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Phenotypic and Molecular Characterization of Two Enterobacter Strains, Isolated from a Phytodepuration-Based Wastewater Treatment Plant in Prato (Tuscany, Italy)

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Abstract

Environmental pollution and presence of pollutants in water environments are main concerns for society worldwide. Environmental-friendly methods for the clearance of these compounds are continuously developed and improved to provide efficient and economical solutions. Phytodepuration has been exploited over the years, since it is based on the remedying abilities of plants and their rhizospheric microorganisms. In this preliminary work, we describe the phenotypic and molecular characterization of two *Enterobacter* strains isolated from a wastewater treatment plant, which uses the common reed *Phragmites australis*. Both strains were able to resist against multiple antibiotics, growing in the presence of a wide range of pH and several pollutants (such as those containing metals and metalloids). They were also able to produce indole-3-acetic acid and to use diesel fuel as the sole carbon and energy source. In conclusion, this work represents the beginning for a deep characterization of the *P. australis* microbiota used for phytodepuration, to discover new metabolic abilities. These results might be used for the engineering of super-degrader strains with advantageous features such as a broad degradation activity against recalcitrant compounds and ability to grow in polluted environments.

Keywords: *Enterobacter*; *Phragmites australis*; Phytodepuration; Wastewater

Abbreviations

WW: Wastewater; CW: Constructed Wetlands; LFL: Landfill Leachate; WWTP: Wastewater Treatment Plant; MBR: Membrane Bio-Reactor; RAPD: Random Amplified Polymorphic DNA; RDP: Ribosomal Database Project; MIC: Minimal Inhibitory Concentration; TSB: Tryptic Soy Broth; TSA: Tryptic Soy Agar; IAA: Indole-3-Acetic Acid; CMC: Carboxymethyl Cellulose; EcC: *Enterobacter cloacae* Complex; MDR: Multi-drug Resistant; SWW: Synthetic Wastewater

Introduction

Environmental pollution, and above all water pollution, represents an issue of considerable relevance worldwide. In this context, phytodepuration, the group of technologies that utilize plants and their rhizospheric microorganisms for the removal and/or transformation of contaminants present in water and Wastewater (WW) [1], is considered as both an environmental-friendly and a valuable solution for environmental cleanup, especially in the case of wastewater treatment. Moreover, phytodepuration is appreciated because it is cost effective, presents aesthetic advantages, and allows long-term applicability.

Constructed Wetlands (CW) are engineered systems that mimic the self-purification properties of natural wetlands. CW have been successfully exploited in WW treatment and are considered as an environment-friendly management option for treatment of WW [2]. Indeed, they have been used successfully for removal of urban, rural, and industrial WW pollutants [3]. This ability relies on the interactions involving plants, microorganisms, soil, and pollutants [4], with the rhizosphere being the principal effector involved [5]. To this purpose, *Phragmites australis* (globally known as "common reed") is among the most employed plant species. It provides several advantages, such as its ability to grow well in marshy areas and its resistance to both heavy metals (i.e. As, Ni, and Fe) [6] and high salt concentrations [7]. It has recently been demonstrated that the endophytic bacteria associated with the roots of *P. australis* are beneficial to plants growing in contaminated sites, promoting the degradation of xenobiotic compounds [8].

To date, however, only a few studies have been carried out to understand and to better evaluate the role of the endophytic microorganisms associated to *P. australis* in the CW, especially to prove whether this association really represents an advantage. In literature, the effectiveness of the use of CW in the treatment of sewage containing heavy metals [9] and high salinity is reported. In this regard, the pilot plant in Calice (Prato, Italy), managed by G.I.D.A. SpA, has the goal to characterize and improve the remediation properties in tertiary treatment of Landfill Leachate (LFL) [10].

This paper describes the phenotypic and molecular characterization of two bacterial strains belonging to the genus *Enterobacter*, isolated from the root of *P. australis*. The two strains

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were chosen as they exhibited the ability to grow in the presence of different environmental pollutants, which represent critical molecules in wastewater treatment [11]. This work aims to identify new bacterial strains with the potentiality of degrading recalcitrant compounds, an issue that is of major concern due to the increasing interest in the utilization of environmental-friendly remedies for pollutant clearance. Hence, the characterization of bacterial strains isolated from extreme environments, such as wastewaters, might lead to the identification of new potentially useful metabolic abilities.

Materials and Methods

Bacterial Strains

The *Enterobacter* strains studied in this work were isolated from the roots of *P. australis* growing in the G.I.D.A. SpA CW pilot plant located in Calice (Prato, Italy), and were referred to as H5R7 and V5R24 [12].

Site Description

P. australis plants were collected from the CW pilot plant, which is managed by G.I.D.A. SpA within the Wastewater Treatment Plant (WWTP) of Calice (Prato, Italy). The CW of Calice has been intended for the tertiary treatment of LFL. CW is downstream to a Membrane Bio-Reactor (MBR) used for the pretreatment of a mixture of LFL before its discharge in the main line of a full-scale WWTP.

CW is a two-stage subsurface flow system, formed by a horizontal system followed by a vertical one. The maximum hydraulic load supplied to the entire system was 95 m3/day, which corresponds to a 1.9-day Hydraulic Retention Time for the horizontal stage [10].

Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD profiles of the two isolates were obtained using two different primers, i.e. 1253 and RF2 (Table 1). The reaction mix was performed in a 25 µl-reaction volume with 1X DreamTaq Buffer (Thermo Scientific), 200 µM dNTPs, 500 ng of primer, 1 U of DreamTaq DNA Polymerase (Thermo Scientific) and 2 µl of thermal lysate used as template. The PCR cycling adopted was set up in a Bio-Rad T100 thermal cycler as follows: 90°C for 1 min followed by 45 cycles of 95°C 95 sec, 36°C for 1 min, 75°C for 2 min, then 75°C for 10 min and finally 60°C for 10 min. Amplicons were visualized on a 2% w/v agarose gel.

Amplification and sequencing of 16S rRNA and *gyr*B genes

PCR reactions were performed to amplify the 16S rRNA coding gene and a region of the gyrB gene. To this purpose, 2 µl of thermal lysate were used as template for a PCR in in a final volume of 25 µl with 1X DreamTaq Buffer (Thermo Scientific), 200 µM dNTPs, 0.2 µM primers (Table 1), 2 U of DreamTaq DNA Polymerase (Thermo Scientific). The PCR cycling for 16S rRNA gene amplification consisted of 95°C for 3 min followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 10 min. A gyrB portion was amplified using the following program: 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 45 sec, 72°C for 1 min, and finally 72°C for 10 min. A Bio-Rad T100 thermal cycler was used in both cases. Amplicons were cleaned-up using ExoSAP-IT Express PCR Product Cleanup (Applied Biosystems) and were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and run on an ABI 3130xl sequencer (Applied Biosystems) (Table 2).

Table 1: Primers used in this work.

Primer	Sequence (5' > 3')	Amplicon	Reference
P0	GAGAGTTTGATCCTGGCTCAG	rDNA 16S	[13]
P6	CTACGGCTACCTTGTTACGA		
PC3	GAAGTSGCGATGCAGTGGAACGA	*gyrB*	[14]
PC8r	AGCGGCGGCTGCGCRAT		
1253	GTTTCCGCCC	RAPDs	[15]
RF2	CGGCCCCTGT		
TS2S	AAYAGAGCTCAYGARVTRGGTCAYAAG	*alkM*	[16]
Deg1RE	GTGGAATTCGCRTGRTGRTC5GARTG		

Table 2: Accession numbers of 16S rDNA and *gyrB* sequences used in this work.

	H5R7	V5R24	Reference
16S rDNA	MN545624	MN545623	[12]
gyrB	MN556965	MN556966	This work

Table 3: Composition of SWWs. Concentration is expressed in mg/L.

Compound	1X SWW	2X SWW	3X SWW
H_3BO_3	20	40	60
$FeCl_2 \cdot 4H_2O$	15	30	45
Na_2SeO_3	0.03	0.06	0.09
NaCl	5000	10000	15000

Taxonomic and Phylogenetic Analyses

Taxonomic affiliation of the two isolates was determined by alignment of full-length 16S rDNA sequences with those of type strains, available in the Ribosomal Database Project (RDP) [17], using BioEdit [18]. Alignment was used to build a phylogenetic tree using MEGA7 [19], and applying the Neighbor Joining algorithm with a 1000-bootstrap resampling. A phylogenetic tree was also generated with the same settings of MEGA7 reported above using a region of the *gyr*B gene. In this case, sequences from H5R7 and V5R24 were aligned with the top-100 scoring BLAST hits using BioEdit. Accession numbers of sequences are reported in Table 2.

Antibiotic Resistance

Minimal Inhibitory Concentration (MIC) of antibiotics was evaluated according to the broth microdilution protocol [20]. Bacteria were inoculated in 96-well plates by diluting freshly grown broth cultures at the concentration of 5×10^5 CFU/mL. The two strains were tested for their resistance/sensitivity against 12 different antibiotics (i.e. ampicillin, cefepime, ceftazidime, chloramphenicol, ciprofloxacin, ertapenem, kanamycin, meropenem, rifampicin, streptomycin, tetracycline, and trimethoprim) at the following concentrations: 0.20 – 0.39 – 0.78 – 1.56 – 3.13 – 6.25 – 12.50 – 25.00 – 50.00 – 100.00 µg/mL. A positive control, consisting of bacteria grown in tryptic soy broth (TSB) medium (Bio-Rad) lacking any antibiotic and a negative control were also assessed for each strain. The MIC value for each antibiotic was considered as the lowest concentration able to inhibit visible bacterial growth. Each test was performed in triplicate.

Table 4: Environmental pollutant resistance assay. Concentration is expressed in mM.

Compound	Concentration							
$FeCl_3 \cdot 6H_2O$	0.79	1.57	3.13	6.25	12.5	25	50	100
$NiCl_2$	0.4	0.79	1.57	3.13	6.25	12.5	25	50
$CuCl_2$	0.4	0.79	1.57	3.13	6.25	12.5	25	50
$ZnSO_4$	0.4	0.79	1.57	3.13	6.25	12.5	25	50
$Cd(NO_3)_2$	0.4	0.79	1.57	3.13	6.25	12.5	25	50
H_3BO_3	0.98	1.96	3.91	7.82	15.63	31.25	62.5	125
KH_2AsO_2	0.2	0.4	0.79	1.57	3.13	6.25	12.5	25
$NaAsO_2$	0.2	0.4	0.79	1.57	3.13	6.25	12.5	25
Na_2SeO_3	$2.5x10^{-3}$	$5x10^{-3}$	0.01	0.02	0.04	0.07	0.13	0.25

Salt Tolerance Assay

Growth at different NaCl concentrations was evaluated according to the broth microdilution protocol [20]. Bacteria were inoculated in the 96-well plates by diluting freshly grown broth cultures at the concentration of 5×10^5 CFU/mL. *Enterobacter* strains were assayed for their ability to grow in the presence of 0.39 – 0.78 – 1.56 – 3.13 – 6.25 – 12.50 – 25.00 – 50.00 – 100.00 g/L NaCl. A positive control, with bacteria grown in TSB medium (Bio-Rad) without the addition of NaCl, and a negative one were also assessed for each strain. The MIC value was considered as the lowest concentration of NaCl that inhibited visible growth of the two strains. Each test was performed in triplicate.

pH Tolerance Assay

Growth at different pH was tested by microplate assay, performing the test in triplicate. 90 µl of TSB medium (Bio-Rad) at different pH (adjusted by adding either HCl or NaOH) and 10 µl of freshly grown broth culture of bacteria were added in each well, to reach the final concentration of 5×10^5 CFU/mL. Tester strains were screened for their ability to grow at pH 3, 4, 5, 6, 7, 8, 9, 10 and 11. Tester strains grown in TSB medium at pH 7 were used as positive control. For each condition, a negative control consisting of the medium lacking bacteria inoculum was also assessed. Outcome of the assay was evaluated as presence or absence of bacterial growth after incubation at 30°C for 24h.

Plasmid Detection

The presence of plasmids in the two *Enterobacter* strains was evaluated through alkaline lysis method [21] and by NucleoSpin Plasmid extraction kit (Macherey-Nagel) starting, in both cases, from 3 ml of an overnight-grown liquid culture in TSB medium at 30°C under shaking. The obtained DNA preparation was checked through electrophoresis on a 0.8% w/v agarose gel.

Resistance against Environmental Pollutant Elements

Metal resistance was evaluated by microdilution protocol [20]. Bacteria were inoculated in 96-well plates by diluting freshly grown broth cultures at the concentration of 5×10^5 CFU/mL. Both strains were screened for their ability to grow in presence of eight different concentrations of $FeCl_3 \cdot 6H_2O$, $NiCl_2$, $CuCl_2$, $ZnSO_4$, $Cd(NO_3)_2$, H_3BO_3, KH_2AsO_2, $NaAsO_2$ and Na_2SeO_3 (Table 4). A positive control, with bacteria grown in TSB medium without any additional element, and two negative controls, consisting of either the medium or each pollutant solution, were also assessed. The MIC value for each tested compound was considered as the lowest concentration of the compound that inhibited the visible growth of the tested isolate.

Growth on Diesel Fuel as the Sole Carbon Source

Bacterial strains were grown overnight at 30°C on Tryptic Soy Agar (TSA) medium (Bio-Rad). Colonies were resuspended in 100 µl of saline solution (0.9 % w/v NaCl), washed three times and a 10 µl drop of the bacterial suspension was streaked on Minimum Davis medium (2 g/L $(NH_4)_2SO_4$, 14 g/L K_2HPO_4, 4 g/L KH_2PO_4, 1 g/L Na_3-citrate $\cdot 2H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$) containing 0.4% v/v diesel fuel B7 (purchased from a diesel fuel station) as the sole carbon source. Diesel fuel was previously filtered with a syringe filter (Sartorius) having pore size of 0.2 µm for sterilization and particle removal. As positive control of bacterial growth, colonies were also streaked on Minimum Davis medium containing 1% w/v dextrose as carbon source. Plates were incubated at 30°C for 48 h. Positivity to this assay was assessed as presence or absence of visible growth.

PCR amplification of *alk*M gene

Primers TS_2S and Deg1RE (Table 1) were used to amplify the partial coding sequence of alkane hydroxylase AlkM [16]. PCR was carried out using a Bio-Rad T100 thermal cycler in a 1X DreamTaq Buffer (Thermo Scientific) containing 200 µM of each dNTPs, 1 µM of each primer, 0.5 U of DreamTaq DNA Polymerase (Thermo Scientific), and 2 µl of cellular thermal lysate in a final volume of 20 µl. The PCR cycling was 95°C for 4 min followed by 25 cycles of 95°C for 45 sec, 40°C for 1 min 72°C for 1 min, then a final extension at 72°C for 5 min.

Indole-3-Acetic Acid (IAA) Production Assay

This test was performed as previously described [22]. 5 ml of a 1:10 dilution of TSB medium (Bio-Rad), supplemented with 1 mg/mL L-tryptophan, were inoculated with 200 µl of bacterial liquid culture as described previously in [24]. After overnight incubation at 30°C, 100 µl of the culture were dispensed in a 96-well plate, in triplicate, to measure absorbance at 600 nm. 50 µl of Salkowsky reagent (35% v/v perchloric acid, 10 mM $FeCl_3$) were added to 50 µl of the spent medium (obtained through centrifugation of the bacterial cultures for 3 min at 10000 x g) and OD530 was measured after 30 min of incubation at room temperature [22]. IAA production was estimated comparing the OD530/OD600 ratio of the assayed isolates with those ones of the positive control (Escherichia coli DH5µ) and the negative one (medium only).

Biofilm Formation

This assay was performed according to Checcucci et al. [24]. Briefly, 5 ml of TSB (Bio-Rad) were inoculated with a single colony. After incubation overnight at 30°C with shaking, absorbance at 600 nm was measured and adjusted to 0.1. Then, 100 µl of suspension were dispensed in triplicate and incubated for further 24 h at 30°C without shaking. OD_{600} was measured with a microplate reader and 20 µl of a 0.1% w/v crystal violet filtered solution were added. Upon incubation at room temperature for 10 min, samples were rinsed three times with water and left to dry for 15 min. 100 µl of 95% v/v ethanol were finally added and, after incubation at room temperature for 15 min, OD_{540} was determined. Biofilm formation was relatively evaluated through the comparison of OD_{540}/OD_{600} ratios.

Table 5: Antibiotic resistance assay. MIC values are reported as µg/mL. Symbols: + and – indicate growth and no growth in the presence of all antibiotic concentrations assayed, respectively. MIC values reported in bold denote resistance against the assayed antibiotic.

Antibiotic	Class	Target	Strain		MIC breackpoint [30][1]
			H5R7	V5R24	
Ampicillin	Penicillins	Cell wall synthesis	+	+	8
Chloramphenicol	Phenicols	Ribosome	25	12.5	8
Cefepime	Cephems	Cell wall synthesis	6.3	6.3	4
Ceftazidine	Cephems	Cell wall synthesis	+	25	4
Ciprofloxacin	Fluoroquinolones	Topoisomerases	-	0.39	0.5
Ertapenem	Carbapenems	Cell wall synthesis	1.56	1.56	0.5
Kanamycin	Aminoglycosides	Ribosome	50	25	nr
Meropenem	Carbapenems	Cell wall synthesis	1.56	12.5	8
Rifampicin	Ansamycins	RNA polymerase	25	12.5	*
Streptomycin	Aminoglycosides	Ribosome	25	25	nr
Tetracyclin	Tetracyclines	Ribosome	6.25	6.25	*
Trimethoprim	Diaminopyrimidines	DNA replication	6.25	3.13	4

[1]Values were retrieved from [30] and are referred to Enterobacteriales order. nr: not reported in [30]; *: no breakpoint, susceptibility testing is not recommended.

Cellulose Degradation

The protocol described below was adapted from Saha et al. [25]. Bacterial strains were grown overnight at 30°C on TSA medium (Bio-Rad). Freshly grown broth cultures were diluted at the concentration of 5×10^5 CFU/mL and 20 µl bacterial suspension of each strain were spotted on TSA medium, with the addition of 0.1% w/v Carboxymethyl Cellulose (CMC). *Bacillus subtilis* KACC10111 and *E. coli* DH5α were used as positive and negative controls, respectively. Petri dishes were incubated at 30°C for 48 h, then 10 ml of a 0.15 w/v Congo Red solution were added over each plate and incubated for 30 min at room temperature. Then, plates were washed three times with 10 ml of 1 M NaCl. Cellulolytic activity was observed as a white halo around the bacterial colonies.

Result

The two bacterial strains described in this work were isolated from the roots of *P. australis* exploited for phytodepuration of wastewater in the WWTP in Calice, and they were named H5R7 and V5R24 [12]. When cultivated at 30°C overnight on TSA medium, both H5R7 and V5R24 formed raised yellowish circular colonies with entire margin and viscid consistency. Moreover, surface of colonies was translucent and smooth. Gram staining classified both of them as Gram-negative bacteria.

We firstly performed a RAPD analysis using two different primers (1253 and RF2, [15]) to check whether the two isolates corresponded to the same strain. Data obtained (Figure 1) revealed that the two isolates did not share the same RAPD profile(s) demonstrating that they did not correspond to the same strain.

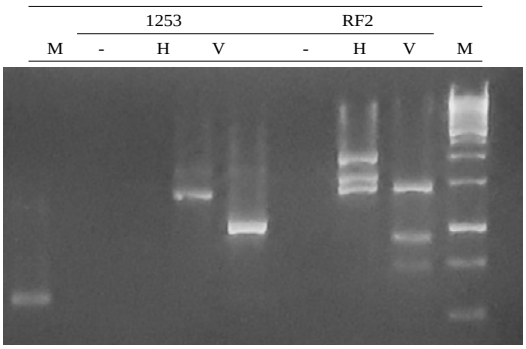

Figure 1: RAPD analysis using primers 1253 and RF2. M, -, H and V indicate GeneRuler 1 kb DNA Ladder (Thermo Scientific), negative control (no template), H5R7 and V5R24, respectively.

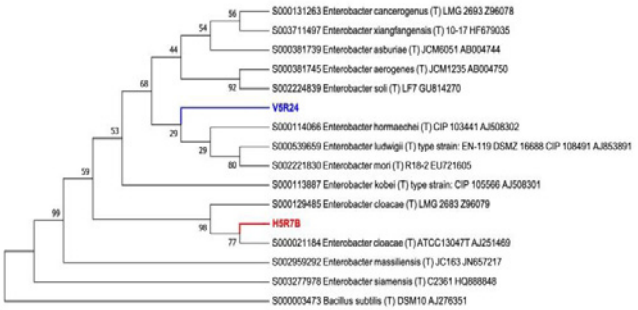

Figure 2: Phylogenetic analysis of *Enterobacter* strains based on 16S rRNA gene sequences.

The taxonomic affiliation was determined through phylogenetic analysis of the full-sequenced 16S rRNA (Figure 2) and *gyr*B coding genes (Figure 3), as described in Materials and Methods. A previous BLAST analysis performed using the four sequences as query retrieved at the lowest e-values *Enterobacter* orthologous sequences, strongly suggesting that they belong to this genus. Moreover, the analysis of the phylogenetic trees revealed that both strains belong to the *Enterobacter cloacae* Complex (EcC) whose members are able to colonize medical devices and are responsible of nosocomial infections, such as pneumonia, urinary tract infections and peritonitis [26-27]. However, in the two phylogenetic trees the two strains joined different branches strongly suggesting that they belong to different *Enterobacter* species. Moreover, in both trees strain H5R7 was very close to *E. cloacae*, whereas V5R24 was close to *Enterobacter*

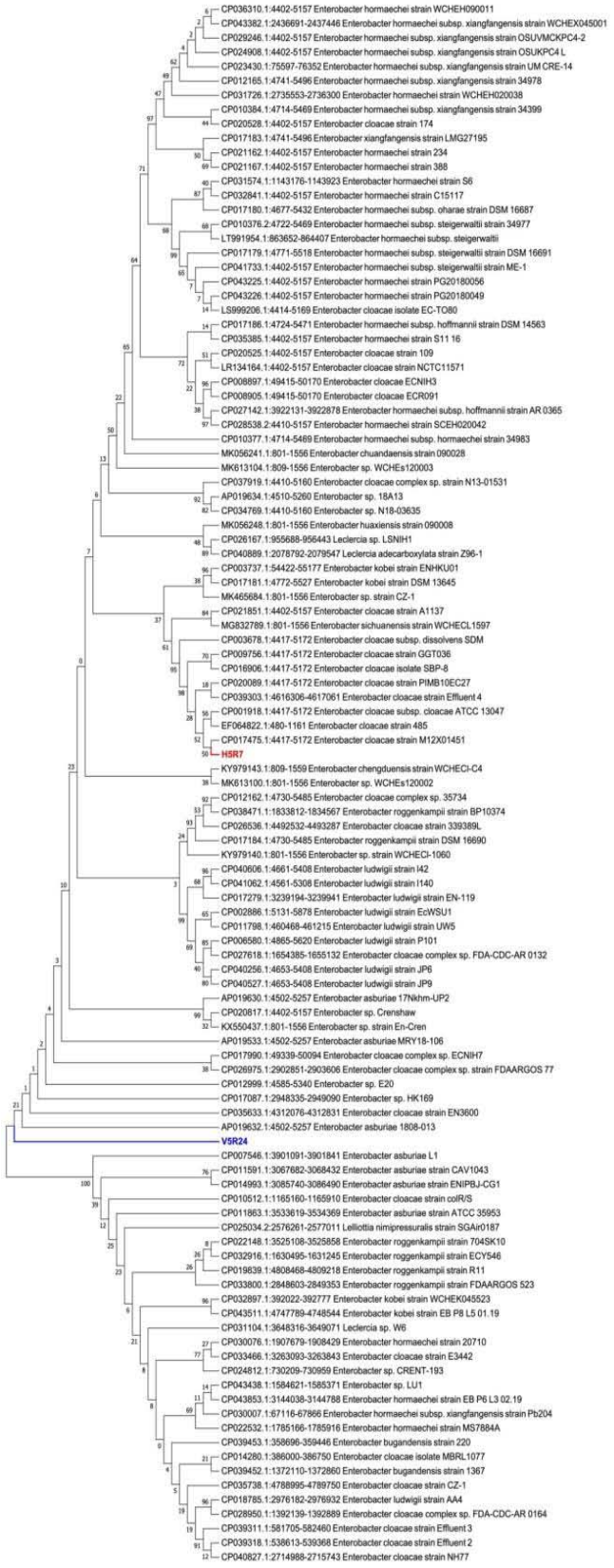

Figure 3: Phylogenetic analysis of *Enterobacter* strains based on partial *gyrB* gene sequences.

hormaechei, Enterobacter ludwigii and *Enterobacter mori* in the 16S rRNA gene tree and joined a larger group of *Enterobacter* strains, including *E. hormaechei*, and *E. cloacae* in the *gyr*B tree.

Antibiotic Resistance Profile

To better characterize their phenotypic traits, the two strains were firstly assayed for their ability to grow in the presence of different concentrations of twelve antibiotics. These compounds were chosen because they belong to different chemical classes and/or they have different targets. In detail, ampicillin was used here as positive control because *Enterobacter* strains are intrinsically resistant against this antibiotic due to the presence of either chromosome- or plasmid-encoded β-lactamase genes (e.g. *ampC*) [28-29], which are usually expressed at basal level and whose expression is induced by the presence of β-lactams. Other clinically relevant β-lactams, i.e. cefepime, ceftazidime, ertapenem, and meropenem, were also tested since they are exploited to fight Gram-negative infections in humans. Chloramphenicol, kanamycin, streptomycin, and tetracyclin were chosen because they bind ribosome subunits and inhibit protein synthesis; ciprofloxacin was assayed because it targets topoisomerases. Lastly, rifampicin and trimethoprim were tested because of their ability to inhibit DNA transcription through binding of RNA polymerase, and to indirectly alter DNA replication through the inhibition of tetrahydrofolic acid formation, respectively. Data obtained (Table 5) revealed that both isolates, as expected, showed resistance against ampicillin; moreover, they were able to grow in the presence of almost all the tested antibiotics. Ciprofloxacin was the most effective antibiotic since it completely inhibited the growth of H5R7 and was strongly effective against V5R24 at the second lowest concentration tested (0.39 μg/mL). In general, these two isolates had similar MIC values with the main exception represented by meropenem whose MIC values differed by almost 10-fold magnitude: indeed, they resulted 1.56 and 12.50 μg/mL for H5R7 and V5R24, respectively. The presence of simultaneous resistances against antibiotics belonging to at least three different categories allowed the classification of these two *Enterobacter* strains as Multi-Drug resistant (MDR) strains [31]. The analysis of plasmid profiles revealed that the resistance of these two *Enterobacter* strains to the tested antibiotics was not very likely conferred by genes embedded in low molecular weight plasmids, since no plasmid DNA was extracted by alkaline lysis method using either standard protocols or commercial kits (data not shown).

The resistance profiles of H5R7 and V5R24 are in line with those of *Enterobacter* strains isolated from clinical samples [32] even though they were not isolated from hospitals. Hence, considering that even civil wastewaters are treated in the plant of Calice, the presence of these two (and likely other) strains might represent an example of how the spreading of resistance genes in the environment can be easy, thus constituting a serious health threat worldwide.

Tolerance to pH Variation and Pollutants

Tolerance to different pH and salt concentrations of the two *Enterobacter* strains was then evaluated as described in Materials and Methods. Both strains were able to grow at pH values ranging from 4 to 11 and only pH 3 exerted an inhibitory effect on their growth. Similarly, they showed the same tolerance profile against salt concentration, being able to proliferate in all tested conditions, except

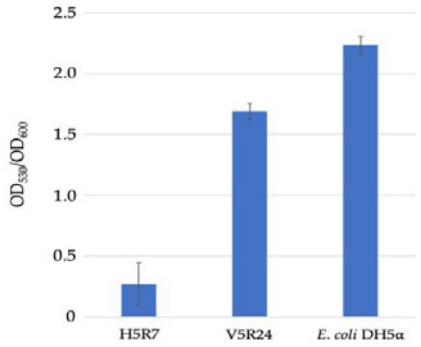

Figure 4: IAA production by *Enterobacter* strains H5R7 and V5R24.

Table 6: Environmental polluant resistance assay. MIC values are reported in mM. + indicates growth in the presence of all concentrations tested.

Pollutant	Strain	
	H5R7	V5R24
$FeCl_3 \cdot 6H_2O$	100	50
$NiCl_2$	6.25	6.25
$CuCl_2$	12.5	12.5
$ZnSO_4$	25	+
$Cd(NO_3)_2$	+	+
H_3BO_3	125	62.5
KH_2AsO_2	+	+
$NaAsO_2$	0.4	0.2
Na_2SeO_3	+	+

at the highest NaCl concentration assayed (100 mg/mL).

In the case of H5R7, the ability to tolerate the presence of chemicals usually contained in wastewater was previously checked through the use of a Synthetic Wastewater (SWW) having a composition resembling the one of the real wastewater treated in the WWTP [12]. Resistance against SWW prompt us to test whether these two *Enterobacter* strains are able to prosper even in stressful and polluted conditions. Hence, a broader panel of compounds, including metals, metalloids, and nonmetals (i.e. Fe, Ni, Cu, Zn, Cd, B, As and Se) was tested, even though some of these compounds are not usually present in the wastewater treated at the WWTP in Calice. MIC values obtained are reported in Table 6 and showed that H5R7 and V5R24 share a similar resistance profile. The main difference between the two strains was that the latter grew in the presence of ZnSO4 at all tested concentrations, whereas H5R7 had 25.00 mM as MIC for this chemical.

Occurrence of heavy metal resistance has already been described at different levels in *Enterobacter* iaceae from polluted environments [33-34]. Presence of resistance against both antibiotics and heavy metals is of main concern if we take in mind that the latters are used as antimicrobials and growth promoters in livestock too. Moreover, it has been reported that the occurrence of multiple heavy metal resistance genes is associated with that of antibiotic ones [27, 35-36]: indeed, resistance cassettes against both antibiotics and heavy metals can be localized in the same mobile genetic elements, such as plasmids. So, the accumulation of heavy metals in the environment can cause the selection of antibiotic resistant species.

Interestingly, both H5R7 and V5R24 were also able to grow on minimal medium containing diesel fuel as the sole carbon and energy source (not shown), suggesting their ability to degrade at least some aliphatic and/or aromatic hydrocarbons.

IAA production, cellulose degradation and biofilm formation

The whole body of data reported in the previous paragraphs suggested that the two *Enterobacter* strains might exert a promoting effect on plant growth. To check this hypothesis, they were assayed for production of IAA. Results revealed that both strains produced IAA under the experimental conditions described in Materials and Methods, even though at a different extent. Indeed, V5R24 showed a 3-fold higher production when compared to H5R7 (Figure 4). These two *Enterobacter* strains were able to synthesize IAA, which may be favorable for *P. australis* growth and, to this purpose, *in vitro* tests will be conducted to further characterize this plant-growth-promoting activity.

Besides IAA production, ability to degrade cellulose was investigated to further characterize the relationship between these two isolates and their host *P. australis*. The assay based on the use of CMC as substrate did not show any cellulolytic activity. This feature was not surprising: indeed, the presence of cellulolytic activity in *Enterobacter* strains seems to be dependent on the selection operated by the environment from whom strains were isolated and the ability to produce cellulases may differ in *Enterobacter* of diverse origins, as reported previously [37-40]. Similarly, a test to assay the formation of biofilm by both strains was performed, resulting negative. This feature might be considered as an advantage in case of future applications of these isolates in the WWTP. Indeed, the lack of biofilm formation would prevent the possible clogging of pipes in the plant, making the maintenance easier.

Conclusion

In conclusion, in this work we described the phenotypic and molecular characterization of two bacterial strains isolated from the CW in Calice (Prato, Italy). The RAPD analysis indicated that the two isolates actually corresponded to different strains. Sequencing of the full 16S rRNA gene allowed to taxonomically affiliate the two strains to the EcC. Sequence analysis allowed to identify H5R7 as *E. cloacae*, while in case of V5R24 its identification was uncertain. Same results were obtained through the phylogenetic analysis of *gyrB* sequences. From a phenotypic viewpoint, these two isolates shared similar features, suggesting that the selective pressure acting in the CW can select strains belonging to different species but sharing the same (or very similar) phenotypic traits. They showed a broad antibiotic resistance, being resistant against almost all antibiotics tested, allowing their classification as MDR strains. The ability of these strains to resist to a wide plethora of antibiotic compounds permits their use for the implementation of phytodepuration processes in G.I.D.A. SpA WWTP, where the presence of antimicrobial molecules of anthropic origin cannot be excluded since the nature of the leachate entering the plant. This preliminary work represents the basis for a future work aimed to characterize the microbiota of the G.I.D.A. SpA

WWTP and how its composition can impact the performances of the plant itself. The identification of these bacterial strains is the first step for the implementation of the process. Indeed, knowledge about the metabolic pathways could be exploited for the construction of super-degrading strains with augmented remediation properties against a wide plethora of environmental pollutants.

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