was used as non-specific hybridization control. It should, however, be noted that standard FISH protocols were employed in our experiments, including PFA fixation and centrifugation steps that might have interfered with floc structure.

As expected, no hybridization signal was obtained using the EUB-mix (a mixture of probes EUB-338 I, II and III) as 16S rDNA gene sequences of members of the WWE3 bacteria show at least two mismatches with EUB-338 probes. Superposition of SYTO 9 and WWE3-specific probes showed an unusual ring-shaped localization pattern of the probe-specific hybridization signals, suggesting a hypothetical compartmentalization of the cytoplasm. Interestingly, analogous arrangements have already been reported for members of the Planctomycetales (Fuerst, 2005) and Poribacteria divisions (Fieseler et al., 2004).

The size of the WWE3 population exhibited extensive variation from sample to sample. Moreover, for a given sample, unequal distribution of probe-positive cells from floc to floc was recorded. Overall, the estimated proportion of WWE3 population varied from undetectable levels to up to 5% of the SYTO 9-stained biomass.

Discussion

During the last few decades, rRNA gene sequence comparison has been the classical way of examining microbial
A new bacterial candidate division: WWE3
Table 4. Annotation of the DIGA11YD11 predicted genes using the MaGe annotation system.

| Label       | Begin | End  | Gene     | Product                                                                 | EC number | Cellular role                          |
|-------------|-------|------|----------|--------------------------------------------------------------------------|-----------|----------------------------------------|
| WVE3-TFM_1  | 58    | 2190 | WWE3-TFM_1 | Putative UvrD/REP helicase                                                |           | DNA metabolism                         |
| WVE3-TFM_2  | 2202  | 3302 | WWE3-TFM_2 | Putative DNA recombination protein                                         |           | DNA metabolism                         |
| WVE3-TFM_3  | 3337  | 3702 | WWE3-TFM_3 | Hypothetical protein                                                      |           | DNA metabolism                         |
| WVE3-TFM_4  | 3781  | 5196 | WWE3-TFM_4 | Methionyl-tRNA synthetase (Metionine-tRNA ligase) (MetRS)                 | 6.1.1.10  | Protein synthesis                      |
| WVE3-TFM_5  | 5214  | 6134 | WWE3-TFM_5 | Putative S-3’ exonuclease                                                 |           | DNA metabolism                         |
| WVE3-TFM_6  | 6208  | 6861 | WWE3-TFM_6 | Endonuclease III [DNA-(apurinic or apyrimidinic site) lyase]               | 4.2.99.18 | DNA metabolism                         |
| WVE3-TFM_7  | 6882  | 7403 | WWE3-TFM_7 | Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_8  | 7537  | 8484 | WWE3-TFM_8 | Putative sugar kinase                                                     |           | Unknown function                       |
| WVE3-TFM_9  | 8487  | 9485 | WWE3-TFM_9 | Putative transketolase C-terminal section (TK)                            | 2.2.1.1   | Central intermediary metabolism        |
| WVE3-TFM_10 | 9507  | 10115| WWE3-TFM_10| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_11 | 10120 | 10968| WWE3-TFM_11| Putative transketolase N-terminal section (TK)                            | 2.2.1.1   | Central intermediary metabolism        |
| WVE3-TFM_12 | 11277 | 12545| WWE3-TFM_12| Multifunctional protein [Ribulose-phosphate 3-epimerase; unknown domain] | 5.1.3.1   | Central intermediary metabolism        |
| WVE3-TFM_13 | 12555 | 13004| WWE3-TFM_13| Putative ribose-5-phosphate isomerase B (Phosphoriboisomerase B)          | 5.3.1.6   | Central intermediary metabolism        |
| WVE3-TFM_14 | 13001 | 13753| WWE3-TFM_14| lgt Prolipoprotein diacylglycerol                                         | 2.4.99.-  | Protein fate transferase               |
| WVE3-TFM_15 | 13770 | 14351| WWE3-TFM_15| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_16 | 14547 | 14837| WWE3-TFM_16| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_17 | 14870 | 16672| WWE3-TFM_17| Putative DNA ligase                                                       | 6.5.1.1   | DNA metabolism                         |
| WVE3-TFM_18 | 16719 | 17711| WWE3-TFM_18| Conserved hypothetical protein                                             |           | Unknown function                       |
| WVE3-TFM_19 | 18223 | 19281| WWE3-TFM_19| recF DNA replication and repair protein RecF                               |           | Unknown function                       |
| WVE3-TFM_20 | 19306 | 20187| WWE3-TFM_20| mutM Formamidopyrimidine-DNA glycosylase (Fapy-DNA glycosylase) [DNA-(apurinic or apyrimidinic site) lyase mutM] | 3.2.2.23, 4.2.99.18 | DNA metabolism                        |
| WVE3-TFM_21 | 20180 | 21304| WWE3-TFM_21| Putative glycosyltransferase                                              |           | Unknown function                       |
| WVE3-TFM_22 | 21407 | 21919| WWE3-TFM_22| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_23 | 22015 | 22503| WWE3-TFM_23| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_24 | 22518 | 24944| WWE3-TFM_24| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_25 | 25073 | 26404| WWE3-TFM_25| Conserved hypothetical protein                                             |           | Unknown function                       |
| WVE3-TFM_26 | 26575 | 28006| WWE3-TFM_26| 16S rRNA                                                             | 16S rRNA | DNA metabolism                         |
| WVE3-TFM_27 | 28621 | 29046| WWE3-TFM_27| Putative NUDIX hydrolase                                                 |           | Unknown function                       |
| WVE3-TFM_28 | 29132 | 29761| WWE3-TFM_28| Hypothetical protein                                                      |           | Unknown function                       |
| WVE3-TFM_29 | 29746 | 29997| WWE3-TFM_29| Conserved hypothetical protein                                             |           | Unknown function                       |
| WVE3-TFM_30 | 30580 | 30655| WWE3-TFM_30| tRNA-ile                                                              |           | Protein synthesis                      |
| WVE3-TFM_31 | 30876 | 31865| WWE3-TFM_31| tRNA-ile                                                              |           | Protein synthesis                      |
| WVE3-TFM_32 | 31210 | 32177| WWE3-TFM_32| tRNA-Ala                                                               |           | Protein synthesis                      |
| WVE3-TFM_33 | 32570 | 35588| WWE3-TFM_33| 23S rRNA                                                              |           | Protein synthesis                      |
| WVE3-TFM_34 | 35719 | 35837| WWE3-TFM_34| SS rRNA                                                              |           | Protein synthesis                      |
| WVE3-TFM_35 | 36123 | 37367| WWE3-TFM_35| rpsA 30S ribosomal protein S1                                           | 30S rRNA | Protein synthesis                      |
| WVE3-TFM_36 | 37367 | 38689| WWE3-TFM_36| DNA repair protein radA homologue                                        |           | DNA repair protein radA homologue     |
|             |       |      |          | (DNA repair protein sms homologue)                                       |           | DNA metabolism                         |

Diversity in natural environments (Rappé and Giovannoni, 2003). The number of 16S rDNA sequences deposited in the databases is still increasing very rapidly, as well as the number of archaeal and bacterial phyla. These 16S rDNAs were affiliated with almost one hundred bacterial divisions [Greengenes database (http://greengenes.lbl.gov) (DeSantis et al., 2006)]. More recently, metagenomic approaches applied to the study of environmental samples have led to the discovery of novel microorganisms with an important role in the biological carbon and nitrogen cycles (Treusch et al., 2005; Hallam et al., 2006; Leininger et al., 2006). A similar metagenomic approach permitted the discovery of a new archaeal division that was not previously detected when using classical primer sets (Baker et al., 2006). The primers usually used to obtain almost full-length sequences were designed on the basis of rDNA sequences from cultured organisms (Weisburg et al., 1991). However, a number of phylogenetic groups remain undetected because they show more than one mismatch with the commonly used PCR primers (Baker et al., 2003).

In this study, we tested whether new bacterial phyla could be discovered by a metagenomic approach using a DNA–DNA hybridization procedure. This approach, based...
on the sequence identity and not on PCR, allowed us to find several clones bearing a 16S rDNA representative of a newly defined bacterial candidate division we named WWE3. These WWE3 16S rDNA sequences have at least two mismatches with the commonly used PCR primers and probes, explaining in part the reason behind the quasi-absence of representatives of this group in public databases.

According to commonly accepted criteria (Hugenholtz et al., 1998; Rappé and Giovannoni, 2003; Harris et al., 2004), the WWE3 group of sequences constitutes a new bacterial candidate phylum: (i) more than three distinct WWE3 sequences were obtained from independent PCR products, (ii) WWE3 sequences are of a minimum of 1 kb in length, and (iii) phylogenetic analysis of more than 1600 nearly full-length 16S rDNA gene sequences retrieved from 12 different environmental samples showed that WWE3 sequences, when compared with representatives of known bacterial phyla, form a monophyletic group branching apart from the other bacterial divisions, with a percentage of intradivergence of 20% [the cut-off used to distinguish a new bacterial phylum being 85% according to Hugenholtz and colleagues (1998)].

Apart from WWE3 sequences, H17 deletion was only observed in sequences AB193897 and AY193166, for which affiliation is unclear.

Polymerase chain reaction screening using WWE3-specific primers documented the diversity of this bacterial candidate division and permitted the detection of bacteria from this group in a number of different ecosystems (anaerobic sludge digesters in many different locations worldwide, swine lagoon slurries and freshwater biofilms). The use of additional WWE3-PCR primers to test other terrestrial and aquatic environments may enable us to have a broader view of this phylum and provide some hints on its metabolic lifestyle.

In situ hybridization indicated that WWE3 bacteria were embedded in sludge flocs and that they may present cytoplasmic compartmentalization as has been shown for

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Fig. 3. Epifluorescence micrographs of WWE3 bacteria in sludge samples from the anaerobic digester of Evry.
A and D. Cy3-labelled DIGA11YD11-21-specific probe (red).
B. FITC-labelled Eub338 probe mix (green).
C. Colour combination of (A) and (B), WWE3 bacteria were not labelled by the Eub338 mix probe and then did not appear yellow.
E. SYTO 9 staining (green).
F. Colour combination of (D) and (E), WWE3 bacteria appear yellow.
some members of the Planctomycetales (Fuerst, 2005) and Poribacteria (Fieseler et al., 2004). Study of this compartmentalization by transmission electron microscopy will require isolation of WWE3 representatives.

In the studied sample, WWE3 has been identified as a significantly abundant group of microbes (29 out of the 570 16S rDNA-bearing fosmids) that was undetectable through PCR approach using ‘universal primers’. A metagenomic approach has thus proven to be effective in discovery of yet undescribed microbial groups. With decreasing costs, sequencing may represent a valuable alternative for a nearly exhaustive identification of prokaryotic divisions in natural environments.

Experimental procedures

Metagenomic library screening

Construction and screening of part of the fosmid metagenomic library, using genomic DNA extracted from the anaerobic mesophilic digester of Evry (France), was performed as described by Pelletier and colleagues (2008). Briefly, fosmid DNA was extracted from 27,648 clones (384 × 72) and spotted in duplicate onto 20 × 20 cm nylon membranes (Hybond N+, GE Healthcare Europe GmbH, Saclay, France). Membranes were successively hybridized with 32P-labelled 16S rDNA probes representatives of the different archaeal and bacterial lineages described in the WWTP of Evry (Chouari et al., 2003; 2005a,b). Positive clones were picked and their 16S rDNA was directly sequenced using a set of four internal primers (Table 2). For 29 clones, the 16S rDNA sequence was not obtained. HindIII fingerprints of these 29 clones were performed and Southern blot was hybridized with the same 16S rDNA-targeting probes. One of these fosmids, DIGA11YD11, was shotgun sequenced.

Sample collection

DNA was extracted from 64 different samples and tested by PCR for the presence of WWE3 bacteria. A total of 48 anaerobic sludge digesters samples are described in Table 3. Ten swine lagoon sludges as well as six freshwater biofilms were also tested. All freshwater biofilms were obtained from a river (Rû de Balory, close to Evry, France).

Primer and probe design

The 16S rDNA sequence obtained from the fosmid DIGA11YD11 was used to design PCR primers and FISH probes. All possible 18 bp oligonucleotides were generated and those specific only for the DIGA11YD11 clone were retained. Final PCR primers were checked for their low potential for hairpin formation and FISH probes were chosen by estimating their accessibility to target sites as described (Behrens et al., 2003). Characteristics of the PCR primers are described in Table 1. Primer set number 1 was used to determine the 16S rDNA sequences of the 28 fosmids.

PCR amplification, cloning and sequencing of WWE3 16S rDNA

16S rDNA clone libraries were constructed using DNA extracted from sludge samples obtained from anaerobic digesters in wastewater treatment plants in Cholet, Corbeil, Creil, Evry, Manheim, Palencia and Vic (Table 3), using specific primer sets 1 and 2 and also combinations of DIGA11YD11-specific primers with bacterial and universal primers (sets 5 and 6). The 16S rDNA amplicons were cloned using a TA cloning kit (pGEM-T vector; Promega) in accordance with the manufacturer’s instructions. DNA sequencing was performed using standard methods. Analysis (alignments and secondary structures) of these 16S rDNA gene sequences allowed us to design degenerate primers (set 7) targeting all the known WWE3 16S rDNA sequences. These newly designed degenerate primers were used for 16S rDNA clone library construction using DNA extracted from one swine lagoon sample, three freshwater biofilms and five sludge samples (Casolino, Cholet, Manheim, Palencia and Vic, Table 3).

Phylogenetic analysis

The 16S rDNA sequences obtained were edited and assembled with Phrap (http://www.phrap.org/). For all subsequent phylogenetic analysis, we used sequences containing at least 1200 nucleotides. The resulting 16S rDNA sequences were chimera checked and then compared with BLAST to those available in public databases [GenBank, RDP (http://rdp.cme.msu.edu/), and Greengenes (http://greengenes.lbl.gov)]. The retained sequences were then imported into the ARB database (http://www.arb-home.de) for phylogenetic analyses. An automatic alignment was performed which was manually checked and corrected.

WWE3 16S rDNA sequences were compared with 16S rDNA sequences representative of the main bacterial divisions described in public databases and phylogenetic analyses were performed using representatives of these bacterial phyla (data not shown). Twenty-five 16S rDNA sequences representative of WWE3-defined OTUs, as well as representatives of OP11, W56, OD1 and TM7 candidate divisions were used for tree construction. A modified version of the ‘Lane mask’ was used to choose homologous positions for tree construction (Lane, 1991). Phylogenetic trees were built using three methods provided by PAUP 4.0b10 software (Swofford, 2002): distance (BioNJ), maximum likelihood and maximum parsimony. For all the sequence sets studied, models of nucleotide substitution were evaluated with MODELTEST 3.0 (Posada and Crandall, 2001) to identify the model that best fit the data. Distance- and maximum likelihood-based phylogenetic trees were constructed with the General Time Reversible (Tavaré, 1986) nucleotide substitution model. The heterogeneity of nucleotide substitution rates among sites was approximated by a gamma distribution and an assumption of invariable sites. Maximum-likelihood analyses were carried out with a heuristic search strategy to find the best trees. The maximum-parsimony trees were built with the full heuristic search and the tree bisection-reconnection (TBR) branch-swapping option. A strict consensus tree was drawn when multiple best trees
were obtained. Statistical confidence levels for maximum-likelihood, maximum-parsimony and BioNJ trees were evaluated by the non-parametric bootstrap method based on 100 re-samplings. Bootstrap for maximum-likelihood analysis was performed without branch swapping to reduce computational time.

The radA annotated gene from DIGA11YD11 fosmid was aligned along with bacterial (sequences subset extracted from family HBG000623) and archaeal and eukaryotic (family HBG049531) radA homologues obtained from HOGENOM (http://pbl.univ-lyon1.fr). Phylogenetic analysis were performed using PhyML (Guindon and Gascuel, 2003).

**WWE3 distribution**

The presence of the WWE3 bacteria was checked by PCR amplification using DIGA11YD11-specific primer sets 1, 2, 3 and 4, on 23 DNA samples extracted from the anaerobic digester of Evry (recovered from 2000 to 2006), six samples from Corbeil and three from Creil. The other digester samples are described in Table 3. Swine lagoon and freshwater biofilm samples were tested using specific primer sets and degenerate primers (set 7).

**FISH experiments**

Sludge aliquots from the anaerobic digester of Evry were prepared for FISH experiments by washing in PBS and then by paraformaldehyde fixation (Amann et al., 1995). Fluorescence in situ hybridization experiments were performed as previously described (Manz et al., 1992). DIGA11YD11-specific oligonucleotides were tested for FISH and the best results were obtained with probe DIGA11YD11-21 (5′-TAGGACCTACGCTGAACC-3′) labelled with Cy3. This probe was used in combination with a mixture of probe Eub338-I, II and III (labelled with FITC) which detects most bacterial divisions (Daims et al., 1999). The Cy3-labelled nonsense probe DIGA11YD11-21 was used as a negative control. SYTO 9 (Molecular Probes) was used to stain total biomass. An inverted Zeiss confocal laser scanning microscope (CLSM, LSM510-META), equipped with three lasers (Argon 488 nm, Helium-Neon 543 nm, Helium-Neon 633 nm), was used for recording probe-conferred fluorescence signals.

**rRNA secondary structure construction**

Secondary structure of 16S rRNA of the DIGA11YD11 fosmid was calculated by the crss software (P. Daegelen, unpublished) using the E. coli secondary structure as reference. The resulting secondary structure was then used as reference to build secondary structures of representative 16S rRNA for each OTU.

**Fosmid annotation**

Gene prediction was conducted using the AMIGene software (Bocs et al., 2003). A total of 36 coding sequences was predicted and annotated (Barbe et al., 2004; Vallenet et al., 2006). Fosmid annotation is available at https://www.genoscope.cns.fr/agc/mage/wwwE3scope/.

**Nucleotide sequences accession numbers**

Sequences determined in this study were deposited in the EMBL database under Accession No. CU367853 to CU367881 for sequences obtained from fosmid clones and CU392752 to CU392838 for sequences obtained from 16S rDNA clone libraries. DIGA11YD11 complete fosmid sequence was submitted under Accession No. CU367853.

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### Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Maximum-likelihood phylogenetic tree showing the relationship of the environmental WWE3-radA gene to representatives of bacterial and archaeal ones. Amino acid sequences were aligned using MUSCLE. The phylogenetic tree was conducted using PhyML software using the Jones–Taylor–Thornton (JTT) model of amino acid substitution. Heterogeneities between sites were estimated under a gamma law-based model of substitution and 100 bootstrap replicates were made. Only bootstrap values over 50% are shown for the internal branches. The scale bar indicates the number of amino acid substitutions per site.

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