Diversity and activity of bacterial biofilm communities growing on hexachlorocyclohexane

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Abstract

γ -Hexachlorocyclohexane (γ -HCH) persists in the environment and is recalcitrant to microbial degradation. To determine the extent of the microbial potential for the degradation of γ -HCH the diversity of bacteria from 12 soil samples collected around insecticide and pesticide producing factories in Egypt were assessed and compared with biofilm communities grown on γ -HCH microcrystals. From all samples highly diverse microbes were isolated, able to grow on γ -HCH as sole source of carbon. The same soil samples were used to inoculate γ -HCH microcrystals on a substratum in microcosms to grow biofilm communities. All soil samples formed multi-species biofilms on γ -HCH

. Biofilms stained with Nile Red showed distinct cell clusters of high hydrophobicity and it is speculated that these aggregates have a substantial role in the degradation of the hydrophobic substrate. While many *Bacillus* species were isolated, this group was almost absent in the different biofilm communities. The finding of cells with highly hydrophobic envelopes together with the differences in species composition between isolates and interacting microbial communities point to fundamental differences in the interaction with hydrophobic substrates of single strains and microbial communities.

Introduction

Most bacteria living in the environment are organized in biofilms (Hall-Stoodley et al., 2004). A biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface. Biofilms can contain many different types of microorganism, e.g. bacteria, archaea, protozoa, and fungi; each group performing specialized metabolic functions. Biodegradation is a process whereby microbial communities contribute extensively to the attenuation, mineralization and transport of organic (carbon-based compounds) contaminants in the environment. The development of biofilms by microbial communities is often a key factor contributing to the overall efficiency of these processes. The potential of bioremediation (remediation using biological processes) as an alternative to physical and chemical remediation strategies has resulted in a significant amount of research effort on degradative biofilms. Biofilms have industrially been used, e. g. for bioremediation of hazardous materials and waste sites, biofiltration of industrial waste water or industrial air (Edwards and Kjellerup, 2013). There is ample evidence that microbial interactions are important for the functioning of microbial communities, especially when challenged with complex substrates (Macedo et al., 2005). Two key properties of degradative biofilms are: (1) the spatial organization of cells and (2) the establishment of a stable microenvironment through the production of extracellular polymeric substances (EPS). These characteristics promote the assemblage of larger and more diverse genetic pools in confined microniches, thereby expanding the range of substrates that can be degraded.

In this study the potential of biofilm communities and isolates, directly obtained from the same soil, for the degradation of γ -hexachlorocyclohexane (γ -HCH) or lindane was assessed. HCH is insecticidal, toxic and considered as potential carcinogenic (Philipps et al., 2005). Due to persistence and recalcitrance, however, HCH continues to pose a serious toxicological problem at industrial sites where past production of lindane along with unsound disposal practices has led to serious contamination (Nayyar et al., 2014). In addition, many countries still allow HCH production and use despite localized limitations. Abiotic factors may degrade pesticides in soil or water

ecosystem, however, the microorganisms present in soil and water are a major factor in the degradation of these pesticides (Singh et al., 2000). γ -HCH is degraded under both aerobic and anaerobic conditions, but it is mainly mineralized under aerobic conditions. Many γ -HCH-degrading aerobic bacteria have been isolated and characterized (Boltner et al., 2005; Lal et al., 2006; Mohn et al., 2006). Isolation of lindane-degrading microorganisms by enrichment culture has confirmed the ability of specific species of bacteria to degrade HCHs either aerobically or anaerobically (Alvarez et al., 2012). HCH-degrading *Brevundimonas vesicularis* P59 was isolated in the Netherlands (Bachmann et al., 1988) by enrichment culture from contaminated soil slurries. *Sphingomonas paucimobilis* UT26 is capable of aerobically degrading α -, γ - and δ -HCH isomers and using γ -HCH as a sole carbon source.

It can be assumed that in γ -HCH polluted habitats not only single strains but bacterial consortia are adapted to this carbon source and that these consortia handle the pollutant differently to single strains. To gain access to these consortia biofilm communities derived from several soil samples were grown on γ -HCH microcrystals and their community composition was compared with strains obtained from the same soil and isolated on agar plates containing γ -HCH as sole carbon source.

Materials and Methods

Collection of soil samples. A total of twelve soil samples (approx. 50 g) were collected from Egypt at different governorates located in the delta of the Nile (Alexandria (4 sites), Kafr El-Sheikh (1 site), Gharbia (1 site), Qalyubia (2 sites) and Monufia (4 sites)), around factories which produce chemicals, insecticides, and pesticides. The samples were collected in sterile plastic bags, homogenized and stored at 4°C until use (Harry et al., 2000).

Isolation and purification of the γ -HCH-degrading bacteria. One gram of soil was incubated in 250 ml Erlenmeyer flasks containing 100 ml of M9 medium (Sambrook and Russell, 2001) with γ -HCH (2 mM) as the sole source of carbon and energy. After one month of cultivation at 30°C and shaking on a rotary shaker operated at 150 rpm, bacteria were isolated from soil through serial dilutions in PBS buffer. One hundred microliters of the dilution were spread onto M269 minimal medium (per litre: 2g (NH₄)₂SO₄ 100 mg KCl, 500 mg K₂HPO₄, 500 mg MgSO₄ x 7 H₂O) agar plates supplemented with crystals of γ -HCH in the lid of the plate. After 7 days of incubation, colonies were picked and transferred to new plates by repeated subculturing and streaking on R2A medium or LB agar plates until pure cultures were obtained. For stock cultures a loop of a pure culture was added to 750 μ l sterile LB or R2A medium depending on the isolate in a 2 ml cryo-vial and incubated for one day at 30°C. Then 500 μ l of sterile glycerol was added, the vial was mixed by vortexing and frozen at -20°C.

Sequencing of bacterial 16S rRNA genes. DNA was obtained for the amplification of the 16S rRNA gene by polymerase chain reaction by boiling single colonies in 100 μ l of TE buffer for about 10 min. A nearly complete 16S rRNA gene sequence was obtained as described previously (Abraham et al., 1999). The reactions were evaluated on an Applied Biosystems 377 genetic analyzer and the final contig was assembled

using the program SEQUENCHER™ Version 4.0.5 (Gene Codes Corporation, USA). The sequences together with those of the closest type strains were aligned using Clustal Omega software (Sievers et al., 2011) and the phylogenetic analysis was performed using MEGA 6 software (Tamura et al., 2013). Tree topologies were reconstructed with neighbour-joining algorithm with 1000 bootstrap replications using the sequences of type strains obtained from the EMBL database. The determined 16S rRNA gene sequences have been deposited in GenBank (accession numbers are given in figures 2-4).

Assessment of HCH-degradation of the isolates. Bacterial strains from frozen stock cultures were streaked on R2A or LB agar plates and incubated at 30°C until the formation of the colony was visible. Single colonies were inoculated in liquid culture (LB or R2A medium). The culture was incubated overnight in 100 ml Erlenmeyer flasks with 20 ml medium, pH 7.0 at 30°C with orbital shaking at 150 rpm. To determine HCH-degradation a volume of 300 µl of the culture showing $OD_{600} = 0.5 - 0.7$ was transferred into 20 ml of the minimal liquid culture (M269) which contain 2 mM of γ -HCH as the sole carbon source. This culture was incubated at the same conditions and sampled every 24 h until day 9. The bacterial cell density was quantified by measuring OD_{450} (Mosmann, 1983) and the viability of the cells was determined by using Water-soluble Tetrazolium salt (WST-1) reagent, starting at 0 incubation time. One hundred and sixty microliters of culture were incubated with 20 µl of WST-1 in a 96-well plate for 30 min with shaking at 650 rpm and at 30°C. For negative control the strains were also inoculated in mineral medium without HCH, no growth of these isolates was observed.

Microcosm experiments. About five grams of homogenised soil sample were placed in a 100 ml stoppered glass vessel and 80 ml of sterile tap water were added (Figure 1). Twenty mg of γ -HCH were dissolved in 1 ml of dichloromethane (DCM). Droplets of 25 µl of the γ -HCH solution were placed on sterile Permanox™(Nunc, USA) plastic slides (100 x 20 mm) and DCM was allowed to evaporate. One slide, loaded with 8 droplets of the compound, was placed in the microcosm, sides with γ -HCH crystals downwards facing the water surface of a reservoir. The microcosms were maintained at room temperature. The biofilm communities were harvested weekly with a sterile cotton swap from 4 spots of γ -HCH microcrystals, transferred to columns provided in the commercially available FastDNA® SPIN® Kit for Soil (Bio 101, La Jolla, CA) according to the manufacturer's instructions. Pieces with 4 spots of γ -HCH microcrystals per slide were cut off and immediately examined by confocal laser scanning microscope (CLSM).

Single strand conformational polymorphism (SSCP) fingerprint analysis. The primers chosen for amplification of bacterial 16S rRNA genes were the forward primer Com1 (5'CAGCAGCCGCGGTAATAC3') and the reverse primer Com2-Ph (5'CCGTCAATTCCTTTGAGTTT3' with 5'-terminal phosphate group) as published (Schmalenberg et al., 2001). The phosphorylated strand of the PCR products was digested by lambda exonuclease (New England Biolabs, Schwalbach, Germany), proteins were removed by the Mini-elute kit (QIAGEN, Hilden, Germany) as recommended by the manufacturer, and the remaining single-stranded DNA was dried under vacuum. The DNA was then re-suspended in denaturing SSCP loading buffer (47.5% formamide, 5 mM sodium hydroxide, 0.12% bromophenol blue, and 0.12% xylene cyanol) and subjected to electrophoresis (Schwieger and Tebbe, 1998).

Gels were run at 400 V for 17 h at 20°C in a MacroPhor electrophoresis unit (LKB, Bromma, Sweden) and subsequently silver stained (Bassam and Gresshoff, 2007).

Sequence determination of SSCP bands. Bands were excised from the gel, eluted in buffer (10 mM Tris buffer, 5 mM KCl, 1.5 mM MgCl₂ · 6H₂O, 0.1% Triton X-100, pH 9.0), and extracted at 95°C for 15 min. Extracts were centrifuged, and the supernatant was used as a DNA template in the PCR with the primers described above. The PCR product was purified (Mini-elute kit; QIAGEN, Hilden, Germany) and sequenced with a sequencing kit (DYEnamic ET Terminator cycle sequencing kit; Amersham Biosciences, Freiburg, Germany) and both primers. The product was cleaned with the Dye Ex Spin kit (QIAGEN, Hilden, Germany), and the sequence was analyzed on an ABI PRISM 337 DNA sequencer and an ABI PRISM 3100 genetic analyzer. The sequences were analyzed as described above.

Microscopy analysis - biofilm staining. Samples were stained with SYTO9 for nucleic acids (bacteria) and with Nile Red (Sigma, St. Louis, MO) for hydrophobic compounds (HCH, hydrophobic lipids) (Andrews et al., 2010). Cells were first stained with SYTO9 (Molecular Probes, Eugene, OR) for 10 min, rinsed and then stained with Nile Red. For this purpose a stock solution of 2 mg Nile Red in 1 ml acetone-water (1:1, vol/vol) was diluted 1:1,000 in demineralized water. After staining for 15 min, the sample was carefully rinsed twice. Alternatively, live and damaged cells in biofilms were stained with the BacLight kit (Molecular Probes) as described by the manufacturer. All samples were incubated in the dark and examined immediately after staining using CLSM. Laser scanning microscopy was performed using the model TCS SP attached to an upright microscope. The instrument was controlled by Leica Confocal software. The system was equipped with three visible lasers: an Ar laser (458, 476, 488, and 514 nm), a laser iodide (561 nm), and a He-Ne laser (633 nm). The spectrophotometer feature allowed flexible and optimal adjustment of sliders on the detector side. The following settings were used for excitation and recording of emission signals (ex/em), respectively: Nile Red (488 and 550/700 nm), SYTO9 (488/500 nm) and propidium iodide (490/635 nm). Biofilm samples were observed with 10x 0.3-numerical aperture (NA), 20x0.5-NA, and 63x0.9-NA water-immersible lenses.

Results and Discussion

Phylogeny and characteristics of bacterial isolates from Egyptian localities that were able to grow in the presence of γ -HCH

The sequence of the 16S ribosomal RNA genes of the isolated strains from several localities, compared to the database of the National Centre for Biotechnology Information (NCBI) and sequences of their closest type strains (Euzéby, 2014), revealed the bacteria able to grow in the presence of γ -HCH as a nutrient. In the present study, 68 different bacterial strains were isolated from soil samples from Egyptian locations. Bacteria recorded in this investigation could be classified into 3 phyla: Proteobacteria, Firmicutes and Actinobacteria according to their 16S rRNA gene sequences. The phylum with the highest frequency was Proteobacteria, the phylum with moderate frequency and diversity was Firmicutes which contained the genera *Bacillus*, *Oceanobacillus* and *Paenibacillus*. The

rarest phylum was Actinobacteria which contained 7 different species belonging to *Agromyces*, *Gordonia*, *Microbacterium*, *Micromonospora*, and *Rhodococcus*. The highest number of bacterial isolates was obtained from samples collected from the Monufia location and this was the only site heavily dominated by *Bacillus* species.

The Alexandria samples comprised a community which was mainly composed of the genera *Achromobacter*, *Agromyces*, *Bacillus*, *Lysinibacillus*, *Microbacterium*, *Ochrobactrum*, *Pseudomonas*, *Rhodococcus* and *Starkeya*. The phylogenetic tree showed the diversity and the bacterial relationship of these isolates (Figure 2). Contrary to the Alexandria site the bacterial communities of the Monufia samples were dominated by a broad diversity of *Bacillus* species (Figure 3). It was found that the bacterial strains which were isolated from Gharbia samples were again different from the previous samples. They consisted mainly of bacterial strains of the genera *Aquamicrobium*, *Bacillus*, *Gordonia*, *Mesorhizobium*, *Micromonospora* and *Rhodococcus*. Only three bacterial genera, *Achromobacter*, *Lysobacter* and *Pseudomonas*, could be identified in the Kafr El-Sheikh sample. From Qalyubia samples *Bacillus*, *Brevundimonas*, *Luteimonas*, *Ochrobactrum*, *Pseudomonas*, *Rhodanobacter* and *Rhodococcus* strains were isolated (Table 1).

Growth of the bacterial isolates on γ -HCH

The metabolization and degradation of γ -HCH and bacterial growth was carried out for 15 days. Not all of the isolated bacterial species from soil samples grew well in the presence of γ -HCH. Screening or selection of the most suitable bacterial species was based on the growth rate in the medium containing γ -HCH. The results revealed that the most active bacterial isolates were *Rhodococcus ruber* Qalyubia2S12, *Pseudomonas balearica* Alexandria4S10, *Bacillus sp.* Monufia7S7, *Mesorhizobium shangrilense* Gharbia4S11 and *Lysobacter daejeonensis* Kafr3S9. Growth of *Rhodococcus ruber* Qalyubia2S12 reached its maximum after the 2nd day of cultivation. *Pseudomonas balearica* Alexandria4S10, *Bacillus sp.* Monufia7S7 and *Mesorhizobium shangrilense* Gharbia4S11 gradually reached their maximum at 3rd day of incubation. The growth of *Lysobacter daejeonensis* Kafr3S9 was at its maximum after the 4th day of cultivation. After that the growth of all isolates gradually decreased over time (Figure 4). The species found here to grow best in the medium containing HCH as sole carbon source are different to the ones usually used to study HCH degradation. Sahu *et al.* studied the degradation of γ -HCH by a *Pseudomonas sp.* isolated from sugarcane rhizosphere soil. The authors demonstrated the almost complete disappearance of the pesticide within 24 h of incubation with a concomitant release of Cl^- almost in stoichiometric amounts (Sahu *et al.*, 1990). Lindane was totally consumed within 72 h by a consortium of bacteria isolated from a river sediment (Benimeli *et al.*, 2006). Further work is needed to characterize the degradation pathways and the optimal conditions for HCH degradation for any of the above isolates.

Analysis of bacterial biofilm community compositions developing on γ -HCH microcrystals

After isolation and identification of the most active degraders for γ -HCH, the objective of the present work was to compare the diversity and activity of the isolates with microbial biofilm communities colonizing γ -HCH crystals. Microbial communities organized in biofilms show a multitude of interactions, including carbon sharing (Nielsen *et*

al., 2000), interspecies communication (Schachter, 2003), and steep physicochemical gradients and are very well protected against environmental stress factors such as toxic compounds, water stress, or grazing (Matz et al., 2004). To take advantage of the special conditions in biofilms, soil samples were taken as inocula to grow biofilm communities on the pollutant. Biofilms developing on the γ -HCH microcrystals were harvested at different time points (7, 14, 21, 28, 35 and 42 days). To allow a fast overview over the diversity of biofilm communities and their dynamics 16S rRNA gene fingerprinting using SSCP was applied. SSCP community profiling showed highly diverse and distinct bacterial communities for γ -HCH microcrystals with biofilm from the soil samples. Although some changes could be seen the majority of species in the biofilm communities remained constant as the bands in the SSCP profiles remained constant over time (Figure 5). Analysis of bacterial biofilm structure from Alexandria location by SSCP revealed considerable diversity in the bacterial communities. By comparing the sequences of 11 excised bands, 8 different operational taxonomic units (OTUs) could be identified. The phylogenetic tree (Figure 5) presents the closest related species to each sequence obtained. The majority of the identified OTUs were members of the phylum Alphaproteobacteria followed by Betaproteobacteria and Gammaproteobacteria.

For all samples it was possible to grow biofilms on the HCH crystals and the individual biofilm communities differed considerably between the different sites, a phenomenon already described for other biofilm communities (Macedo and Abraham, 2009). This underlines that different communities are capable of HCH degradation and that probably conditions specific for the site control community composition as has been shown before in the case of PCB degradation (Macedo et al., 2007). From bacterial biofilm communities 38 operational taxonomic units (OTUs) were identified. Fifteen OTUs belonged to the phylum Proteobacteria but the phyla Firmicutes and Cyanobacteria contained only one OTU each. *Sphingomonas*, *Serratia*, *Pseudomonas* and *Burkholderia* were the most frequent genera. The members of the individual biofilm communities turned out to be different from those isolated through classical microbiological methods from the same soil samples. This again underlines both the strong selection pressure applied by isolation and the tight interactions between different species in such biofilm communities.

This is an interesting finding because most of the HCH-degrading aerobic bacterial strains reported until now are gram-negative and members of the family Sphingomonadaceae. A few other HCH-degrading isolates such as *Rhodanobacter lindaniclasiticus* and *Xanthomonas sp.* were also reported (Nalin et al., 1999). Only very few gram-positive strains, such as *Microbacterium sp.* and *Bacillus sp.*, have been shown to degrade HCH (Elcey and Kuhni, 2010). Previous studies have demonstrated that the individual strains do not act isolated from the other community members but have strong interactions with each other (Pelz et al., 1999). A simple comparison of the isolates with the activity of the biofilm communities is therefore not possible. Furthermore, a mere combination of the isolates is not feasible to gain activities and robustness comparable to the one observed in the biofilms. Probably other techniques as meta-transcriptomics or stable isotope analyses are required to get a much deeper understanding of HCH degradation in biofilms communities (Tillmann et al., 2005).

Structure of the biofilm changes along the pollution gradient

The biofilm originating from soil sample was treated with Live/Dead stain to determine the rate of live to damaged cells over 42 days and it was found that damaged cells could be preferentially found on the HCH-crystals and towards the end of the experiment. After 7 days of incubation a biofilm was detected on the Permanox™ slide close to the crystals but only few cells were observed on the crystals directly. Subsequently, after 14 days a substantial biofilm accumulation on the margins of the pollutant was observed. Furthermore, after 14 days the number of live cells was higher than those of the defective cells. After 21 days when the biofilm showed the highest number of species in the SSCP profiles large microbial aggregates encircling the microcrystals of γ -HCH could be seen. Twenty-eight days after incubation, the bacterial population on the Permanox™ substratum was somewhat reduced and the crystals started to break up. The 35 and 42 days old biofilms revealed the dominance of damaged cells and the complete disappearance of the γ -HCH crystals. Finally, the aggregates of pollutants almost disappeared and almost all cells were damaged. One reason could be metabolites inhibiting or damaging cells in the biofilm leading to a higher ratio of damaged to living cells (Macedo et al., 2005).

In order to follow the dynamics in biofilm architecture the biofilm was also monitored using the hydrophobic dye Nile Red and, interestingly, this dye stained also some aggregates of bacteria indicating highly hydrophobic cell surfaces (Figure 1). The role of these highly hydrophobic microcolonies in the degradation process is not clear. One can speculate that these microcolonies are the ones preferentially taking up HCH for the initial degradation step and nourish the less hydrophobic microcolonies with intermediates.

Generally, the bacterial biofilms in the 2nd and 3rd weeks were more prominent and diverse, but remained relatively constant in the 4th week and no new significant SSCP bands appeared. In the 5 and 6 weeks old biofilms, most of the microbial communities changed. The HCH crystals were never heavily colonized by bacteria, instead they were surrounded by bacteria probably taking advantage of the diffusion gradient of HCH dissolving in water. An astonishing phenomenon is the fragmentation and dissolution of the HCH microcrystals at the end of the experiment which was not seen before in any of these microcosms. Microcosms are closer to the situation in the field than isolated microorganisms; however, they are still not the same than the situation *in situ*. Nevertheless, valuable insights into the degradation process by microbial communities and its dynamics can be gained by such experiments. The discovery of highly hydrophobic microcolonies is one example.

Conclusions

Among the isolates found using HCH as carbon source, *Bacillus*, *Pseudomonas* and *Rhodococcus* were the most frequent and diverse genera and their species have been reported frequently to grow on γ -HCH. However, some rare genera were also among the isolates (e.g. *Achromobacter*, *Cupriavidus*, *Starkeya*). The study presented here enlarges the number of genera and species able to use HCH offering novel possibilities in using bacteria for HCH degradation. The results of our study revealed diverse microbial communities in Egyptian soil samples which were able to colonize γ -HCH crystals. Remarkable is the large dichotomy between the taxonomic composition of the

isolates and the species detected in the biofilm community growing on HCH-crystals. This highlights two facts: that still a number of bacteria species are difficult to isolate and that different species interact in biofilm communities achieving the degradation of recalcitrant substrates. This is supported by the detection of highly hydrophobic microcolonies within the biofilm and we speculate that these microcolonies have a key role in the degradation process within the biofilm.

The method applied was very effective in selecting communities of γ -HCH degraders, which could metabolize its microcrystals. Despite the fact that some members of the communities disappeared during incubation, the most abundant members tend to stay over time. The approach used proved to be a good method to follow the dynamics of biofilm communities composed of uncultured bacteria. The current work on the diversity of HCH degraders and their dynamics in the biofilm communities is therefore a step forward in understanding the role of different microorganism and their communities in the degradation of HCH. The findings presented here support and help to optimize *in situ* bioremediations using biofilm communities.

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Conflict of interest: The authors declare that they have no conflict of interest.

Figure legends

Figure 1 Left: Scheme of the microcosm used to grow biofilms; right: Biofilm grown from the Gharbia sample after 7 days on γ -HCH microcrystals stained with SYTO9 (green) and Nile Red showing areas with medium hydrophobicity in yellow and high hydrophobicity in red. A γ -HCH microcrystal can be seen in the lower right corner of the micrograph. Grid size = 20 μ m

Figure 2 Phylogeny of bacterial isolates from Alexandria samples able to grow on γ -HCH and their closest type strains (Neighbor joining clustering of 16S rRNA gene sequences; GenBank acc. no. in brackets; outgroup: *Ferroplasma acidiphilum* DSM 12658^T [AJ224936]). Bar represents 2% sequence dissimilarity

Figure 3 Phylogeny of bacterial isolates from Monufia samples able to grow on γ -HCH and their closest type strains; GenBank acc. no. in brackets; outgroup: *Ferroplasma acidiphilum* DSM 12658^T [AJ224936]. Bar represents 2% sequence dissimilarity. The high prevalence of *Bacillus* isolates compared to the Alexandria samples (Figure 2) is striking

Figure 4 Growth curves of isolates on M269 medium with γ -HCH as sole carbon source. Rhombus: *Mesorhizobium shangrilense* Gharbia4S11, circle: *Pseudomonas balearica* Alexandria4S10, triangle: *Bacillus* sp. Monufia7S7, square: *Lysobacter daejeonensis* Kafr3S9, diamond: *Rhodococcus ruber* Qalyubia2S12. All these strains had their maximum after around 2 days after inoculation and then OD₄₅₀ slowly decreased

Figure 5 Composition of γ -HCH bacterial biofilm communities obtained from two soil samples from Qalyubia analyzed by 16S rRNA gene based community fingerprint (SSCP). Numbers on top of the gel correspond to sampling time in weeks and lanes M show the marker; numbered bands in the gel belong to OTUs shown in the phylogenetic tree. The phylogenetic tree based on the sequences of the SSCP bands differs considerably from the isolates from the different sites (Figures 2 and 3). Bar represents 0.02 substitutions per nucleotide position

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