

Detection of Multiple Resistances, Biofilm Formation and Conjugative Transfer of *Bacillus cereus* from Contaminated Soils

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Abstract The purpose of this study was to detect microbial resistances to a set of antibiotics/pesticides (multi-resistance) within pesticide and antibiotic-contaminated alluvial soils and to identify the corresponding antibiotic resistance genes (ARGs). To assess whether identified multi-resistant isolates are able to construct biofilms, several biofilm formation and conjugation experiments were conducted. Out of 35 isolates, six strains were used for filter mating experiments. Nine strains were identified by 16S rDNA gene sequence analyses and those were closely related to *Pseudomonas* sp., *Citrobacter* sp., *Acinetobacter* sp., *Enterobacter* sp., and in addition, *Bacillus cereus* was chosen for multi-resistant and pesticide-tolerant studies. Antibiotic-resistant and pesticide-tolerant bacterial strains were tested for the presence of ARGs. All nine strains were containing multiple ARGs (*ampC*, *ermB*, *ermD*, *ermG*, *mecA*, *tetM*) in different combinations. Interestingly, only strain WR34 (strongly related to *Bacillus cereus*) exhibited a high biofilm forming capacity on glass beads. Results obtained by filter mating experiments demonstrated gene transfer frequencies from 10^{-5} to 10^{-8} . This study provides evidence that alluvial

soils are hot spots for the accumulation of antibiotics, pesticides and biofilm formation. Particularly high resistances to tetracycline, ampicillin, amoxicillin and methicillin were proved. Apparently, isolate WR34 strongly correlated to a pathogenic organism had high potential to deploy biofilms in alluvial soils. Thus, we assume that loosened and unconsolidated soils investigated pose a high risk of an enhanced ARG prevalence.

Introduction

The rise of antibiotic resistances is a rapidly evolving problem due to growing inefficiencies of antimicrobial agents to treat infectious diseases. Therefore, a steadily increase of antibiotic resistances is an enhanced public health concern of great urgency. Increased resistances are mainly caused by the propagation of antibiotic resistance genes affiliated to an overuse of antimicrobials in humans, animals and agriculture [14]. Bacteria are able to adapt rapidly to new environmental conditions such as the presence of antimicrobial molecules, and consequently, (multi) resistances increase by an irresponsible usage of antibiotics [3, 20].

The development process of pesticide tolerances is a complex physiological and genetic phenomenon in microorganisms, which is based on the variation of temporary and permanent tolerances [9]. In particular, horizontal gene transfer (HGT) by conjugation seems to be of particular importance under environmental conditions, as resistance genes are often located on plasmids which are either transferable or mobilizable. Especially plasmid-mediated HGT plays an important role in the emergence of new pathogens [3, 10, 25]. Since soil has high potential to

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enrich antibiotic-resistant genes (ARGs) continuously, it is important to investigate the microbial ecosystem play according to the transmission of ARGs [4, 20]. Furthermore, the natural microbial production of antibiotics in ground is another potential source for the selection for antibiotic resistance genes. Even the acquisition of new genetic traits may enhance the transcription of necessary genes required for the formation of biofilm communities and quorum sensing [19].

Also the transformation impacts the dissemination of transfer integron- and antibiotic resistances in environmental habitats. It is documented that DNA from integron carrying strains of *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Pseudomonas* and *Salmonella* could confer antibiotic resistance as well as transfer integrons and transposons within one day [7]. Moreover, since groups of soil bacteria encode resistance elements to counteract the effects of the molecules, they produce the networks of selection and resistance that exist in soil are tremendous. Genes conferring resistance to β -lactams, erythromycin, tetracyclines and glycopeptides existed in the environment well before the use of clinical antibiotics. Structural and functional assays on the glycopeptide resistance element *VanA* demonstrated that the ancient soil resistance determinant was similar to the modern clinical resistance element [17]. In this study, bacteria from pesticide and antibiotic-contaminated soils were investigated, near a pesticide industry and animal husbandry. The aim of this study was (a) to detect microbial resistances to one or more antibiotics/pesticides (multi-resistance) and to identify corresponding antibiotic resistance genes, (b) to observe the transfer of resistance genes by filter mating experiments (c) and to identify biofilm forming organisms of bacterial isolates obtained by this study.

Materials and Methods

Soil Sample Collection, Characteristics and Pesticide Analysis

Near a pesticide industry, (India Pesticide Ltd. Chinhat, Lucknow, U.P. India) a 200 m away situated agricultural field was supplied by wastewater discarded from that factory. Nearby also a livestock holding was located. Composite soil samples (15 cm depth) were collected from 5 different agricultural fields which had a relative distance of approximately 50 m to each other. One composite sample was prepared by mixing all five contaminated soil samples together. A control soil sample was taken from nearby agricultural fields supplied by ground water. Characteristics and analysis of pesticides of soils (Table S2) were carried out according to Nawab et al. [16].

Isolation of Bacteria and Multi-Resistance Testing

The isolation of pesticide-tolerant bacteria from contaminated soils was conducted according to Nawab et al. [16]. 35 bacterial isolates were sensitivity tested to antimicrobial agents (Table 3) and pesticides tolerances (Table 2) according to Shafiani and Malik [16], respectively.

Cell Lysate Preparation and Antibiotic Resistance Genes Amplification of Multi-Resistant Bacteria

100 μ l of lysate from positive control strains (Table S1) and 35 overnight cultures (Table 3) were centrifuged (Eppendorf 5417R) at 10,000 g for 2 min. The obtained pellet was resuspended in 20 μ l lysis buffer (50 mM NaOH, 0.25 % SDS) and incubated at 95 $^{\circ}$ C for 20 min. The design of used antibiotic resistance gene primers was prepared according to Böckelmann et al. [4] and Schiwon et al. [20]. For the DNA amplification, 1 μ l DNA template of each bacterial lysate was diluted with milli Q water (1:10). The amplification of antibiotic resistance genes was carried out in 50 μ l of a PCR reaction mixture (Thermo Scientific, Germany) containing a 0.8 μ M concentration (each) of primers, a 0.02 mM concentration of each dNTP, 1.5 U DNA polymerase (Thermo Scientific, Germany), 2 mM $MgCl_2$ and 5 μ l of a 10 \times reaction buffer. The PCR protocol used included an initial DNA denaturation step for 2 min at 95 $^{\circ}$ C, 35 cycles of 95 $^{\circ}$ C for 30 s, annealing at 57–60 $^{\circ}$ C for 45 s and elongation at 72 $^{\circ}$ C for 1 min; 7 min at 72 $^{\circ}$ C.

Identification of Multi-Resistant Bacteria

All 35 isolates were characterized on the basis of morphological, cultural and biochemical characteristics [6]. For an unambiguous identification of bacteria, both DNA-based phylogenetic analyses as well as the DNA independent microscopy were carried out. Thus, a set of nine highly multiple antibiotic-resistant and pesticide-tolerant bacteria was identified by 16S rDNA gene sequencing using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). All obtained sequences were initially analysed employing the NCBI BLAST (n) algorithm and the ribosomal database project. The corresponding sequences and the closest relatives, respectively, were considered for further analyses. The 16S rDNA-gene sequence-based phylogenetic analyses were conducted by the ARB and MEGA software as described by Krakat et al. [13]. Moreover, to clearly identify *Bacillus cereus* and to counter the systematic error of the DNA-based techniques applied, respectively, a non-DNA-based technique (microscopy) was considered. Morphological observations confirmed the assumption obtained by phylogenetic studies, as we identified 1 \times 2–3 μ m, gram-positive, rod-

shaped cells as regularly *B. cereus* sp. exhibit (Fig. S1). In addition to that we also used 3 different highly specific agar media for the confirmation of *Bacillus cereus*; mannitol egg yolk polymyxin agar (MYPA, Merck, Millipore, Germany), polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA, Mast GmbH, Germany) and the brilliance bacillus cereus agar (ThermoScientific, Germany). The pure culture (WR34) was cultivated on agar plates at 30 °C for 24 to 48 h. However, all cultivation procedures were conducted according to the manufacture's guidelines.

Conjugative Plasmid DNA Transfer by Filter Mating Experiments

From nine highly pesticide-tolerant and antibiotic-resistant bacteria, only six strains were resistant to specific selection marker (ampicillin) and thus were chosen for filter mating experiments. WR21, WR22, WR24 and WR33 (Table 3) served as biparental mating donor, while *E. coli* HMS was considered as recipient strain. For triparental mating experiments, WR34 and *E. coli* XL 10 were used as donor 1 and donor 2, respectively, and were tested by the recipient *Streptococcus pneumoniae* T4N517 Novo. Strain WR35 and *E. faecalis* OG1X were used as donor 1 and donor 2, respectively, while *E. faecalis* T9 served as corresponding recipient. Filter mating experiments were conducted according to Schiwon et al. [20].

PCR Verification of Plasmid Transfer

To ascertain conjugative plasmids captured by mobilizable transconjugant plasmids (*gfp/pIP501*-labelled), PCR experiments were performed according to Schiwon et al. [20]. The DNA target length was approximately 1700 bp.

Bioreactor and Biofilm Formation

The biofilm formation was carried out in 6 up flow bioreactors (3 for WR34; 3 for WR35) which were simultaneously operated for a period of 8 days at 37 °C. The glass bioreactors (height 15 cm, width 8 cm, working volume 800 ml) were charged with glass-, polystyrene- or silicon beads to trigger biofilm growth processes. To guarantee the addition of media, all reactors were equipped with an inlet at the bottom and an outlet at the top. The fresh BHI medium addition (25 ml h⁻¹) and the inner medium circulation (of 2.5 l h⁻¹) were achieved by peristaltic pumps. Prior to biofilm formation experiments, all isolates were tested for the ability of biofilm formation according to Schiwon et al. [20]. The ability of biofilm formation was scored as follows: OD < 0.120: no biofilm formation; 0.120 < OD < 0.240: weak biofilm formation; OD > 0.240: strong biofilm formation.

Lectin-Binding-Analysis and Lectin Blocking Assays

All lectin-binding and blocking analyses were carried out according to Bockelmann et al. [5].

Nucleotide Sequence Accession Numbers

All sequences obtained in this study were deposited in NCBI's gene bank database under accession numbers GQ922652, GU384325 to GU384332.

Results and Discussion

Characterization and Determination of Pesticides in Contaminated Alluvial Soils

Organochlorines (OC), lindane and endosulfan are pesticides mainly used in agriculture to preserve the health of crops and to prevent its destruction by diseases and pests infestation [1, 18]. A triplicate approach was used to measure soil pesticides by gas chromatography. The average concentration of α -, β -endosulfan and lindane within soil samples investigated was 422, 421 and 547 ng g⁻¹, respectively, whereas the corresponding concentrations of ground water irrigated soils (control sample) were below 3 ng g⁻¹ (Table S2). Prakash et al. [18] investigated 45 soil samples from pesticide-contaminated sites and industrial areas and reported lindane residues at concentrations between 0.04 and 463 μ g g⁻¹.

Measured pH values, organic carbon contents, water holding capacities and nitrogen concentrations revealed comparable data (Table S2), suggesting that both soil textures were comparable. However, interestingly, the heavy metals concentrations in waste water irrigated soils were notably higher. Accordingly, the concentrations of chromium, zinc, iron, copper and cadmium demonstrated averagely 50 times increased concentration values, while the nickel accumulation was even more prominent (120 times lifted concentrations). Seemingly alluvial soils seem to have district tendencies of pooling heavy metals. Respectively, this study revealed in comparison to other studies [22, 24] an averagely 40-fold increased metal soil impact (Cr, Zn, Ni, Fe, Cu and Cd) of contaminated samples investigated (Table S2).

Multi-Resistances in Bacteria and Identification of Highly Resistant Bacteria

All (35) isolates were multiple resistant to antibiotics (Tables 2, 3) and tolerant to multiple pesticides (Table 1). From 35 isolates, 9 highly multiple-resistant and multiple-tolerant isolates were identified by 16S rDNA gene analyses. Regarding to the prevalence of multiple antibiotic

Table 1 Overview of used pesticides and percentage of tolerant isolates

Pesticides		Concentration ($\mu\text{g/ml}$)	No. of tolerant isolates	Tolerant isolates (%)
Lindane	(ln)	1600	21	60.0
Endosulfan	(end)	1600	22	62.8
Chlorpyrifos	(clr)	400	20	57.2
Monocrotophos	(monc)	400	21	60.0
Captan	(cap)	800	17	48.5
Folpet	(flp)	800	19	54.2

Table 2 Overview of used antibiotics and percentage of resistant isolates

Pesticides		Concentration ($\mu\text{g/disc}$)	No. of resistant isolates	Resistant isolates (%)
Ampicillin	(A)	25	34	97.1
Amoxicillin	(Am)	30	35	100
Cloxacillin	(Cx)	30	12	34.3
Cefaclor	(Cj)	30	32	91.4
Chloramphenicol	(C)	25	10	28.6
Ciprofloxacin	(Cf)	30	0	0
Co-trimoxazole	(Co)	25	10	28.6
Doxycycline	(Do)	30	12	34.3
Erythromycin	(E)	15	10	28.6
Gentamycin	(G)	30	21	60.0
Kanamycin	(K)	30	1	2.9
Methicillin	(M)	30	34	97.1
Nitrofurantoin	(Nf)	30	26	74.3
Neomycin	(N)	30	14	40.0
Nalidixic acid	(Na)	30	15	42.9
Polymyxin B	(B)	30	1	2.9
Rifampicin	(R)	30	24	68.6
Sulfadiazine	(Sz)	30	5	14.3
Streptomycin	(S)	25	30	85.7
Tetracycline	(T)	30	32	91.4

resistances spread in pesticide and antibiotic-contaminated agricultural fields, our results are only partially consistent to those of Matyar et al. [15]. In their study, 236 gram-negative bacterial strains isolated from industrially polluted sampling sites were resistance tested against 16 different antibiotics. They observed sharp resistances to ampicillin (93.2 %) and streptomycin (90.2 %) which is in conformity to our results (Table 2). However, we observed that isolates from pesticide-contaminated soils were resistant to 14 or more antibiotics, while Mayer et al. [15] reported that 56.8 % of all bacterial isolates were resistant by an average of 7 antibiotics.

Moreover, Shafiani and Malik [21] isolated 40 isolates from agricultural soils irrigated with industrial wastewater and domestic sewage and tested antibiotic resistance with 7 different antibiotics. They were able to show that 45, 100, 35, 20, 12.5, 57.5 and 30 % of investigated isolates were resistant against nalidixic acid, cloxacillin, chloramphenicol, tetracycline, amoxicillin, methicillin and doxycycline,

respectively. Interestingly, our values differ significantly, particularly the percentage of resistances to tetracycline, ampicillin, amoxicillin and methicillin was above 90 %, while the percentage of cloxacillin was below 35 % (Table 2). This finding confirms an increased resistance of soil isolates against tetracycline, ampicillin, amoxicillin and methicillin within investigated soils.

Shafiani and Malik [21] also conducted pesticide tolerance experiments against endosulfan, carbofuran and malathion with 40 isolates of *Pseudomonas*, *Azotobacter* and *Rhizobium*. The measured tolerant concentrations of carbofuran, endosulfan and malathion of considered isolates were 1600, 800 and 1600 $\mu\text{g ml}^{-1}$, respectively. The corresponding endosulfan tolerance concentration by this study was twice as high, indicating a comparable enhanced resistance of bacteria (Table 1). However, it has been shown that all pesticide-tolerant isolates were also resistant to one or more antibiotics. Hence our results clearly indicate that highly multiple antibiotic-resistant and pesticide-tolerant

bacterial communities exist in alluvial soils. Such soils seem to be an appropriate matrix for antibiotic and have more capability of inclusion of pesticides. Particularly, high resistances to tetracycline, ampicillin, amoxicillin and methicillin were proven in soils investigated.

Out of 35 isolates, 9 highly multi-resistant bacteria were identified. Accordingly, 16S rDNA analyses identified bacterial strains WR7, WR17, WR19, WR21, WR24, as closely related to *Pseudomonas aeruginosa* and *Pseudomonas* spp. (type GS8, M9J918, CL-3), respectively. WR22—WR33—WR34—WR35—isolates were closely related to *Citrobacter* sp. (AzoR-5), *Acinetobacter lowffii*, *Bacillus cereus* and *Enterobacter radicinitans*. Nevertheless, the sequence length of isolate WR34 covered from 1400 nucleotides (total gene sequence) about 930 nucleotides with minor 3' and 5' mismatches. Accordingly, strain WR34 was assigned to the *B. cereus* sensu lato species group. To clearly identify *Bacillus cereus* and to counter the systematic error of the DNA-based in silico analyses applied, the non-DNA-based optical microscopy was applied. Particularly, according to strain WR34, a high similarity to *B. cereus* could be confirmed as we identified $1 \times 2\text{--}3 \mu\text{m}$, gram-positive, rod-shaped cells as regularly *B. cereus* exhibits (Fig. S1). Also the results of all specific agar media identified strain WR34 as *Bacillus cereus*. For MYPA, sharply pink colonies (5–9 mm) were observed surrounded by a pink precipitation zone. According to PEMBA, we identified blue colonies with a turquoise/blue outer zone. The diameters were about 4 mm and 8 mm, respectively, for the colonies and outer zones. These results were confirmed by the Brilliance *Bacillus cereus* agar, as sharply green blue colonies (2–3 mm) were detected surrounded by white margin zones.

Detection of Antibiotic Resistance Genes in Multi-Resistant Bacteria

A total of 35 antibiotic-resistant and pesticide-tolerant bacterial strains were tested for the presence of ARG. The profiling pattern can be taken from Table 3. Investigations according to ARG obtained by PCR experiments revealed the presence of *ampC*, *tetM* and *ermD* genes in most of the isolates, while the *mecA* gene was detected in 19 isolates. However, the *ermG* and *ermD* genes encoding for rRNA methylase proteins were prevailing in highly multi-resistance bacteria (Table 3). Apparently, the transcription of these genes played a major role in microbial antibiotic resistance mechanisms. This is in accordance with a study of Gupta et al. [8]. They declared that *erm* genes encode for methyltransferase enzymes which modify the DNA and thus reduce the ribosomal binding of corresponding antibiotics and consequently confer resistances to microorganisms.

Nevertheless, in contrast to our study is an antibiotic resistance gene study by Böckelmann et al. [4] in which a frequent presence of *tetO* and *ermB* genes was reported within reclaimed water (Spain, Sabadell and Italy Nardò), while the *mecA* gene was detected only once. Zhang et al. [25] investigated sewage sludge and reported that among the tested 14 tetracycline resistance (*tet*) genes, 9 genes encompassing efflux pumps (*tetA*, *tetC*, *tetE* and *tetG*), ribosomal protection proteins (*tetM*, *tetO*, *tetQ* and *tetS*) and enzymatic modification (*tetX*) were commonly detected, whereas 5 genes *tetB*, *tetD*, *tetL*, *tetK* and *tetA* were not detected. Additionally, 109 lactose-fermenting Enterobacteriaceae (LFE) strains were isolated from the activated sludge. Tetracycline-resistant LFE accounted for 32 % of the total 109 LFE strains. The occurrence frequencies of *tet* genes among all TR-LFE strains varied from 0 to 91 %. Also our study indicated a clear resistance against tetracycline together with ampicillin, amoxicillin, cefaclor and methicillin (percentage 90–100 %), while ciprofloxacin, kanamycin and polymyxin (percentage <3 %) were of minor importance (Table 3). The plasmid-based spread of ARGs in environmental microbial communities is a matter of concern for public health, but it remains difficult to study for methodological reasons [2]. To observe the transfer rate of mobilizing plasmids containing ARGs in agricultural soils, further conjugation experiments by filter mating were performed.

Conjugative Transfer of Plasmid DNA by Filter Mating

Biparental mating investigations revealed that gene transfer frequency of strains WR21, WR22, WR24 and WR33 was 4.55×10^{-6} , 4.53×10^{-7} , 6.00×10^{-8} and 3.56×10^{-5} transconjugants per recipient, respectively.

WR34 and WR35 were selected for triparental matings. The mobilization frequency (frequency of gene transfer) of the mobilizing plasmid pMSP3535-VA-GFP-oriT- pIP501 by WR34 was 2.9×10^{-7} transconjugants per recipient. Hence, the result of this experimental set-up indicated WR34 (closely relative to *Bacillus cereus*) as capable to mobilize the *gfp* tagged shuttle plasmid (pMSP3535VA-GFP) from *E. coli* XL 10 to *Streptococcus pneumoniae* T4N517 Novo recipient strains. However, this study could not provide an indication of gene transfer by strain WR35. The presence of the *gfp-oriT* gene within focused transconjugants was proved by PCR. Also the nisin-induced *gfp*-gene expression exhibited bright green fluorescence signals confirming a successful transfer of mobilizing plasmids. Fluorescence microscopy revealed that after 2–3 h, a *gfp*-gene expression was induced during the exponential growth phase (OD_{600} : 0.5) by nisin.

Table 3 Profiling of pesticides, antibiotics and genotypes

Strains	Pesticides	Antibiotics ^a	Genotype (positive PCR)
WR1	ln	Am Cj Cx Nf	<i>mecA</i>
WR2	clr	A Am Cj Nf M	<i>mecA</i>
WR3	clr	A Am Cj Do M	<i>ampC</i>
WR4	clr	A Am Cj Pb M	<i>mecA</i>
WR5	clr	A Am Cj C R M	<i>mecA</i>
WR6	ln	A Am M Nf	<i>ampC</i>
WR7 ^b	ln	A Am Cj Cx Co Do E G M N Nf R T	<i>mecA</i> , <i>tetM</i>
WR8	ln	A Am M Nf	<i>ampC</i>
WR9	ln	A Am Cj Cx G M Nf S	<i>mecA</i>
WR10	clr, monc	A Am M Nf	<i>ampC</i>
WR11	cap, end	A Am Cj M	<i>ampC</i>
WR12	clr, cap	A Am Cj G M S	<i>mecA</i>
WR13	clr, end, monc	A Am Cj Cx G M Nf S	<i>mecA</i>
WR14	clr, flp, monc	A Am Cj M S	<i>mecA</i>
WR15	cap, end, flp, monc	A Am Cj M S	<i>mecA</i>
WR16	cap, flp, monc	A Am Cj M Nf S	<i>mecA</i>
WR17 ^b	cap, end, flp, monc	A Am Cj Cx C Do G M N Na Nf S	<i>ampC</i> , <i>tetM</i> , <i>ermD</i> , <i>ermG</i> , <i>mecA</i>
WR18	cap, end, flp, ln	A Am Cj M S	<i>mecA</i>
WR19 ^b	cap, clr, end, flp ln, monc	A Am Cj Cx Co Do E G M Na N Nf S	<i>mecA</i> , <i>ermD</i> , <i>ampC</i>
WR20	cap, end, flp ln	A Am Cj E G N M Nf	<i>ermD</i>
WR21 ^b	cap, clr, end, flp, ln, monc	A Am Cj Cx Co Do E G M Na N Nf S	<i>mecA</i> , <i>ermD</i> , <i>ermG</i> , <i>ampC</i>
WR22 ^b	cap, clr, end, flp, ln, monc	A Am Cj Cx Co C Do E G M N Na Nf S	<i>mecA</i> , <i>ermD</i> , <i>ermG</i> , <i>ampC</i>
WR23	cap, end, flp, ln	A Am Cj C G T R M	<i>tetM</i>
WR24 ^b	cap, clr, end, flp, ln, monc	A Am Cj Cx Do E G M N Na Nf S T	<i>mecA</i> , <i>ermD</i> , <i>ampC</i> , <i>tetM</i>
WR25	end, flp, monc, ln	A Am Cj Co G M T Nf	<i>tetM</i>
WR26	end, flp, ln, monc	A Am Cj Co G M T Na Nf	<i>tetM</i>
WR27	clr, end, ln, monc	A Am Cj Cx C G N Na Nf T M	<i>ampC</i> , <i>tetM</i>
WR28	cap, clr, end, flp, monc	A Am Cj T M Na Nf	<i>tetM</i> , <i>ermD</i>
WR29	cap, clr, end, flp, monc	A Am Cj Cx Co T S E G C M N Na Nf	<i>ampC</i> , <i>tetM</i> , <i>ermD</i>
WR30	clr, end, ln, monc	A Am Cj T S R G M Na Nf	<i>tetM</i> , <i>ermD</i>
WR31	end, flp, ln, monc	A Am Cj S T N Na Nf C G M	<i>ampC</i> , <i>tetM</i>
WR32	clr, end, ln, monc	A Am Cj M T C G N Na Nf R	<i>ampC</i> , <i>tetM</i>
WR33 ^b	cap, clr, end, flp, ln, monc	A Am Cj Nf M G Co R Do C Cx N Na S E	<i>mecA</i> , <i>ermD</i> , <i>ermG</i> , <i>ampC</i>
WR34 ^b	cap, clr, end, flp, ln, monc	A Am Cj Nf M G Co R Do C K N Na S T E	<i>mecA</i> , <i>ermD</i> , <i>ermG</i> , <i>ampC</i> , <i>tetM</i>
WR35 ^b	cap, clr, end, flp, ln, monc	A Am Cj Nf M G Co R Do C Cx N Na S T E	<i>mecA</i> , <i>ermD</i> , <i>ermG</i> , <i>ampC</i> , <i>tetM</i>

Pesticides tolerance concentrations [$\mu\text{g ml}^{-1}$](ln): 1600, (end): 1600, (clr): 400, (monc): 400, (cap): 800, (flp): 800. Antibiotic resistance phenotype concentrations [$\mu\text{g disc}^{-1}$](S): 25, (E): 15, (A): 25, (C): 25, (Co): 25, (Am): 30, (Cj): 30, (Cf): 30, (Cx): 30, (Do): 30, (G): 30, (K): 30, (M): 30, (Na): 30, (N): 30, (Nf): 30, (B): 30, (R): 30, (Sz): 30, (T): 30

amp ampicillin, *van* vancomycin, *tet* tetracycline, *mec* methicillin, *erm* erythromycin resistance genes

^a Abbreviations are given in Table 2

^b Highly multi-resistant bacteria containing multiple ARGs, identified by 16S rDNA gene sequencing

In contrast to Timmerly et al. [23] and Jorquera et al. [12], our results differ as lower gene transfer frequencies were noticed. Our findings based on triparental mating experiments lead to the conclusion that isolate WR34 was capable to mobilize the *gfp*-tagged shuttle plasmid (pMSP3535VA-GFP) from *E. coli* XL 10 to *Streptococcus pneumoniae* T4N517 Novo which served as HGT recipient.

The corresponding mobilization transfer frequency of plasmid pMSP3535-VA-GFP-*oriT* pIP501 by strain WR34 was 2.9×10^{-7} transconjugants per recipient.

Moreover, conducted biparental mating experiments revealed gene transfer frequencies of donor strains WR21, WR22, WR24 and WR33 from 10^{-5} to 10^{-8} transconjugants per recipient. These values are not in accordance with

studies of Timmerly et al. [23] and Jorquera et al. [12] as they reported up to 10,000-fold higher gene transfer rates (cow and soy milk 10^{-1} to 10^{-4} , rizospheric plant species 10^{-5} to 10^{-6}).

Studies of Binh et al. [3] discussed the incidence and types of transferable antibiotic resistance plasmids (amoxicillin, sulfadiazine or tetracycline) in swine manure samples and found a gene transfer frequency of 10^{-5} . Our findings compared to those of mentioned above are indicating with a few exceptions that soil samples investigated by this study were less contaminated and the gene transfer capacity was lower, respectively.

Biofilm Formation of Bacterial Isolates in Bioreactor, Lectin-Binding and Lectin Blocking Assays

The present study also focused on biofilm forming bacterial strains from pesticide and antibiotics-contaminated alluvial soils as HGT may occur rapidly in a biofilm, which in consequence cause a favourable environment for the rise of new pathogens. After a bioreactor operation period of 8 days, a biofilm formation of strain WR34 on glass bead surfaces was observed ($OD > 0.240$), whereas no biofilm formation ($OD < 0.120$) was detectable on silicon tubes or polystyrene beads. All remaining isolates exhibited negative responses. The conducted lectin test confirmed strain WR34 as biofilm former (Fig. S1).

Adhesive properties of *Bacillus cereus* sp. and the presence of inert flagella, respectively, might have enabled the capability of an enhanced biofilm forming potential on the glass surface and interactions between bacterial cells and reactor wall. A study by Huang Hsueh et al. [11] announced that in contrast to the wild type, *Bacillus cereus* was much more capable to form a biofilm accompanied by an increased biosurfactant production. On the contrary, polystyrene plates coated with surfactin, a biosurfactant from *Bacillus subtilis*, rescued the deficiency in biofilm formation by the wild type. Houry et al. [10] investigated the role of the *Bacillus cereus* flagellar apparatus during the biofilm formation. They suggested that motility is necessary for the bacteria to reach surfaces suitable for biofilm formation, especially for the biofilm formation in glass tubes. Also, motility seemed to be involved in the spread of the biofilm on glass surfaces. Therefore, our results also showed that isolate WR34 strongly related to the *B. cereus* has high potential to form biofilms on glass surfaces and hence also to build biofilm matrices in soils highly mineralized with silica and hitherto less described alluvial soils, respectively.

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